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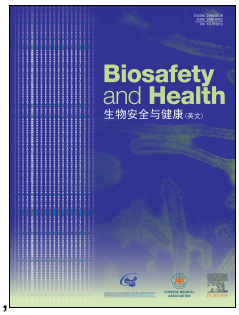

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Human-animal interactions and bat coronavirus spillover potential among rural residents in Southern China

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Abstract

Human interaction with animals has been implicated as a primary risk factor for several high impact zoonoses, including many bat-origin viral diseases; however, the animal-to-human spillover events that lead to emerging diseases are rarely observed or clinically examined, and the link between specific interactions and spillover risk is poorly understood. To investigate this phenomenon, we conducted biological-behavioral surveillance among rural residents in the Yunnan, Guangxi, and Guangdong provinces of Southern China, where we have identified a number of SARS-related coronaviruses in bats. Serum samples were tested for four bat-borne coronaviruses using newly developed enzyme-linked immunosorbent assays (ELISA). Survey data were used to characterize associations between human-animal contact and bat coronavirus spillover risk. A total of 1,596 residents were enrolled in the study from 2015 to 2017. Nine participants (0.6%) tested positive for bat coronaviruses. 265 (17%) participants reported severe acute respiratory infection (SARI) and/or influenza-like illness (ILI) symptoms in the past year, which were associated with poultry, carnivore, rodent/shrew, and bat contact, with variability by family income and province of residence. This study provides serological evidence of bat coronavirus spillover in rural communities in Southern China. The low seroprevalence observed in this study suggests that bat coronavirus spillover is a rare event. Nonetheless, this study highlights associations between human-animal interaction and zoonotic spillover risk. These findings can be used to support targeted biological behavioral surveillance in high-risk geographic areas in order to reduce the risk of zoonotic disease emergence.

Key words

Bat coronavirus, human-animal interaction, disease emergence, Southern China, rural community

Highlights

Scientific question

What are the behavioral risks in human-animal interactions that could lead to the emergence of bat coronaviruses in human population.

Evidence before this study

Bat borne coronaviruses have caused several emerging infectious disease outbreaks of global significance, including SARS. Novel SARS-related coronaviruses have been discovered in bat populations in South China, some of which have the capacity to infect human cells. Human-animal interactions are thought to be critical for the emergence of bat coronaviruses, however the specific interactions linked to animal-to-human spillover remain unknown.

New Findings

This study found serological evidence for bat-borne coronavirus transmission to people. Direct contact with bats was not identified as a risk factor. However, self-reported severe acute respiratory infection (SARI) and/or influenza-like illness (ILI) was linked to human interaction with other wildlife and livestock, suggesting that there may be other zoonotic exposures leading to clinical illness in these populations.

Significance of the study

Findings from this study suggested that an integrated biological and behavioral surveillance in healthy community settings can help identify potential zoonotic disease spillover events or target surveillance to at-risk populations. This approach represents a potential early-warning system that could be used under non-outbreak conditions to identify potential zoonotic emerging diseases prior to largescale outbreaks.

1. Introduction

In the highly biodiverse southern region of China, interactions among humans, wildlife, and livestock are likely to be common, and are hypothesized to be a risk factor in the emergence of zoonotic infectious diseases [1-3]. Human-animal interactions may pose a particular public health threat in rural communities where frequent contact with animals occurs and where disease prevention measures are likely less well-developed [4]. Although human-animal interactions are thought to be associated with zoonotic disease emergence, few studies have addressed the nature of specific interactions that occur between animals (particularly wild animals) and humans that lead to pathogen spillover.

Bats (order Chiroptera) are reservoirs of a large number of zoonotic viruses, including coronaviruses (CoVs) that have caused disease outbreaks in human and livestock populations [5-13]: Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), the causative agent of the SARS outbreak affecting 32 countries in 2002-3, infecting 8,096 people and causing 774 deaths [14]; Middle East Respiratory Syndrome coronavirus (MERS-CoV), which has caused 823 deaths from 2,374 human cases in 27 countries by the end of February 2019, and is thought to have originally spilled over from bats into camels, in which is it now endemic [15-18]; and Severe acute diarrhea syndrome coronavirus (SADS-CoV) which emerged in the pig population of Southern China and caused the deaths of more than 20,000 piglets in 2017 and 2018 [5].

A large diversity of coronaviruses, including SARS-related Coronaviruses (SARSr-CoVs) have been discovered in bats, and phylogenetic and pathogenesis studies of these suggest a high capacity for transmission across species barriers [9, 11, 13, 18-22]. However, few studies have analyzed bat-to-human spillover events in non-outbreak conditions, likely due to the rarity of

these events, and difficulties in identifying at-risk populations or target geographies. Additionally, the symptoms of novel bat coronavirus infection in the human population may not be clinically recognized at the time of emergence due to lack of adequate surveillance, or confusion with other diseases. This represents a significant biosafety risk due to the large and increasing number of coronaviruses discovered in bats [23, 24] and the wide distribution of bat populations in rural regions such as Southern China [25].

In this paper we report on a study designed to characterize the bat coronavirus spillover potential associated with presumed high-risk human behavior in rural communities of Southern China [26]. We collected data from community serological and behavioral surveillance to understand the driving factors of bat coronavirus spillover provide evidence for community-based strategies to help prevent zoonotic disease emergence.

2. Materials and Methods

2.1 Study Location and Target Population

We conducted a cross-sectional study in the provinces of Yunnan, Guangxi, and Guangdong, China which are known for their high levels of animal biodiversity, active animal trade activity, and historic zoonotic disease emergence events [3, 5, 10, 14, 22, 24, 27]. Eight study sites were selected in areas where we have previously reported diverse coronaviruses in bat populations [24] close (within five kilometers) to human dwellings. The study targeted human populations that are highly exposed to bats and other wildlife, including people who visit or work around bat caves, work in local live animal markets, raise animals, or are involved in trading wild animals (e.g., wild animal harvest, trade, transportation, and preparation), as identified by previous exploratory ethnographic interviews.

2.2 Recruitment and Sampling

Prior to recruitment and sampling, project staff who received human subject research training visited each participating site to introduce the project to the community with assistance from officials from provincial and city-level Centers for Disease Control and Prevention. To generate interest and develop recruitment strategies, project staff held meetings with village committees to discuss topics relevant to their daily contact with animals and any health issues in the community that were particularly concerning for them. With permissions from local authorities, community leaders conducted house visits and broadcast announcements a week before data collection took place to inform community residents about the study and its recruitment plan. All information was communicated in local dialects using simple language to convey the study purpose, eligibility and inclusion guidelines, potential risks and benefits of participation, and the time and locations at which the study would take place.

We aimed to obtain a minimum sample size of 400 participants from each of the three provinces (Yunnan, Guangxi, and Guangdong), for a total sample size of over 1,200 participants. A snowball sampling method was used because the population size at selected sites and the people who were highly exposed to wild animals were difficult to elucidate [28]. During each house visit, we requested information about potential eligible participants from the residents' networks, and we then followed their referrals to recruit from the community. Only one person per household was recruited to participate in this study, and no participants were recruited from clinics or healthcare settings. We made every effort to include participants across a range of demographic indices including gender, age, and socioeconomic status, as well as to ensure that any contribution was voluntary and involved minimal risk to the participants.

2.3 Data collection and management

Following the completion of the informed consent process, a standardized Mandarin questionnaire was administered by study staff in local dialects. The interview was conducted in a private environment where confidentiality was maintained, and interviewers and participants were paired by sex. Children aged 10 to 18 years were interviewed with the permission and in the presence of a parent or guardian.

The questionnaire included five sections consisting of demographics, living circumstances and livelihood, travel, and types of contact with animals, as well as unusual illness symptoms in the past 12 months. The survey assessed symptoms including fever with cough and shortness of breath or difficulty breathing (severe acute respiratory infection [SARI] symptoms) and fever with muscle aches, cough, or sore throat (influenza-like illness [ILI] symptoms) (Appendices). SARI and ILI symptoms were included in the survey in anticipation of potentially low coronavirus sero-positivity rates. These symptoms are commonly used as metrics in emerging infectious respiratory disease surveillance and are known to be associated with coronavirus infections (e.g., MERS-CoV, SARS-CoV) [29]. Therefore, SARI and ILI symptom histories can be analyzed in addition to serological testing to maximize our understanding of bat coronavirus spillover risk.

After the questionnaire interview, participants were asked to provide a blood sample (2.5-5 mL stored in a serum-separating tube) and an oropharyngeal swab (stored in a cryotube with viral transport medium). Samples were collected by study staff from local clinics. All samples were stored in liquid nitrogen immediately after collection and transferred to an ultralow (-80°C) freezer within 48 hours.

A unique alphanumeric identification code was assigned to each questionnaire and biological specimen collected from each participant. No personal identifying information was collected.

Only authorized study personnel who received human subject research training were allowed access to the questionnaire and biological data.

2.4 Serological testing

Serum samples collected from study participants were analyzed using newly developed IgG enzyme-linked immunosorbent assays (ELISA) based on selected nucleocapsid proteins (NP) expressed and purified in *E. coli* for four specific coronaviruses: SARSr-CoV (DQ071615, Bat SARS coronavirus Rp3, NP), HKU10-CoV (sample 3740, NP), HKU9-CoV (MG762674, BatCoV_HKU9-2202, NP), and MERS-CoV (JX869059, Human betacoronavirus 2c EMC/2012, NP). Micro-titer plates were coated with recombinant and purified NP (100ng/well); samples were tested at 1:20 dilution; and an anti-Human IgG-HRP conjugated monoclonal antibody (Kyab Biotech Co., Ltd, Wuhan, China) was used as the secondary antibody with different dilution ratios for different coronaviruses. 100 serum samples collected from healthy people in Wuhan were tested using this ELISA kit to set up the cutoff value, and positive test results were determined by the cut-off value in each run for each of the four coronaviruses, as the product of the mean of all serum samples' optical density (OD) values plus three standard deviations, and confirmed by Western blot test [30].

2.5 Questionnaire data analysis

Questionnaire data were entered into an Excel database with quality control for data cleaning and validation. The glmnet package in R version 3.6.0 was used to fit a least absolute shrinkage and selection operator (LASSO) regression to characterize associations between animal contact and SARI and/or ILI symptoms in the preceding 12 months [31, 32]. The bat coronavirus serology testing outcome was not analyzed in the LASSO due to low rates of sero-positivity. The LASSO regression is an adaptation of the generalized linear model (GLM) and was selected because it is effective at minimizing prediction error for datasets with many predictor

variables. The model identifies subsets of predictors that are associated with the outcome of interest by applying a shrinkage operation to regression coefficients and shrinking some coefficients to exactly 0. The LASSO is often utilized for its variable selection capabilities for sparse datasets including surveys and questionnaires. Demographic variables (age, gender, province, and income) were included in the model as independent and interaction terms in order to account for potential confounding. Because the LASSO does not generate confidence intervals, we repeated the model using bootstrapping to instead calculate bootstrap support, i.e., the proportion of times a predictor variable is selected into the model [33-36]

Chi-Square and fisher exact tests were also conducted to explore the associations between potential risk factors in local demographics, behaviors, and attitudes (independent variables) and bat CoV serological evidence (dependent variables), with effect size examined. However, due to the low positivity rate (9/1,497), the results were not robust and are not reported in this paper.

3. Results

From October 2015 to July 2017, a total of 1,596 residents from eight sites in Yunnan (n=761), Guangxi (n=412), and Guangdong (n=423) provinces were enrolled in this study. Of these, 1,585 participants completed the questionnaires and 11 participants withdrew from the questionnaire interview due to scheduling reasons. After the interviews, 1,497 participants provided biological samples for lab analysis.

3.1 Demographics

More female (62%) than male (38%) community members participated in this study. Most participants were adults over 45 years old (69%) and had been living in the community for more than 5 years (97%) with their family members (95%). A majority relied on a comparatively low

family annual per capita income less than 10,000 RMB (86%), which is below the national mean of per capita disposable income of rural households from 2015 to 2017 (11,422 - 13,432 RMB) [37]. Most participants (98%) had not received a college education and were making a living on crop production (76%). 9% of participants frequently traveled outside the county as migrant laborers. Some participants were working in sectors where frequent human-animal contacts occur, such as the animal production business (1.7%), wild animal trade (0.5%), slaughterhouses or abattoirs (0.5%), protected nature reserve rangers (0.4%) or in wildlife restaurants (0.3%). It was common for participants to have multiple part-time jobs as income sources (Table 1)

234

Variable		Total	
		N	Valid %
Gender (n= 1,574)	Female	968	61.5
	Male	605	38.4
	Other	1	0.1
Age (n=1,582)	Under 18 years	71	4.5
	18 to 44 years	420	26.5
	45 to 64 years	780	49.3
	Age 65 or older	311	19.7
Province (n=1,585)	Guang Dong	420	26.5
	Guang Xi	412	26.0
	Yun Nan	753	47.5
Residence time (n=1,568)	< 1 month	4	0.3
	1 month – 1 year	12	0.8
	1 year – 5 years	26	1.7
	> 5 years	1,526	97.3
Family annual PCI (n=1,565)	<1000 yuan	271	17.3
	1001-10000 yuan	1067	68.2
	>10000 yuan	227	14.5
Livelihood since last year	Extraction of minerals, gas, oil, timber (n=1,566)	5	0.3
	Crop production (n=1,569)	1,196	76.2
	Wildlife restaurant business (n=1,564)	5	0.3
	Wild/exotic animal trade/market business (n=1,566)	8	0.5
	Rancher/farmer animal production business (n=1,566)	27	1.7
	Meat processing, slaughterhouse, abattoir (n=1,567)	8	0.5
	Zoo/sanctuary animal health care (n=1,565)	1	0.1
	Protected area worker (n=1,567)	7	0.4
	Hunter/trapper/fisher (n=1,565)	3	0.2
	Forager/gatherer/non-timber forest product collector (n=1,566)	4	0.3
	Migrant laborer (n=1,567)	144	9.2
	Nurse, doctor, healer, community health worker (n=1567)	7	0.4
	Construction (n=1,564)	41	2.6
	Other (n=1,568)	293	18.7
Education (n=1,570)	None	428	27.3
	Primary School	632	40.3
	Secondary school/Polytechnic school	479	30.5
	College/university/professional	31	2.0
Live with family (n=1,564)	No	73	4.7
	Yes	1491	95.3

235 Table 1 Demographics of study participants. Total counts differ due to missing responses.

236 **3.2 Animal contact and exposure to bat coronaviruses**

237 Serological testing of serum samples from 1,497 local residents revealed that 9 individuals

238 (0.6%) in four study sites were positive for bat coronaviruses, indicating exposure at some point

239 in their life to bat origin SARSr-CoVs (n=7, Yunnan), HKU10 CoV (n=2, Guangxi), or other

coronaviruses that are phylogenetically closely related to these. All individuals who tested positive (male=6, female=3) were over 45 years old, and most (n=8) were making a living from crop production. None of those participants reported any symptoms in the 12 months preceding the interview.

Due to the low rate of sero-positivity, we did not obtain robust results from the statistical comparisons of animal-contact behavior by coronavirus outcome. Figure 1~~2~~ shows animal contact rates in the previous 12 months among the survey population (n= 1,585) and among seropositive individuals (n=9). Participants reported common contact with poultry and rodents/shrews, and most animal contact occurred in domestic settings through animal raising or food preparation activities.

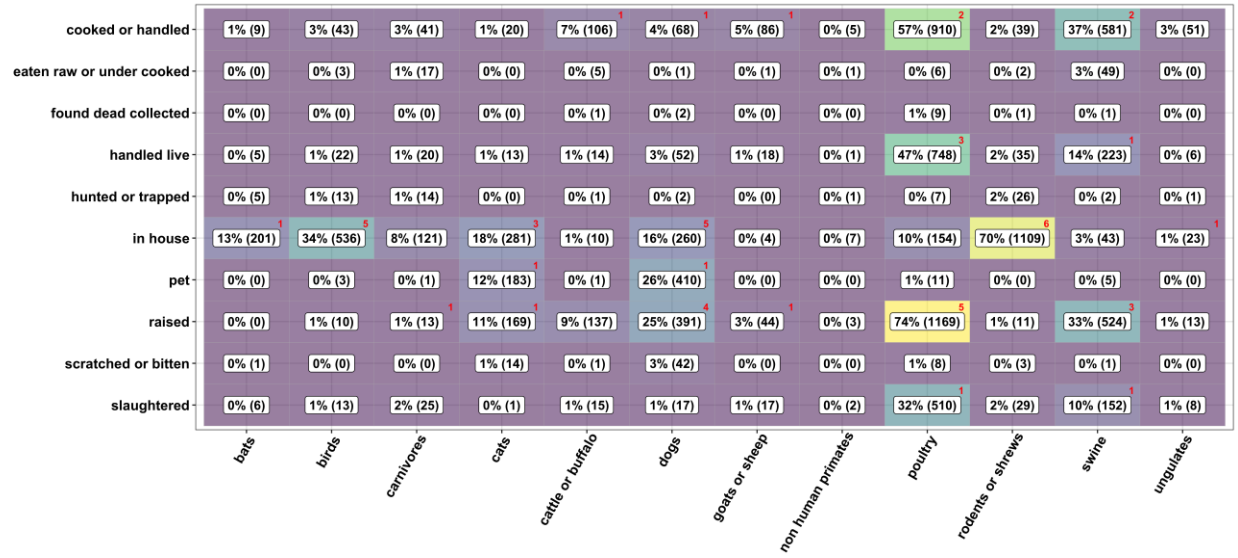

Figure 1~~2~~. Animal contact by taxa and activities. Values and shading represent the survey population; red numbers in the upper-right corners of the cells indicate the number of seropositive individuals with the given contact.

3.3 Self-report SARI/ILI symptoms and animal contact

Among the 1,585 participants who responded, 265 (17%) reported experiencing SARI (n = 73) and/or ILI (n = 227) symptoms in the last year. The LASSO regression showed that eating raw or undercooked carnivores in the preceding 12 months was the most salient predictor of self-reported SARI and/or ILI symptoms over the same time period (odds ratio [OR] = 1.6; bootstrap support = 0.67). Additional salient predictors were slaughtering poultry as a resident of Guangxi province (OR = 1.4; support = 0.68), having an income below 10,000 RMB as a resident of Guangxi province (OR = 1.3; support = 0.84), domestic contact with bats (OR = 1.3; support = 0.63) and domestic contact with rodents or shrews as a resident of Guangdong province (OR = 1.2; support = 0.63) (Figure 2~~3~~).

Some demographic variables were associated with self-reported SARI and/or ILI symptoms as either independent or interactive terms. For example, respondents aged 41 to 60 and residents of the YunNan province were less likely to report symptoms. Slaughtering poultry was positively associated with the outcome only in GuangXi residents, whereas the association was negative in GuangDong residents. Family income also showed interactions, with family income less than 10,000 RMB being positively associated with the outcome in respondents who raised poultry but negatively associated in respondents who cooked or handled poultry. Gender was not found to be salient in either direction.

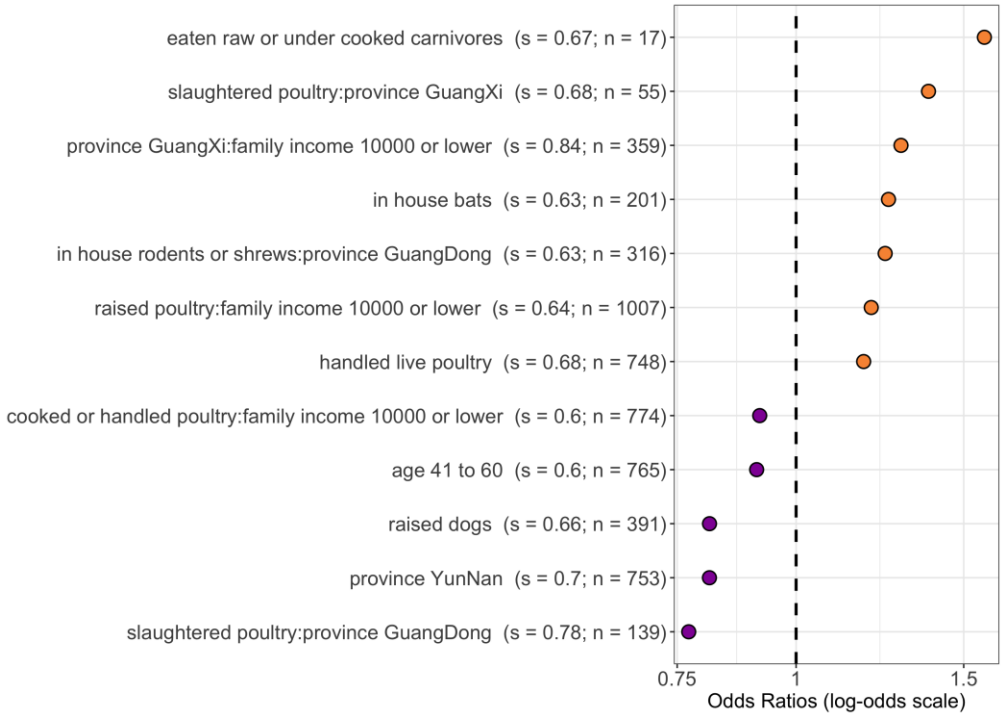

Figure 2~~3~~. Most salient predictors of self-reported ILI and/or SARI symptoms in the last year (s = bootstrap support; n = count positive out of 1,585 respondents). Bootstrap support values ≥ 0.6 are demonstrated here, meaning they were identified as associated with the outcome for 60% or more of the bootstrap iterations. Odds ratios > 1 (orange) are positively associated with the outcome, and odds ratios <1 (purple) are negatively associated with the outcome.

3.4 Attitudes towards zoonotic diseases emergence

When asked about animals and disease transmission, more than half of the study participants believed that animals could spread disease (n=871, 56%) and were worried about disease emergence from animals at wet markets (n=810, 52%). Of those worried about disease emergence, 46% (n=370) still purchased animals from wet markets in the past 12 months. Among all participants who purchased animals from wet markets in the past 12 months (n=502, 32%), some (n=194, 39%) took protection measures or strategies such as washing hands, purchasing live animals less often (n=153, 30%), or purchasing meat at supermarkets instead of

live animal markets (n=148, 29%). Very few participants considered wearing a mask (n=7, 1%) or gloves (n=7, 1%) while visiting the markets.

4. Discussion

We used a novel human surveillance approach to integrate serological and behavioral data to characterize associations between human-animal contact and zoonotic disease spillover risk in Southern China. This study provides the first serological evidence of bat-origin SARSr-CoVs and HKU10 CoV transmission to people and highlights potential spillover pathways through animal contact. Given the high diversity and recombination rate of bat coronaviruses, and close relationship of SARSr-CoVs to SARS-CoV, it is possible that exposure to these coronaviruses may lead to disease emergence in human populations. Continuous surveillance of both human and bat populations, as well as further pathogenesis studies of these viruses, are important to determine the extent of the disease risk.

Contact with animals was prevalent among the survey population. Raising poultry and having rodents/shrews in the house were the most common types of contact. Correspondingly, contact with poultry and rodents/shrews, as well as with carnivores, was identified in the LASSO regression as being associated with self-reported ILI and/or SARI symptoms, with results varying by income and province. It's important to note that the questionnaire used broad classification of the type of animals for these exposures due to the presumed variability in respondent's capacity to identify species or genera of wildlife. It is likely that the most significant exposure we identified (to carnivores) reflects animals as diverse as civets, porcupines, ferret badgers and animals that respondents identified as non-rodent and non-shrew. This study also assessed health risks from human interaction activities for each study participant in the survey based on their travel history and the health history of people who they lived with, to minimize the

possibility of human-to-human transmission of other pathogens causing ILI and/or SARI symptoms. We did not find evidence supporting a direct relationship between bat contact and bat coronavirus sero-positivity in the human population. However, there is frequent contact with domestic animals in these communities and it is known that other bat-origin viruses have been transmitted to humans via livestock (e.g. henipavirses and filoviruses) [38-41]. It is possible that these findings reflect indirect exposure to bat CoVs via these pathway, or fomites and future surveillance may benefit from including a wide range of livestock and peri-domestic animals in viral and serological studies to identify potential spillover pathways [42-45].

While it is known that bias can occur in self-reported illness data, this approach has been widely used in previous disease surveillance and risk factor studies [46-49]. It may be particularly useful as an early warning system during non-outbreak conditions to assess broad categories of high-risk within communities for further longitudinal surveillance. This may be particularly important in rural communities, where people have high levels of contact with domestic and wild animals but may not seek diagnosis or treatment in a timely fashion, slowing early detection and response.

While the majority of survey respondents believed that animals could spread disease and were worried about disease emergence from animals at wet markets, many did not take measures to protect themselves from exposure. Further work on what drives these local attitudes to risk may help in developing risk-mitigation behavior change programs. A number of affordable and readily adaptable measures could be targeted to these at-risk populations, including use gloves and masks while killing or butchering animals, and handwashing.

The low levels of sero-positivity found in the study could reflect a number of factors: 1) the rarity of spillover and bat-to-human transmission, as has been reported for other virus-host systems

[50-54]; 2) the use of a snowball technique for sample selection that could have biased the population sampled; 3) the limited diversity of CoVs that this study tested for; 4) the possibility that these infections cause high mortality rates and therefore the number of survivors and number of seropositive people is low, although this seems unlikely because the mortality rate from SARS was >10% during an outbreak that included hospital exposure and therefore likely high infectious doses [55, 56]; and 5) that antibodies to these viruses wane rapidly in humans. The latter hypothesis is supported by findings that antibodies to SARS decline rapidly (2-3 years) after illness [57]. Expanding this approach to a larger cohort of subjects, using a longitudinal (repeated sampling) approach, and targeting selection to people who are in the higher risk categories we have identified may provide a larger number of sero-positives and more critical information on what drives spillover risk. However, despite the small sample sizes, this study suggests that there are a substantial number of people in rural Southern China who are exposed to bat-origin viruses, and that this exposure is likely within normal practices for rural communities, rather than specific high risk groups (e.g. wet market workers). Considering the proven potential of some SARSr-CoVs currently circulating in bats in southern China, to infect human cells, cause clinical signs in humanized mouse models, and lead to infections that cannot be treated with monoclonal therapies effective against SARS-CoV [58-60], this represents a clear and present danger to our biosafety and public health. Further studies to determine the relationship between SARSr-CoV and HKU10-CoV exposure and illness in people may help elucidate this risk and provide critical mitigation strategies.

Author contribution: All authors read and approved the final manuscript. PD, ZS, MM, EH, SL, HY, and AC designed the study, developed the research tools, and obtained ethical approval; SL, HY, HH, and GZ implemented the field data collection; WZ and NW conducted the serological testing; HL, CZ, EM, EH, and NR contributed to the data management, analysis, and writing; and PT, MF, and PD edited and approved the final version.

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Competing interests: None declared

Ethical approval: This study was approved by Wuhan University School of Health Sciences Medical Ethics Committee; Institutional Review Board Administration of University of California, Davis (No. 804522-6); and Hummingbird IRB (No. 2014-23).

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Highlights

Scientific question

What are the behavioral risks in human-animal interactions that could lead to the emergence of bat coronaviruses in human population.

Evidence before this study

Bat borne coronaviruses have caused several emerging infectious disease outbreaks of global significance, including SARS. Novel SARS-related coronaviruses have been discovered in bat populations in South China, some of which have the capacity to infect human cells. Human-animal interactions are thought to be critical for the emergence of bat coronaviruses, however the specific interactions linked to animal-to-human spillover remain unknown.

New Findings

This study found serological evidence for bat-borne coronavirus transmission to people. Direct contact with bats was not identified as a risk factor. However, self-reported severe acute respiratory infection (SARI) and/or influenza-like illness (ILI) was linked to human interaction with other wildlife and livestock, suggesting that there may be other zoonotic exposures leading to clinical illness in these populations.

Significance of the study

Findings from this study suggested that an integrated biological and behavioral surveillance in healthy community settings can help identify potential zoonotic disease spillover events or target surveillance to at-risk populations. This approach represents a potential early-warning system that could be used under non-outbreak conditions to identify potential zoonotic emerging diseases prior to largescale outbreaks.