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 worldwide. This new emerging pathogen is highly transmittable and can cause fatal disease. More than 35 million cases have been confirmed and the fatality was about 2.9% up to October 9 2020. However, the original and intermediate hosts of SARS-CoV-2 remain unknown. Here, a total of 3160 poultry samples collected from 14 provinces between September and December 2019 in China were tested for the purpose of traceable surveillance for SARS-CoV-2 infection. The results indicated that all samples were SARS-CoV-2 negative, and a total of 593 avian coronaviruses were detected, including 485 avian infectious bronchitis viruses, 72 duck coronaviruses and 36 pigeon coronaviruses. The positive rates of avian infectious bronchitis virus, duck coronavirus, and pigeon coronavirus were 15.35%, 2.28% and 1.14%, respectively. Our surveillance demonstrated the diversities of avian coronaviruses in China, and higher prevalence were also recognized in some regions. The possibility of SARS-CoV-2 originating from the known avian-origin coronaviruses can be preliminarily ruled out. More surveillance and research on avian coronaviruses should be strengthened for better understanding the diversity, distribution, cross-species transmission and clinical significance of these viruses.

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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 worldwide. This new emerging pathogen is highly transmittable and can cause fatal disease. More than 35 million cases have been confirmed and the fatality was about 2.9% up to October 9 2020. However, the original and intermediate hosts of SARS-CoV-2 remain unknown. Here, a total of 3160 poultry samples collected from 14 provinces between September and December 2019 in China were tested for the purpose of traceable surveillance for SARS-CoV-2 infection. The results indicated that all samples were SARS-CoV-2 negative, and a total of 593 avian coronaviruses were detected, including 485 avian infectious bronchitis viruses, 72 duck coronaviruses and 36 pigeon coronaviruses. The positive rates of avian infectious bronchitis virus, duck coronavirus, and pigeon coronavirus were 15.35%, 2.28% and 1.14%, respectively. Our surveillance demonstrated the diversities of avian coronaviruses in China, and higher prevalence were also recognized in some regions. The possibility of SARS-CoV-2 originating from the known avian-origin coronaviruses can be preliminarily ruled out. More surveillance and research on avian coronaviruses should be strengthened for better understanding the diversity, distribution, cross-species transmission and clinical significance of these viruses.

Key words: SARS-CoV-2; Avian-origin Coronaviruses; Surveillance; Diversity; Cross-species Transmission

1 INTRODUCTION

Coronaviruses (CoVs) belong to *Nidovirales* , family*Coronaviridae, Orthocoronavirinae* , which are divided into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* and*Deltacoronavirus* (King et al., 2012), and can infect many animals including humans (Cheng et al.,2017; Guan et al., 2003; Rota et al., 2003;Woo et al., 2006; Woo et al., 2009; Marra et al., 2003). Among them, *Alphacoronavirus* mainly infects humans and 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 are mainly originated from the *Gammacoronavirus* and *Deltacoronavirus*of Coronaviridae (King et al., 2011; Jordan et al., 2015). CoVs isolated from domestic poultry such as avian infectious bronchitis virus (IBV) are belonged to *Gammacoronavirus* (King et al., 2012). In the avian CoVs, IBV is more harmful to the poultry industry and were listed as notifiable disease by World Organization for Animal Health (OIE). In addition, duck coronavirus (DuCoV), goose coronavirus and pigeon coronavirus (PiCoV) were also detected and showed highly genetic differences compared with IBVs (Chen et al., 2013; Jonassen et al., 2005; Zhuang et al., 2020).

In December, 2019, an outbreak of unknown pneumonia occurred in Wuhan, China (Zhou et al., 2020). The pathogen was soon identified to be an new emerging coronavirus, named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by International Committee on Taxonomy of Viruses (ICTV), and the disease was designated coronavirus disease 2019 (COVID-19) by World Health Organization (WHO) (Zhou et al., 2020; Chen et al., 2020). The clinical symptoms of COVID-19 mainly 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 the higher basic reproduction number, representing more 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 in the worldwide (Stoecklin et al., 2020; Ghinai et al., 2020; Tuite et al., 2020).

The viruses have been successfully isolated, but the pathogenesis mechanisms and effective vaccines are undergoing extensively study. SARS-CoV-2 belongs to the *Betacoronavirus* , in which SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV) are also included in the same group. The natural host of highly pathogenic SARS and MERS coronaviruses was confirmed as bats, and bats are also thought to be the natural hosts for SARS-CoV-2 based upon genomic sequence analysis (Wang et al., 2020). The transmission of SARS-CoV-2 from bats to humans was suspected to via the direct contact between humans and intermediate host animals (Guo et al., 2020). However, it remains unclear which animals were the intermediate host of SARS-CoV-2. Previous report demonstrated that SARS-CoV can infect ferrets and cats (Martina et al., 2003), implying that these host might be also susceptible to SARS-CoV-2. Since poultry

have very close contact with humans, it is very important to identify the possible source of SARS-CoV-2 from avian, especially in the outbreak areas.

In this study, traceable surveillance was conducted to identify the possibility of SRAS-CoV-2 originated from poultry. Tracheal and cloacal swabs collected for routine surveillance of avian diseases in 2019 were tested by real-time reverse transcription-polymerase chain reaction (RT-PCR)recommended by Chinese Center for Disease Control and Prevention (CDC) to detect the SARS-CoV-2 and universal RT-PCR developed by our laboratory to analysis the molecular characterization of coronaviruses detected from poultry by sequencing respectively. Our study indicated the genetic diversities of avian CoVs and no infection of SARS-CoV-2 in poultry of China in 2019.

2 MATERIALS 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 (CAHEC).

2.2 Sample collection

A total of 3160 swab samples were collected between September to December of 2019 in 14 provinces of China (including Hubei), mainly for routine surveillance on avian diseases, such as avian influenza viruses (AIV), Newcastle disease viruses (NDV), etc. The swab samples were collected through taking smears at both cloacal and oropharyngeal tracts of a bird, and stored in 1.5 mL phosphate buffered saline (PBS, pH 7.2) containing 10% glycerol. The RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions, and stored at -80°C.

2.3 Nucleic Acid Detection of SARS-CoV-2

All RNA samples were identified with nucleic acid detection of SARS-CoV-2 using real-time RT-PCR based on the recommendation by the China CDC (http://ivdc.chinacdc.cn /kyjz/202001/t20200121_211337.html). Briefly, two target genes, including nucleoprotein (N) gene and open reading frame (ORF) 1ab, were simultaneously amplified and tested during the real-time RT-PCR assay. Target 1 (N): forward primer GGGGAACTTCTCCTGCTAGAAT; reverse primer CAGACATTTTGCTCTCAAGCTG; and the probe 5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3'. Target 2 (ORF1ab): forward primer CCCTGTGGGTTTTACACTTAA; reverse primer ACGATTGTGC ATCAGCTGA; and the probe 5'-VIC-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1–3'. The criteria for confirmed diagnosis of SARS-CoV-2 were that both two genes amplified to be positive for N and ORF 1ab gene.

2.4 Nucleic Acid Detection of CoVs using a conserved RT-PCR assay

A conserved RT-PCR and sequencing was used to analysis the genetic characteristics of CoVs circulating in poultry. In brief, the RNA stored above were amplified with the Takara One Step RT-PCR Kit (Takara), using a conserved RT-PCR assay designed by our lab with the primers 5'-GGTTGGGATTAYCCWAARTGYGA-3' (upper) and 5'-YTGTGAACAAAAYTCRTGWGGACC-3'(down). The assay amplifies a 550 bp-nucleotide region in the viral 1ab gene, and this assay has been proved to detect main CoV circulating in animals, including pigs, chickens, ducks, geese, pigeons, etc. The RT-PCR detection was performed in a 25-μl reaction system with incubation at 50 for 30 min and denaturation at 94 for 5 min, followed by 30 cycles at 94 for 30 s, 50 for 30 s and 72 for 45 s. RT-PCR products were purified with an agarose gel DNA extraction kit (Sangon, Shanghai, China), and sequenced directly using the ABI 3730xl DNA Analyzer for the following phylogenetic analysis.

2.5 Phylogenetic analysis

Sequences were aligned and the substitution models and phylogenetic relationships were calculated using the software package MEGA 6.0 (Hall 2013; Tamura et al., 2013). Phylogenetic relationships of sequences were

calculated using the model with the maximum likelihood (ML) method, which was assumed to describe the substitution pattern the best. Gaps were handled by partial deletion and bootstrap values were calculated out of 1000 replicates (Li 1997).

3 RESULTS

3.1 The distribution of the 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), and the geographical distribution of the samples detected in this study were shown in **Figure 1** . All samples including 2238 chicken samples, 669 duck samples, 184 pigeon samples, 59 geese samples, 15 other avian samples (10 from wild geese, 5 from partridges)and originated from 48 retail markets, 18 wholesale markets, 4 slaughterhouse, 18 poultry farms.

3.2 Surveillance of SARS-CoV-2 in poultry

A total of 3160 samples were tested by real-time RT-PCR recommended by China CDC to detect SARS-CoV-2 nucleic acid, and all samples were proved to be negative for SARS-CoV-2.

3.3 Surveillance of CoVs circulating in poultry

A total of 593 CoV positive samples were identified through the conserved RT-PCR and sequenced from the 3160 samples. The accession numbers in GenBank for the sequences of the 593 CoVs are MT944357-MT944949.

3.4 Phylogenetic and diversity analysis of CoVs circulating in poultry

Phylogenetic analysis revealed that the 593 CoVs detected from poultry could be classified into three lineages corresponding to IBV (n=485), DuCoV (n=72), and PiCoV (n=36), respectively (**Figure 2**). The emerging SARS-CoV-2 belongs to the *Betacoronavirus* of*Coronaviridae* , and the detected 593 avian CoVs in this study belongs to the *Gammacoronavirus* . SARS-CoV-2 showed higher genetic distance compared with avian CoV.

The positive rates of IBVs, DuCoVs, and PiCoVs were 15.35%, 2.28% and 1.14%, respectively (**Table1).** Besides, these data suggested that IBVs mainly circulate in chickens, DuCoVs mainly circulate in ducks, and PiCoVs mainly circulate in pigeons. The number of samples and positive rate of three lineages of CoVs in each province were shown in**Table 2.** The results showed that IBV was detected in all provinces investigated in this study, with DuCoV and PiCoV were detected in most provinces. The province with the highest positive rate of CoVs was Hubei (36.20%), with the lowest was Yunnan (5.95%).

The numbers of due-species infections and cross-species infections in the samples collected from living poultry markets (LPMs) were shown in**Table 3.** The results showed that cross-species infections of the CoVs, namely that infections of IBVs in birds other than chickens, infections of DuCoVs in birds other than ducks, and infections of PiCoVs in birds other than pigeons were identified in 23 of the 3160 samples from four sites (6 with IBVs, 9 with DuCoVs and 8 with PiCoVs).

4 DISCUSSION

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

Since SARS-CoV-2 is genetically close to SARS-CoV, it has been proposed that bats could be the natural host (Phan 2020). Recent study shows that the common ancestor of SARS-CoV-2 and SARS-CoV is similar to bat coronavirus HKU9-1 (Zhou et al., 2020). Pangolins was suspected to be direct animal source of

SARS-CoV-2 for humans since the SARS-CoV-2 related CoVs were isolated from Malayan pangolins which shared 97.4% similarity with SARS-CoV-2 in virus receptor binding domain in S gene (Zhang et al., 2020). To date, findings from experimental infection studies suggest that poultry and pigs are not susceptible to SARS-CoV-2 infection (Shi et al., 2020). Of the animal species investigated, cats are the most susceptible species for SARS-CoV-2, which can transmit between cats via respiratory droplets (Jiang et al., 2012). In the laboratory setting ferrets were susceptible to infection. The susceptibility of minks was documented by a report from the Netherlands on 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 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 infection in two dogs from households with human cases of COVID-19 in Hong Kong (Sit et al., 2020).

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

In addition, we conducted a large-scale surveillance of avian CoV for the 3160 samples using a conserved RT-PCR assay. The conserved RT-PCR assay in this study detected avian CoVs, including IBVs, DuCoVs and PiCoVs. Our results demonstrated that IBVs, DuCoVs, and PiCoVs belonged to distinct lineages, even though they all belonged to the*Gammacoronavirus* , with the emerging SARS-CoV-2 belongs to the*Betacoronavirus* of *Coronaviridae.* SARS-CoV-2 showed higher genetic distance compared with the avian CoV.

Our results also suggest that IBV was detected in all provinces investigated in this study, with DuCoV and PiCoV were detected in most provinces. IBV is harmful to the poultry industry and were listed as notifiable disease by OIE, but the pathogenicity of DuCoV and PiCoV to poultry is still unclear, which needs further research. Besides, the positive rates of IBVs, DuCoVs, and PiCoVs were 15.35%, 2.28% and 1.14%, respectively. And these CoVs are of high prevalence in LPMs, and meanwhile also suggest high prevalence in slaughterhouses. Therefore, each links from breeding, marketing to slaughter (poultry farms, LPMs, and slaughterhouses) may likely play an important role in the circulation of CoVs in poultry, as they do in the circulation of AIVs(Jiang et al., 2012).

In conclusion, the possibility of SARS-CoV-2 originating from the known avian-origin CoVs can be preliminarily ruled out according to the above analysis. Moreover, continuous surveillance on animal-origin coronaviruses should be enhanced for better understanding the diversity, distribution, cross-species transmission and clinical significance of CoVs in nature.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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