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Traceable surveillance and genetic diversity analysis of coronaviruses in poultry from China in 2019

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ABSTRACT

Coronavirus disease 2019 (COVID-19), caused by *Severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2), was first reported in Wuhan, China, and rapidly spread throughout the world. This newly emerging pathogen is highly transmittable and can cause fatal disease. More than 35 million cases have been confirmed, with a fatality rate of about 2.9% to October 9, 2020. However, the original and intermediate hosts of SARS-CoV-2 remain unknown. Here, 3160 poultry samples collected from 14 provinces of China between September and December 2019 were tested for SARS-CoV-2 infection. All the samples were SARS-CoV-2 negative, but 593 avian coronaviruses were detected, including 485 avian infectious bronchitis viruses, 72 duck coronaviruses, and 36 pigeon coronaviruses, with positivity rates of 15.35%, 2.28%, and 1.14%, respectively. Our surveillance demonstrates the diversity of avian coronaviruses in China, with higher prevalence rates in some regions. Furthermore, the possibility that SARS-CoV-2 originated from a known avian-origin coronavirus can be preliminarily ruled out. More surveillance of and research into avian coronaviruses are required to better understand the diversity, distribution, cross-species transmission, and clinical significance of these viruses.

1. Introduction

Coronaviruses (CoVs) belong to the order *Nidovirales*, the family *Coronaviridae*, and the subfamily *Orthocoronavirinae*, and are divided into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus*, and *Deltacoronavirus* (King et al., 2012). They can infect many animals, including humans (Cheng et al., 2017; Guan et al., 2003; Rota et al., 2003; Woo et al., 2006, 2009; Marra et al., 2003). Among these genera, *Alphacoronavirus* mainly infects humans, pigs, dogs, cats, bats, etc.; *Betacoronavirus* mainly infects humans, cattle, horses, pigs, mice, bats, and other mammals; *Gammacoronavirus* mainly infects domestic poultry; and *Deltacoronavirus* mainly infects wild birds and pigs. CoVs that can infect birds mainly belong to the genera *Gammacoronavirus* and *Deltacoronavirus* within the family *Coronaviridae* (King et al., 2011; Jordan et al., 2015). CoVs isolated from domestic poultry, such as *Avian infectious bronchitis virus* (IBV), belong to *Gammacoronavirus* (King et al., 2012). Among the avian CoVs, IBV is most harmful to the poultry industry and is listed as a notifiable disease by the World Organization for Animal Health (OIE). Duck coronavirus (DuCoV), goose coronavirus, and pigeon coronavirus (PiCoV) have also been detected and are very genetically different from the IBVs (Chen et al., 2013; Jonassen et al., 2005; Zhuang et al., 2020).

In December 2019, an outbreak of an unknown pneumonia occurred in Wuhan, China (Zhou et al., 2020). The pathogen was soon identified as an emerging coronavirus, designated *Severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2) by the International Committee on the Taxonomy of Viruses (ICTV), and the disease was designated 'coronavirus disease 2019′ (COVID-19) by the World Health Organization (WHO) (Chen et al., 2020). The clinical symptoms of COVID-19 predominantly include asymptomatic infection, mild-to-severe respiratory tract illness, and even death (Huang et al., 2020). Compared with *Severe acute respiratory syndrome coronavirus* (SARS-CoV), SARS-CoV-2 has a

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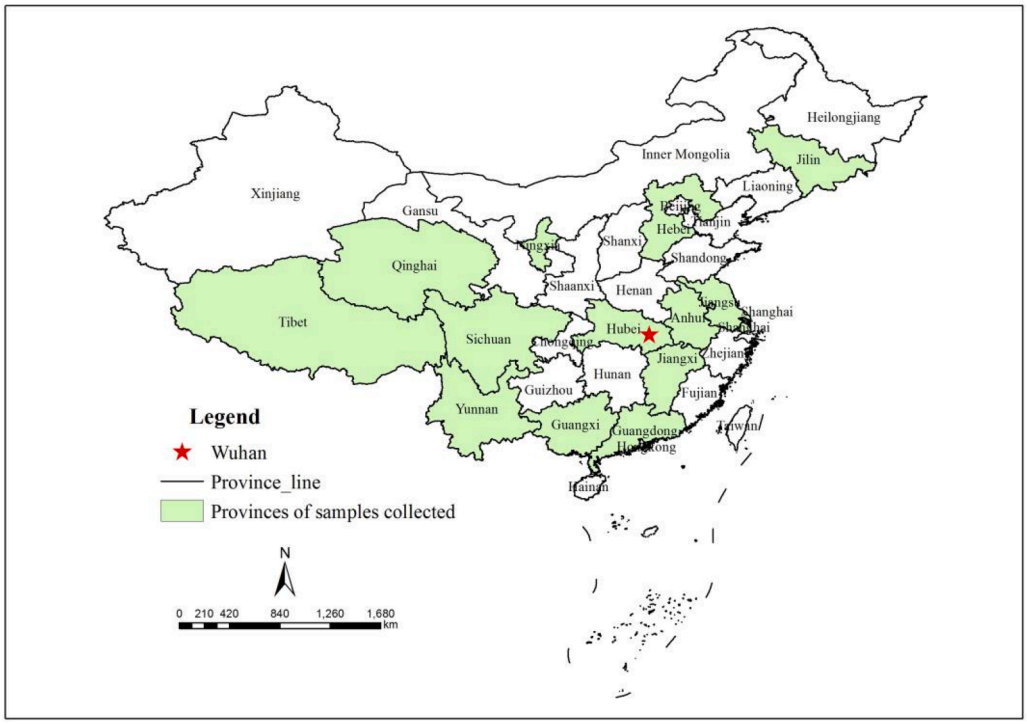

Fig. 1. Geographical distribution of the samples collected in 2019.

higher basic reproduction number, indicating greater transmissibility (Liu et al., 2020). Within a very short period of time, COVID-19 has quickly become a very serious threat to human health, travel, and commerce throughout the world (Stoecklin et al., 2020; Ghinai et al., 2020; Tuite et al., 2020).

The virus has been successfully isolated, but the pathogenic mechanism and effective vaccines are still undergoing extensive study. SARS-CoV-2 belongs to the genus *Betacoronavirus*, which also includes SARS-CoV and *Middle East respiratory syndrome coronavirus* (MERS-CoV). The natural host of the highly pathogenic SARS and MERS coronaviruses has been confirmed as bats, and recombination between bat and pangolin coronaviruses may represent to the origin of SARS-CoV-2 (Zhu et al., 2020). The transmission of SARS-CoV-2 from bats to humans is suspected to have occurred via direct contact between humans and intermediate host animals (Guo et al., 2020). However, it remains unclear which animals are the intermediate hosts of SARS-CoV-2. A previous study demonstrated that SARS-CoV can infect ferrets and cats (Martina et al., 2003), implying that these hosts might also be susceptible to SARS-CoV-2. Because poultry are in very close contact with humans, it is very important to identify any possible source of SARS-CoV-2 in avian species, especially in outbreak areas.

In this study, traceable surveillance was conducted to identify the possibility that SARS-CoV-2 originated in poultry. Tracheal and cloacal swabs collected during the routine surveillance of avian diseases in 2019 were tested with real-time reverse transcription (RT–PCR), as recommended by the Chinese Center for Disease Control and Prevention (CDC) to detect SARS-CoV-2. A universal RT–PCR was developed by our laboratory to analyze and molecularly characterize coronaviruses detected in poultry with DNA sequencing. Our study demonstrates the genetic diversity of the avian CoVs and that no SARS-CoV-2 infection was present in poultry in China in 2019.

2. Material and methods

2.1. Ethics statement

This study was conducted according to the animal welfare guidelines of the World Organization for Animal Health (Thiermann, 2015) and was approved by the Animal Welfare Committee of the China Animal Health and Epidemiology Center.

2.2. Sample collection

A total of 3160 swab samples were collected in September–December 2019 in 14 provinces of China (including Hubei), mainly for the routine surveillance of avian diseases such as avian influenza viruses (AIVs), Newcastle disease virus (NDV), etc. The swab samples were collected by taking smears from both the cloacal and oropharyngeal tracts of each bird, and stored in 1.5 mL of phosphate-buffered saline (pH 7.2) containing 10% glycerol. The RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, and stored at −80 °C.

2.3. Nucleic acid detection of SARS-CoV-2

All RNA samples were examined for SARS-CoV-2 nucleic acid using real-time RT–PCR based on the recommendations of the Chinese CDC (http://ivdc.chinacdc.cn /kyjz/202,001/t20200121_211,337.html). Briefly, two target genes, the nucleoprotein (N) gene and open reading frame (ORF) 1ab, were simultaneously amplified and detected with a real-time RT–PCR assay. The primers used were Target 1 (N): forward primer GGGGAACTTCTCCTGCTAGAAT, reverse primer CAGACATTTTGCTCTCAAGCTG, and probe 5′-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3′; and Target 2 (ORF1ab): forward primer CCCTGTGGGTTTTACACTTAA, reverse primer ACGATTGTGC ATCAGCTGA, and probe 5′-VIC—CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1–3′. The criteria for a confirmed diagnosis of SARS-CoV-2 were the positive amplification of both genes, N and ORF1ab.

2.4. Detection of COV nucleic acids with a conserved RT–PCR assay

A conserved RT–PCR assay and DNA sequencing were used to analyze the genetic characteristics of the CoVs circulating in the poultry. In brief, the stored RNAs described above were amplified with the

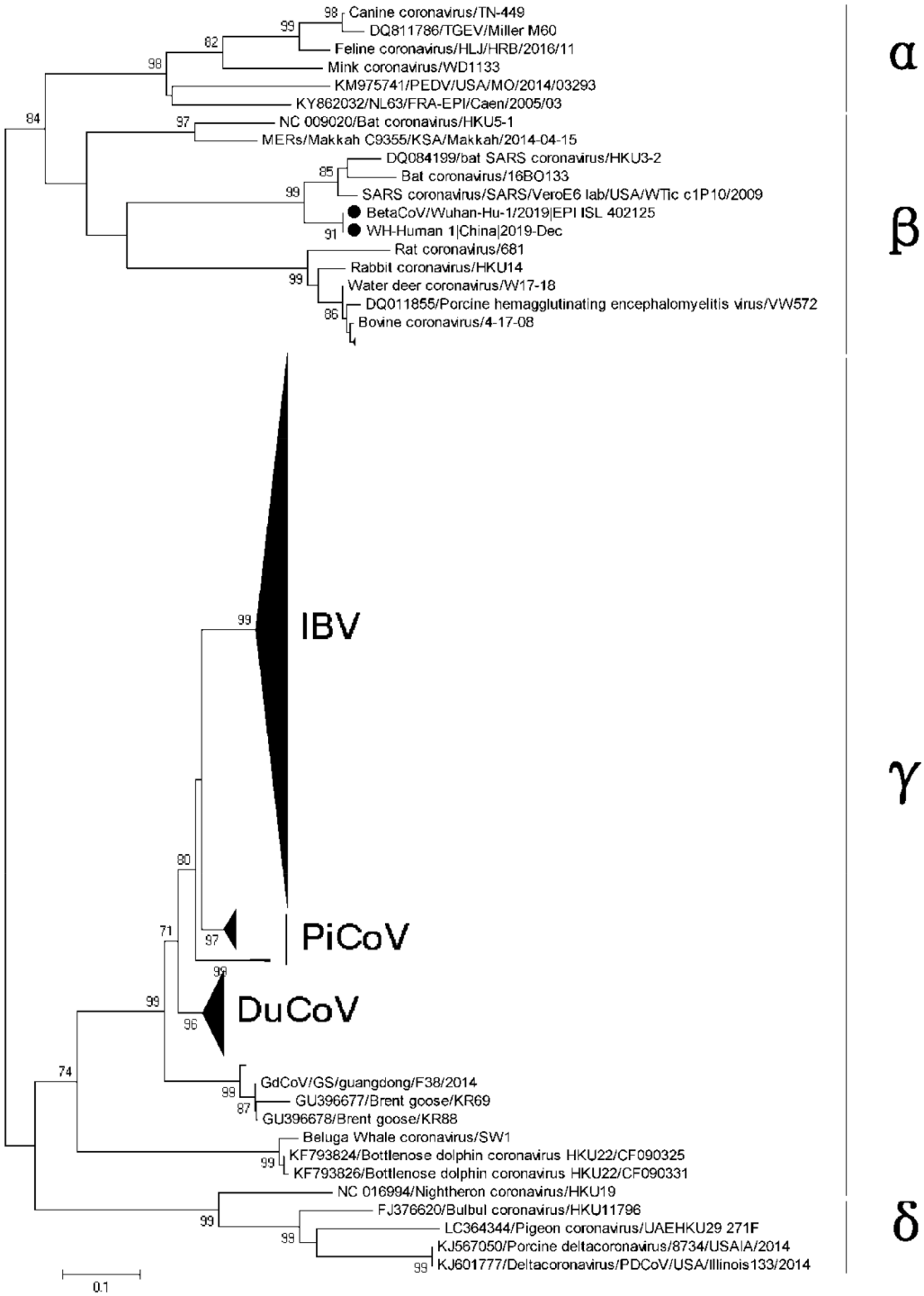

Fig. 2. Phylogenetic trees based on the RdRp gene sequences of coronaviruses. The trees were constructed using the model with the maximum likelihood (ML) method, gaps were handled by partial deletion and bootstrap values were calculated out of 1000 replicates. IBV: avian infectious bronchitis virus; DuCoV: duck coronavirus; PiCoV: pigeon coronavirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2. Two SARS-CoV-2 representative strains were marked with black solid circles (●), which belonged to the *Betacoronavirus* of *Coronaviridae*. A total of 593 avian coronaviruses were detected, including 485 IBVs, 72 DuCoVs and 36 PiCoVs, which all belonged to the *Gammacoronavirus*.

Takara One Step RT–PCR Kit (Takara), using a conserved RT–PCR assay designed in our laboratory, with primers 5′-GGTTGGGATTAYCC-WAARTGYGA-3′ (forward) and 5′-YTGTGAACAAAAYTCRTGWGGACC-3′ (reverse). The amplify region is 14,181–14,780 bp in the RDRP gene of the reference sequence (The GenBanK Accession number is NC_001451). The assay amplifies a 600-bp nucleotide region in the viral ORF1ab, and this assay has been shown to detect the main CoVs circulating in animals, including pigs, chickens, ducks, geese, and pigeons. The RT–PCR was performed in a 25 μl reaction system with incubation at 50 °C for 30 min, followed by denaturation at 94 °C for 5 min, and 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. The PCR products were purified with an agarose gel DNA extraction kit (Sangon, Shanghai, China), and sequenced directly with the ABI 3730xl DNA Analyzer. The sequences were used in a subsequent phylogenetic analysis.

2.5. Phylogenetic analysis

The sequences were aligned and the substitution models and phylogenetic relationships were calculated with the software package MEGA 6.0 (Hall, 2013; Tamura et al., 2013). The phylogenetic relationships of the sequences were calculated with the maximum likelihood (ML) model, which is assumed to best describe the substitution pattern. The gaps were handled by partial deletion, and bootstrap values

Table 1
Positive rates (%) of three lineages of CoVs in the samples collected from four host species in 2019.

	Chickens	Ducks	Geese	Pigeons
IBV	98.97%(480/485)	0.82%(4/485)	0.21% (1/485)	
DuCoV	12.50%(9/72)	87.50% (63/72)		
PiCoV	22.22%(8/36)			77.78% (28/36)

Table 2
Number of samples and positive rate of three lineages of CoVs in each province.

Province	Number of samples	DuCoV	IBV	PiCoV	Total	Rate
Anhui	308	5	44	5	54	17.53%
Guangdong	267	9	12		21	7.87%
Guangxi	256	19	34	3	56	21.88%
Hebei	256	6	42		48	18.75%
Hubei	326	18	100		118	36.20%
Jilin	79		3	4	7	8.86%
Jiangsu	130	3	9	4	16	12.31%
Jiangxi	305	11	86	5	102	33.44%
Ningxia	177		12	2	14	7.91%
Qinghai	105		29		29	27.62%
Shanghai	160		16	7	23	14.38%
Sichuan	317		32	3	35	11.04%
Xizang	222		52	3	55	24.77%
Yunnan	252	1	14		15	5.95%
Total	3160	72	485	36	593	18.77%

were calculated from 1000 replications (Li, 1997).

3. Results

3.1. Distribution of samples detected in 2019

A total of 3160 swab samples were collected in 14 provinces of China in 2019 (Anhui, Guangdong, Guangxi, Hebei, Hubei, Jiangsu, Jiangxi, Jilin, Ningxia, Qinghai, Shanghai, Sichuan, Xizang, and Yunnan). The geographic distribution of the samples detected in this study is shown in **Fig. 1**. The samples included 2238 chicken samples, 669 duck samples, 184 pigeon samples, 59 geese samples, and 15 other avian samples (10 from wild geese, five from partridges), which originated in 48 retail markets, 18 wholesale markets, four slaughterhouses, and 18 poultry farms.

3.2. Surveillance of SARS-CoV-2 in poultry

The 3160 samples were tested with real-time RT–PCR, as recommended by China CDC, to detect SARS-CoV-2 nucleic acid, and all samples were negative for SARS-CoV-2.

3.3. Surveillance of CoVs circulating in poultry

Of the 3160 samples tested, 593 CoV-positive samples were detected with the conserved RT–PCR assay. The viral amplicons (ORF1ab fragments) were sequenced. The GenBank accession numbers for these 593 CoV sequences are MT944357–MT944949.

3.4. Phylogenetic and diversity analyses of CoVs circulating in poultry

A phylogenetic analysis revealed that the 593 CoVs detected in poultry clustered into three lineages, corresponding to IBV ($n = 485$), DuCoV ($n = 72$), and PiCoV ($n = 36$) (**Fig. 2**). Emerging SARS-CoV-2 belongs to the genus *Betacoronavirus* in the family *Coronaviridae*, and the 593 avian CoVs detected in this study belonged to the genus *Gammacoronavirus*. SARS-CoV-2 was highly genetically distant from these avian CoVs.

The positivity rates for the IBVs, DuCoVs, and PiCoVs were 15.35%, 2.28%, and 1.14%, respectively (**Table 1**). Our data suggest that IBVs mainly circulate in chickens, DuCoVs mainly circulate in ducks, and PiCoVs mainly circulate in pigeons. The number of positive samples and the rates of detection of the three lineages of CoVs in each province are shown in **Table 2**. IBV was detected in all provinces investigated in this study, and DuCoV and PiCoV were detected in most provinces. The province with the highest rate of CoVs was Hubei (36.20%), and that with the lowest was Yunnan (5.95%).

The numbers of due-species infections and cross-species infections in the samples collected from live poultry markets (LPMs) are shown in **Table 3**. Cross-species infections of CoVs (i.e., IBV infections in birds other than chickens, DuCoV infections in birds other than ducks, and PiCoV infections in birds other than pigeons) were identified in 23 of the 3160 samples from four sites (six involving IBVs, nine involving DuCoVs, and eight involving PiCoVs).

4. Discussion

So far, seven different human CoVs have been identified: SARS-CoV, MERS-CoV, human coronavirus NL63 (HCoV-NL63), HCoV-229E, HCoV-OC43, HCoV-HKU1, and SARS-CoV-2. Bats are deemed to be the natural hosts of SARS-CoV, MERS-CoV, HCoV-NL63, and HCoV-229E, and rodents may be the natural hosts of HCoV-OC43 and HCoV-HKU1 (Khan et al., 2020). The intermediate hosts of SARS-CoV, MERS-CoV, HCoV-229E, and HCoV-OC43 are palm civets, dromedary camels, alpacas, and cattle, respectively. However, the intermediate host (s) of SARS-CoV-2 remains unknown.

Because SARS-CoV-2 is genetically close to SARS-CoV, it has been suggested that bats are its natural host (Phan, 2020). A study showed that the common ancestor of SARS-CoV-2 and SARS-CoV was similar to the bat coronavirus HKU9–1 (Zhou et al., 2020), while Andersen et al. documented the possible natural origin of SARS-CoV-2 from BatCoV RaTG13 (Andersen et al., 2020). The pangolin is suspected to be a direct animal source of SARS-CoV-2 in humans because SARS-CoV-2-related CoVs have been isolated from Malayan pangolins and shared 97.4% similarity with SARS-CoV-2 in the receptor-binding domain in the viral S gene (Zhang et al., 2020).

Studies have shown that SARS-CoV-2 possibly originated from recombination between bat and pangolin coronaviruses (Zhu et al., 2020). To date, findings from experimental infection studies have suggested that poultry and pigs are not susceptible to SARS-CoV-2 infection (Shi et al., 2020). Of the animal species investigated, cats are the species most susceptible to SARS-CoV-2, and SARS-CoV-2 can be transmitted

Table 3
Numbers of due-species infections and cross-species infections in the samples collected from LPMs.

	Due-species infections			Cross-species infections			Rate
	IBV	DuCoV	PiCoV	IBV	DuCoV	PiCoV	
retail markets($n = 1280$)	97	31	13	3	2	1	11.48%(147/1280)
wholesale markets($n = 1495$)	320	32	15	3	1	1	24.82%(371/1495)
slaughterhouse($n = 245$)	57				6		25.71%(63/245)
Poultry farms ($n = 140$)	6					6	8.57%(12/140)

between cats by respiratory droplets (Jiang et al., 2012). In the laboratory setting, ferrets are susceptible to infection. The susceptibility of minks was documented by a report from the Netherlands of an outbreak of SARS-CoV-2 infection in farmed minks (Oreshkova et al., 2020). Golden Syrian hamsters, as well as cynomolgus and rhesus macaques, can be consistently infected with SARS-CoV-2 and may show clinical signs. Dogs also appear to be susceptible to infection, but appear to be less affected than ferrets or cats. Both virological and serological testing found evidence for natural SARS-CoV-2 infections in two dogs from households with human cases of COVID-19 in Hong Kong (Sit et al., 2020). Also, lions, pumas, and tigers at zoos, all belonging to the same family as cats (Felidae), were infected by asymptomatic and symptomatic patients in two countries (USA and South Africa), with SARS-CoV-2 detected in their fecal samples. In addition to pet and zoo animals, 138 infected mink farms were reported in 11 countries, with about 90% in Europe and 10% in North America (Jia et al., 2021).

Our data show that all the samples collected from domestic poultry in 14 provinces of China (including Hubei) in 2019 were negative for SARS-CoV-2. Shi et al. also found that poultry, such as chickens and ducks, were not susceptible to SARS-CoV-2 (Shi et al., 2020), which is consistent with our results.

We also conducted a large-scale surveillance of avian CoVs in 3160 samples using a conserved RT–PCR assay, which detected avian CoVs such as IBVs, DuCoVs, and PiCoVs. Our results demonstrate that IBVs, DuCoVs, and PiCoVs belong to distinct lineages, although they all belong to the genus *Gammacoronavirus* in the family *Coronaviridae*, whereas SARS-CoV-2 belongs to the genus *Betacoronavirus*. SARS-CoV-2 was highly genetically distant from the avian CoVs.

IBV was detected in all the provinces investigated in this study, and DuCoV and PiCoV were detected in most provinces. IBV presents a risk to the poultry industry and is listed as a notifiable disease by the OIE, but the pathogenicity of DuCoV and PiCoV in poultry is still unclear, and warrants further research. The positive rates of IBV, DuCoV, and PiCoV infections were 15.35%, 2.28%, and 1.14%, respectively. These CoVs are also highly prevalent in LPMs, and our results also suggest their high prevalence in slaughterhouses. Therefore, the sites of poultry breeding, marketing, and slaughter (poultry farms, LPMs, and slaughterhouses) probably play important roles in the circulation of CoVs in poultry, as they do in the circulation of AIVs (Jiang et al., 2012).

Coronaviruses clearly have the capacity to jump species boundaries and adapt to new hosts, making it straightforward to predict that more will emerge in the future, although quite why coronaviruses possess this capacity in comparison to some other RNA viruses is unclear. Critically, the surveillance of animal coronaviruses should include animals other than bats, as the role of intermediate hosts is likely of major importance, providing a more direct pathway for the virus to emerge in humans (Zhang et al., 2020). While our intimate relationship with the animal world means we cannot build impregnable barriers, stronger action against the illegal wildlife trade and removing all mammalian (and perhaps avian) wildlife from wet markets will provide an important buffer.

In conclusion, the possibility that SARS-CoV-2 originated from a known avian-origin CoV can be preliminarily ruled out according to our analysis. However, the continuous surveillance of animal-origin CoVs should be increased to better understand the diversity, distribution, cross-species transmission, and clinical significance of CoVs in nature.

Author statement

Yang Li and Kaicheng Wang contributed to the design of the experimental work and the bioinformatics analysis. Hualei Liu revised the manuscript. Qingye Zhuang and Su-chun Wang contributed to the experimental work and drafting the manuscript. Baoxu Huang, Lijian Jiang, Wenming Jiang, Cheng Peng, Nan Jiang, Fuyou Zhang and Xiaohui Yu revised the manuscript. Suchun Wang, Liping Yuan and Guangyu Hou performed the bioinformatics analysis. Shuo Liu, Jingjing Wang, Jianmin Yu, Jinping Li and Chenglong Zhao performed experimental work. All authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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