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Surveillance and taxonomic analysis of the coronavirus dominant in pigeons in China

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Abstract

Coronaviruses (CoVs) are found in humans and a wide variety of wild and domestic animals, and of substantial impact on human and animal health. In poultry, the genetic diversity, evolution, distribution and taxonomy of CoVs dominant in birds other than chickens remain enigmatic. In our previous study, we proposed that the CoVs dominant (i.e. mainly circulating) in ducks (DdCoVs) should represent a novel species, which was different from the one represented by the CoVs dominant in chickens (CdCoVs). In this study, we conducted a large-scale surveillance of CoVs in chickens, ducks, geese, pigeons and other birds (quails, sparrows and partridges) using a conserved RT-PCR assay. The surveillance demonstrated that CdCoVs, DdCoVs and the CoVs dominant in pigeons (PdCoVs) belong to different lineages, and they are all prevalent in live poultry markets and the backyard flocks in some regions of China. We further sequenced seven *Coronaviridae*-wide conserved domains in their replicase polyprotein pp1ab of seven PdCoVs and found that the genetic distances in these domains between PdCoVs and DdCoVs or CdCoVs are large enough to separate PdCoVs into a novel species, which were different from the ones represented by DdCoVs or CdCoVs within the genus *Gammacoronavirus*, per the species demarcation criterion of International Committee on Taxonomy of Viruses. This report shed novel insight into the genetic diversity, distribution, evolution and taxonomy of avian CoVs.

KEYWORDS

coronavirus, genetic distance, lineage, pigeon, sequence, species, surveillance, taxonomy

1 | INTRODUCTION

Coronaviruses (CoVs) are found in humans and a wide variety of wild and domestic animals, causing mild or severe respiratory, enteric, hepatic and neurological diseases, and resulting in substantial impact on human and animal health (Cheng, Lau, Woo, & Yuen, 2007; Guan et al., 2003; Marra et al., 2003; Peiris et al., 2003; Rota et al., 2003; Rota et al., 2003; Snijder et al., 2003; Woo et al., 2004, 2009). The severe acute respiratory syndrome (SARS) CoV caused an epidemic in 2003 which resulted in 775 human deaths (Woo et al., 2004). The novel coronavirus SARS-CoV-2 emerging in Wuhan, China, in 2019 has spread to dozens of countries and caused thousands of human deaths till the present (WHO, 2020).

CoVs belong to the subfamily *Orthocoronavirinae* in the family *Coronaviridae*. *Orthocoronavirinae* covers four genera, namely *Alpha*-, *Beta*-, *Gamma*- and *Deltacoronavirus* (de Groot et al., 2012). SARS CoV and MERS CoV belong to the genus *Betacoronavirus*. All the CoVs detected from domestic fowls and some CoVs detected from wild birds belong to the genus *Gammacoronavirus*, while some other CoVs detected from wild birds have constituted the

genus *Deltacoronavirus* (de Groot et al., 2012; Jordan, Hilt, Poulson, Stallknecht, & Jackwood, 2015).

The genus *Gammacoronavirus* covers two subgenera, *Igacovirus* which is represented by the species of *Avian coronavirus* including infectious bronchitis virus (IBV) circulating in chickens (Chen et al., 2013; de Groot et al., 2012) and *Cegacovirus* which is represented by CoVs isolated from whales and dolphins (Mihindukulasuriya, Wu, St Leger, Nordhausen, & Wang, 2008; Woo et al., 2014). Infectious bronchitis virus circulates worldwide and causes acute and highly contagious respiratory diseases in chickens of all ages and diminish egg production in hens (Chen et al., 2013; Cook, Jackwood, & Jones, 2012). Additionally, some CoVs detected from turkeys, peafowls and other birds were highly homologous to IBV (Cook et al., 2012; Day et al., 2014; Liu et al., 2005).

Recombination and mutation are frequent in the genomes of CoVs (Liu et al., 2014). This may facilitate rapid adaptation of the viruses to new hosts and ecological niches (Lau et al., 2016; Moreno et al., 2017). Some CoVs distinct from IBVs and mainly circulating in birds other than chickens have been identified (Chen et al., 2013; de Groot et al., 2012). In 2013, we found that CoVs dominant (i.e. mainly circulating) in ducks were phylogenetically distinct from IBVs and may represent a potential novel species within the genus *Gammacoronavirus*, as indicated by the sequences of three regions in the viral 1ab gene (Chen et al., 2013). Then, we sequenced the genomic sequence of the duck-dominant CoV and conducted a surveillance of CoVs in chickens and ducks and found solid evidences to support that the duck-dominant CoVs are distinct from common IBVs and should represent a novel species in the genus *Gammacoronavirus* (Zhuang et al., 2015).

The genetic distances in the following seven *Coronaviridae*-wide conserved domains in the viral replicase polyprotein pp1ab: ADP-ribose-1″-phosphatase (ADRP) in nsp3, nsp5, nsp12, nsp13, nsp14, nsp15 and nsp16 are crucial for newly identified CoVs to be assigned to a species within *Coronaviridae* according to the ninth report of International Committee on Taxonomy of Viruses (ICTV) (de Groot et al., 2012; Woo et al., 2006). According to the ICTV demarcation of CoV species, CoVs that share an overall amino acid (aa) identity of more than 90% in these seven Coronaviridae-wide conserved domains should be regarded as the same species (de Groot et al., 2012).

In this study, we conducted a large-scale surveillance of CoVs in chickens, ducks, geese, pigeons and other birds (quails, sparrows and partridges) and sequenced the aforementioned seven *Coronaviridae*-wide conserved domains in replicase polyprotein pp1ab of multiple CoVs, to further investigate the genetic diversity, distribution and taxonomy of CoVs circulating in poultry, especially those circulating in pigeons.

2 | 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 approved by the Animal Welfare Committee of China Animal Health and Epidemiology Center. The faeces samples, drinking-water samples and swab samples, from poultry farms, backyard flocks and live bird markets, were all collected with permission given by multiple relevant parties, including China Ministry of Agriculture and Rural Affairs, China Animal Health and Epidemiology Center and the relevant veterinary section in the provincial and county government.

2.2 | Designation of lineages and viruses

Viruses were assigned into lineages based on phylogenetic analysis, and the CoVs dominant in pigeons, chickens, ducks and geese were designated as pigeon-dominant coronavirus (PdCoV), chicken-dominant coronavirus (CdCoV), duck-dominant coronavirus (DdCoV) and geese-dominant coronavirus (GdCoV), respectively. It should be noted that PdCoVs may cover some CoVs isolated from the birds other than pigeons, and not all CoVs isolated from pigeons are PdCoVs, and this is the same with CdCoVs and DdCoVs. Virus strains were designated in the format of lineage/host/place/number/year, and hosts were abbreviated as 'PG' for pigeon, 'CK' for chicken, 'DK' for duck and 'GS' for goose in the designations, for example PdCoV/PG/Guangdong/1507/2014.

2.3 | Sample collection for surveillance

A total of 5,249 samples were collected from seventeen provinces (Anhui, Fujian, Guangdong, Guizhou, Hainan, Hebei, Henan, Hubei, Hunan, Jilin, Jiangsu, Jiangxi, Shanxi, Shanghai, Sichuan, Zhejiang and Chongqing) of China, including 4,539 swab samples (2,496 from chickens, 1,187 from ducks, 437 from geese, 403 from pigeons, 11 from partridges and 5 from quails), 605 faeces samples (264 from pigeons, 201 from ducks, 66 from chickens, 40 from geese, 10 from sparrows, 14 from partridges and 10 from quails) and 105 drinking-water samples (63 from ducks, 20 from pigeons, 17 from geese and 5 from chickens). These 5,249 samples were collected from 66 LPMs, 14 duck farms and 22 backyard flocks, for surveillance of avian influenza viruses, Newcastle disease viruses and CoVs circulating in poultry in China in these 3 years, 2013 (n = 737), 2014 (n = 4,309) and 2018 (n = 93).The swab samples were collected through gently taking smears at both cloacal and oropharyngeal tracts of a bird. The faeces samples were collected through taking approximately 0.5 ml wet and fresh faeces on the ground or cages. The drinking-water samples were collected through taking approximately 3.5 ml drinking water from the water trough for a group of birds in LPMs. The swab samples were stored in 1.5 ml phosphate-buffered saline (pH 7.2) containing 10% glycerol, and the faeces samples were stored in 3.5 ml phosphate-buffered saline (pH 7.2) containing 10% glycerol, and the drinking-water samples were stored with 0.4 ml glycerol. The samples were stored at 4°C and detected in three days after collection. The samples were stored at −80°C after detection. The surveillance data of 3,583 of the 4,309 samples collected in 2014 have been published with the focus on DdCoVs rather than on PdCoVs in this study (Zhuang et al., 2015).

2.4 | Detection of swab samples

The swab samples were clarified by centrifugated at 10,000 *g* for 5 min, and the supernatants were inoculated in 10-day-old specific-pathogen-free (SPF) chicken embryonated eggs via the allantoic sac route. The SPF embryonated eggs were purchased from Shandong Healthtec Laboratory Animal Breeding Company (Jinan, China). The inoculated eggs were further incubated for 2 days and checked twice each day during the incubation period. Dead ones were picked out and stored in a refrigerator. After the incubation period, the allantoic fluids of live embryos were examined using the routine haemagglutination assay. All the haemagglutination-positive allantoic fluids of live embryos and the allantoic fluids of all dead eggs were investigated by RT-PCR for detection of avian influenza virus (AIV) and Newcastle disease virus (NDV) (data not shown). They were also examined further by a conserved RT-PCR assay for detection of CoVs, as described below.

2.5 | Detection of faeces and drinking-water samples

The faeces and drinking-water samples were clarified by centrifugated at 10,000 *g* for 5 min, and then, the supernatants were detected through two ways. One was as the same as the detection for swab samples, and the other was detected directly using the following conserved RT-PCR assay for detection of CoVs.

2.6 | Detection of CoVs using a conserved RT-PCR assay

The RNA in the collected faeces and drinking-water samples or in the allantoic fluids was extracted using an RNeasy Mini Kit (Qiagen) and amplified with the One Step RT-PCR Kit (Qiagen), using a conserved RT-PCR assay designed by ourselves with the primers 5′-GGTTGGGAYTAYCCYAAGTGTGA-3′ (upper) and 5′-GAATCIGCCATAWAAACATTRTT-3′ (down) (Zhuang et al., 2015). The assay amplifies a 545-nucleotide region in the viral 1ab gene, and we have found that the conserved RT-PCR assay can detect some CoVs circulating in humans, pigs, chickens, ducks, geese and pigeons. The RT-PCR detection was performed in a 25-μl reaction system with incubation at 42°C for 30 min and denaturation at 94°C for 60 s, followed by 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. RT-PCR products were purified with an agarose gel DNA extraction kit (Sangon) and sequenced directly using the ABI 3730xl DNA Analyzer for the following phylogenetic analysis.

2.7 | Phylogenetic analysis

Sequences were aligned using the software MUSCLE (Edgar, 2004). Bayesian Information Criterion scores of substitution models and phylogenetic relationships were calculated using the software package MEGA 7.0 (Hall, 2013; Kumar, Stecher, & Tamura, 2016). Phylogenetic relationships were calculated using the model with the lowest Bayesian Information Criterion score which is assumed to describe the substitution pattern the best. Gaps were handled by partial deletion, and bootstrap values were calculated out of 1,000 replicates (Hall, 2013).

2.8 | Genome sequencing in seven conserved regions and genome analysis

Seven conserved regions in genomes of the three PdCoVs were amplified through RT-PCR with a series of primers (Table 1). The primers were designed according to the conserved regions in the genomes of the CoVs in genus *Gammacoronavirus* (Chen et al., 2013). The RT-PCR reactions were performed in a 50-μl reaction system with incubation at 42°C for 30 min and

TABLE 1 The primers used for amplification of the conserved regions of PdCoV

Pairs	Amplified region[a]	Forward primer sequence	Reverse primer sequence
1	3,090–4,085	TCATTWTCCATCTGGTGAWGA	CCATCTTCCGTAAGRTAAA
2	8,866–9,786	GCTGGTTTTAAAAAGTTTGTTTC	CTGCAACTTAACACCACCTAC
3	12,313–13,818	GAACCTAATGTTAGCACCAATG	CAACTTGACTGGCTGGTATACAAC
4	12,948–14,206	GTAGTTACACTTGATAACCAGGACC	CTATCACACTTAGGATAATCCCATC
5	14,003–15,637	TGTTGCTGGTGTTTCAATCCT	GGCTCCCACGACAGAATAAGT
6	15,064–16,197	ACAAGGGTAGTAARTTTTGGGA	TGTACTTTTTGCCTGTRTCATTAG
7	16,006–17,591	CTATAGGTTTGGCAGCGTACTTTAG	GTGTGGAAGAATTGAAGGTAGTAG
8	16,744–18,598	CTCAAGGTTCAGARTATGAYTATGT	TCAATAACAAAAACTTTRTCTCCA
9	18,226–20,047	GTAATTTGAAAGCTATGCCATTYT	TCATACCAACTTGTCTCTGTTA
10	19,324–20,379	ACAAGCAAGTSTGYACTGTTGT	CGTTGCCAACATCTCTWACMA

[a]Nucleotides were numbered per the IBV sequence with GenBank accession number NC_001451.

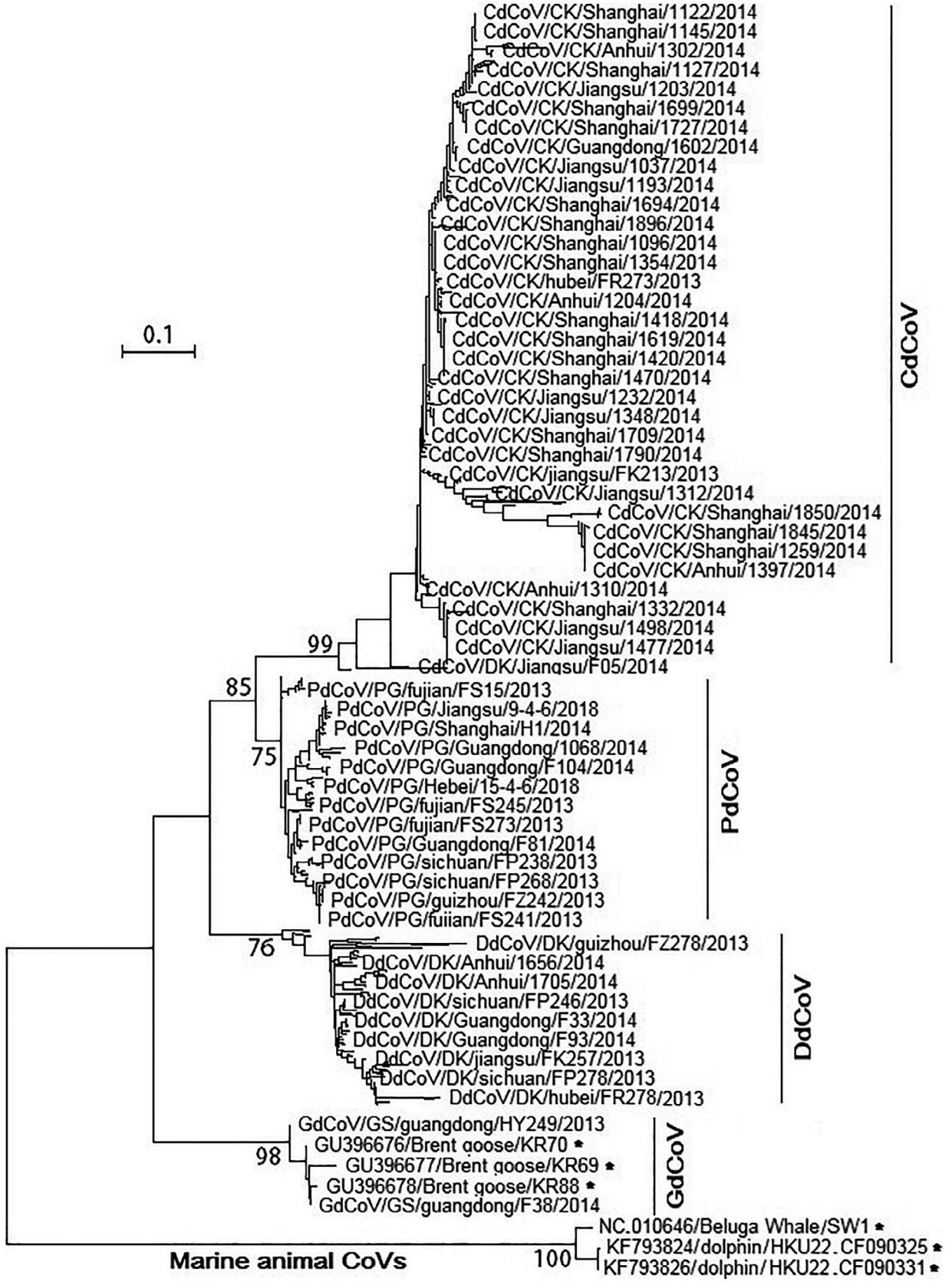

FIGURE 1 Phylogenetic relationships among some CoVs identified through this study based on the sequences in the replicase gene amplified by the conserved RT-PCR. Six reference sequences were marked with asterisks, and many sequence names were hided due to space limitation

denaturation at 94°C for 60 s, followed by 30 cycles at 94°C for 30 s, 50–55°C (largely depending on the Tm values of the primers) for 30 s and 72°C for 1–4 min (depending on the length of the amplicons). RT-PCR products were purified with an agarose gel DNA extraction kit (Sangon). The amplicons were purified using an agarose gel DNA extraction kit (Takara) and ligated into the pEASY-T1 cloning vector (TransGen). Positive clones were sequenced by the ABI 3730xl DNA Analyzer using the pair of M13 primers from both senses. Sequences were assembled and edited manually to generate the whole genome sequence which was further compared with those of IBVs and were annotated manually.

2.9 | Nucleotide sequences

A total of 725 sequences were used for phylogenetic analysis of this study including 306 original sequences of the 1ab gene of CoVs (MK983498–MK983520, MK983524–MK983531, KP032646–KP032664, KP033041–KP033042, KP033080–KP033137, KT222456–KT222651), 49 original sequences of the seven conserved domains of PdCoVs (KT254267–KT254269, KT254272–KT254274, KT254277–KT254279, KT254282–KT254284, KT254287–KT254289, KT254292–KT254294, KT254297–KT254299, MN025297–MN025324), and 468 reference sequences (KM454473, KP006677–KP006687, KP032640–KP032645, KP032665–KP033040, DQ834384, FJ888351, KP033043–KP033079, NC_010800, AY641576, KT254265–KT254266, KT254270–KT254271, KT254275–KT254276, KT254280–KT254281, KT254285–KT254286, KT254290–KT254291, KT254295–KT254296, NC_001451, JQ977698, DQ001339, JQ977697, JQ088078, DQ646405, KF574761, FN430415, GU396668–GU396671, GU396674–GU396679, GU396681, GU396683, GU396685, GU396687–GU396689, NC_010646, KF793824 and KF793826.

3 | RESULTS

3.1 | Surveillance of CoVs in poultry

A total of 736 CoV positive samples were identified through the conserved RT-PCR assay from the 5,249 samples collected in the 3 years. The corresponding RT-PCR amplicons were sequenced, and 725 sequences corresponding to 725 CoVs were clearly revealed, and they were used for further analysis regarding the viral diversity, distribution and phylogenetic relationships.

As shown in Figure 1, phylogenetic analysis of the RT-PCR amplicons suggested that these 725 viruses could be classified into four lineages corresponding to CdCoVs (n = 446), DdCoVs (n = 107), PdCoVs (n = 170) and GdCoVs (n = 2), respectively.

The positive rate of CdCoVs in the samples of chickens, 15.43% (396/2567), was significantly higher than that in the samples of pigeons (2.77%, 19/687), geese (2.23%, 11/494), ducks (1.31%, 19/1451) and other birds (1.08%, 1/93), with $p < .01$ by the chi-square test. These data suggested that CdCoVs mainly circulate in chickens and circulate in other birds at a much lower prevalence.

The positive rate of DdCoVs in the samples of ducks (6.82%, 99/1451) was significantly higher than that in the samples of pigeons (0.87%, 6/687), geese (0.40%, 2/494) and chickens (0.04%, 1/2567), with $p < .01$ by the chi-square test, suggesting that DdCoVs mainly circulate in ducks and circulate in other birds at a much lower prevalence.

As shown in Figure 2, the positive rate of PdCoVs in the samples of pigeons, 23.14% (159/687), was significantly higher than that in the samples of ducks (0.55%, 8/1451), geese (0.20%, 1/494) and chickens (0.08%, 2/2567), with $p < .01$ by the chi-square test. These data suggested that PdCoVs mainly circulate in pigeons and circulate in other birds at a much lower prevalence.

The positive rate of GdCoVs in the samples of geese, 0.40% (2/494), was significantly higher than that in other avian samples (0.00%, 0/4882) in this study, with $p < .01$ by the chi-square test. These data suggested that GdCoVs mainly circulate in geese.

PdCoVs were found in 62.5% (10/17) of the provinces, 46.97% (31/66) of the LPMs and 9.09% (2/22) of the backyard flocks where the samples were collected. CdCoVs were found in 76.47% (13/17) of the provinces, 59.09% (39/66) of the LPMs and 36.36% (8/22) of the backyard flocks where the samples were collected. DdCoVs were found in 64.71% (11/17) of the provinces, 42.42 (28/66) of the LPMs, 9.09% (2/22) of the backyard flocks and 7.14% (1/14) of the duck farms where the samples were collected. These data suggested that PdCoVs, CdCoVs and DdCoVs were significantly more prevalent in LPMs than in backyard flocks and/or poultry farms in some regions in China with $p < .01$ by the chi-square test.

Of the aforementioned 170 PdCoVs, 159 were from pigeon samples, and 11 were from non-pigeon samples. The prevalence of PdCoVs in pigeon swab, faeces and drinking-water samples was 4.96% (20/403), 50.00% (132/264) and 35.00% (7/20). All the collected pigeon faeces and drinking-water samples were found to be CoV negative when they were detected through inoculation in chicken embryonated eggs followed by the conserved RT-PCR assay. This indicates that PdCoVs replicate poorly in chicken embryonated eggs. As for the eleven non-pigeon-origin PdCoVs, 2 were from chicken swab samples, 8 from duck faeces samples and 1 from goose drinking-water samples.

The positive rates of CdCoVs, DdCoVs, PdCoVs and GdCoVs in the faeces samples collected in the 3 years were given in Table 2. This table suggested that CdCoVs, DdCoVs and PdCoVs were all prevalent in their dominant hosts in the 3 years, while GdCoVs were not that prevalent in geese in these 3 years.

Of the detected 5,249 samples, 4,667 were collected from LPMs, 153 were from backyard flocks, and 429 were from poultry farms. Due-species infections of the CoVs, namely that infections of CdCoVs in chickens, infections of DdCoVs in ducks, infections of PdCoVs in pigeons and infections of GdCoVs in geese, were identified in numerous samples collected in all the three types of poultry sites (Table 3). In contrast, cross-species infections of the CoVs, namely that infections of CdCoVs in birds other than chickens, infections of DdCoVs in birds

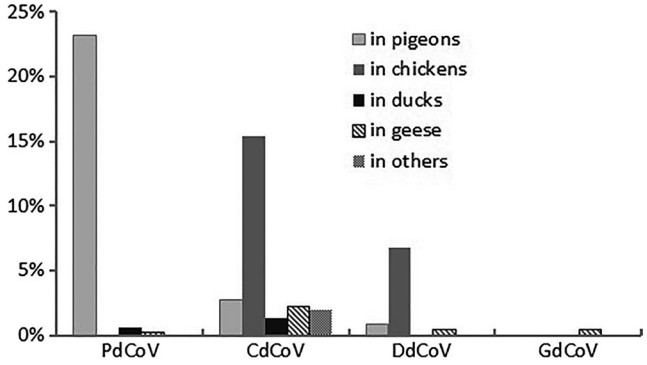

FIGURE 2 The positive rates of PdCoVs, CdCoVs, DdCoVs and GdCoVs in pigeon, chicken, duck and goose samples

other than ducks, infections of PdCoVs in birds other than pigeons and infections of GdCoVs in birds other than geese, were identified in 68 of the 5,249 samples from LPMs (49 with CdCoVs, 8 with DdCoVs and 11 with PdCoVs), and in none of the 153 samples from backyard flocks and the 429 samples from poultry farms.

3.2 | Analysis of the sequences of seven conserved domains

We detected in this study the sequences of the seven conserved domains in the viral replicase gene (namely ADRP in nsp3, nsp5, nsp12, nsp13, nsp14, nsp15 and nsp16) of seven PdCoVs: PdCoV/PG/Guangdong/1418/2014, PdCoV/PG/Guangdong/1068/2014, PdCoV/PG/Guangdong/1507/2014, PdCoV/PG/Jiangsu/9-1-3/2018, PdCoV/PG/Jiangsu/9-4-6/2018, PdCoV/PG/Jiangsu/9-7-9/2018 and PdCoV/PG/Hebei/15-4-6/2018.

As compared to CdCoV and DdCoV sequences, thousands of substitutions and dozens of insertions or deletions occurred in PdCoVs in the seven conserved domains. For example, as shown in Figure 3, two obvious insertions or deletions were found in the Nsp12 domain of PdCoVs as compared with the sequences of CdCoVs and DdCoVs.

Phylogenetic analysis of the sequences of the seven domains combined together, as shown in Figure 4, suggested that the PdCoVs, DdCoVs and CdCoVs belong to different lineages. The PdCoVs, DdCoVs and CdCoVs also belong to different lineages based on each of these seven conserved domains, with the exceptions that DdCoV/DK/Guangdong/2014 is located in the same lineage with the 12 CdCoVs based on the nsp5 gene sequences and the nsp16 gene sequences (Zhuang et al., 2015). The exceptions might be caused by genomic recombination (Zhuang et al., 2015).

As given in Table 4, the identity between seven PdCoVs reported herein and twelve randomly selected CdCoVs (ten from chickens, one from turkeys and one from peafowl) and three DdCoVs in the combined amino acid sequences of the seven conserved domains in the viral replicase gene is 83.6%–85.1% between PdCoVs and CdCoVs, 86.7%–89.8% between PdCoVs and DdCoVs and 85.5%–89.0% between CdCoVs and DdCoVs. In contrast, the corresponding amino acid identity is 97.0%–98.5% among the seven PdCoVs, 94.7%–97.3% among the twelve CdCoVs and 94.6%–98.9% among the three DdCoVs. Therefore, according to the sole species demarcation criterion for CoVs that the viruses sharing more than 90% amino acid sequence identity in these seven conserved domains belong to the same species (de Groot et al., 2012), the PdCoVs, DdCoVs and CdCoVs should belong to three different species.

TABLE 2 Positive rates (%) of four lineages of CoVs in the samples collected from four host species in 2013, 2014 and 2018

	Geese		Pigeons			Chickens			Ducks		
	2013	2014	2013	2014	2018	2013	2014	2018	2013	2014	2018
CdCoV	2/35	9/459	9/287	10/388		22/30	359/2492	15/45	7/349	12/1090	
DdCoV		2/459	6/287						48/349	48/1090	3/12
GdCoV	1/35	1/459									
PdCoV		1/459	97/287	57/388	5/12		2/2492		4/349	4/1090	

TABLE 3 Numbers of due-species infections and cross-species infections in the samples collected from three types of sites

	Due-species infections				Cross-species infections			
	CdCoVs	DdCoVs	PdCoVs	GdCoVs	CdCoVs	DdCoVs	PdCoVs	GdCoVs
LPMs (n = 4,667)	381	91	154	2	50	8	11	0
Backyard flocks (n = 153)	15	3	5	0	0	0	0	0
Poultry farms (n = 429)	0	5	0	0	0	0	0	0

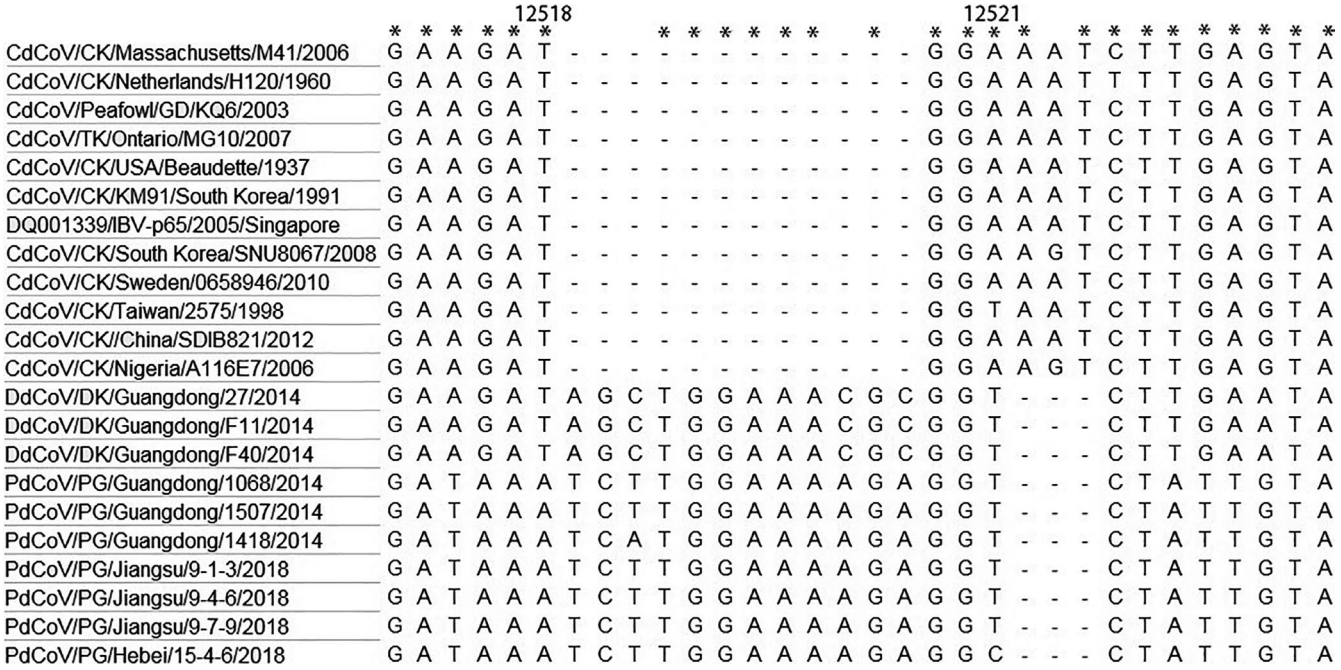

FIGURE 3 Alignment of partial sequences of the Nsp12 domain of some PdCoVs, CdCoVs and DdCoVs. The sites were numbered as per the first sequence, and those sites conserved in two lineages were marked with asterisks

4 | DISCUSSION

The large-scale surveillance of this study revealed that PdCoVs, DdCoVs and CdCoVs mainly circulate in pigeons, ducks and chickens, respectively, and they belong to different phylogenetic lineages. According to the official criteria of ICTV definition for demarcation of CoV species, the genetic distances between PdCoVs, DdCoVs and CdCoVs in the seven conserved domains are large enough to separate them into different species in the genus *Gammacoronavirus* (de Groot et al., 2012). Classifying PdCoVs, DdCoVs and CdCoVs into different species also meets the definition of virus species that a virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche (de Groot et al., 2012).

Currently, the subgenus *Igacovirus* of the genus *Gammacoronavirus* is represented by the species of *Avian coronavirus* covering IBVs in chickens and related CoVs in turkeys and other birds (de Groot et al., 2012). The designation has become questionable because some CoVs in wild birds have been identified to be members of another genus *Deltacoronavirus* (Woo et al., 2012). In this report, we further challenged the designation of *Avian coronavirus* as we provided evidence in supporting that the CoVs dominant, respectively, in pigeons, ducks and chickens likely belong to different species.

This study indicates that PdCoVs likely replicate poorly in embryonated chicken eggs, and this is consistent with a previous report which showed no replication in embryonated chicken eggs could be achieved for pigeon or goose coronaviruses (Jonassen et al., 2005). Therefore, the detection procedure with inoculation in chicken embryonated eggs used in this study is not suitable for the detection of PdCoVs, although it is suitable for surveillance of AIVs, NDVs and CdCoVs. Consequently, the prevalence of PdCoVs in pigeon swab samples detected in this study with inoculation in chicken embryonated eggs could be underestimated.

Our surveillance results suggest that PdCoVs, CdCoVs and DdCoVs are significantly more prevalent at LPMs than in backyard flocks and farms in some regions in China. Cross-species infections of the CoVs were only identified in some samples from LPMs, and none were identified from backyard flocks and poultry farms. Therefore, LPMs likely play an important role in the circulation of a diversity of CoVs in poultry, as they do in the circulation of AIVs (Jiang et al., 2012).

Recently, researchers identified a CoV from pigeons which has been regarded as a member of *Deltacoronavirus* (Lau et al., 2018), and this CoV was phylogenetically distinct from all avian CoVs within *Gammacoronavirus*. It remains unknown whether this CoV is prevalent in pigeons or other birds.

Pigeons are widely distributed in many countries mainly for pigeon racing and food consumption (Ashton, 1984; Stenzel, Pestka, Tykalowski, Smialek, & Koncicki, 2012). Although we identified in this study that PdCoVs are prevalent in pigeons in China, it remains unknown about pathogenicity of PdCoVs which is worth studying in the future.

In conclusion, this study shed novel insight into the genetic diversity, distribution, evolution and taxonomy of avian CoVs. Continuous surveillance and studies on avian CoVs will help to better understand the diversity, distribution, cross-species transmission and clinical significance of these viruses.

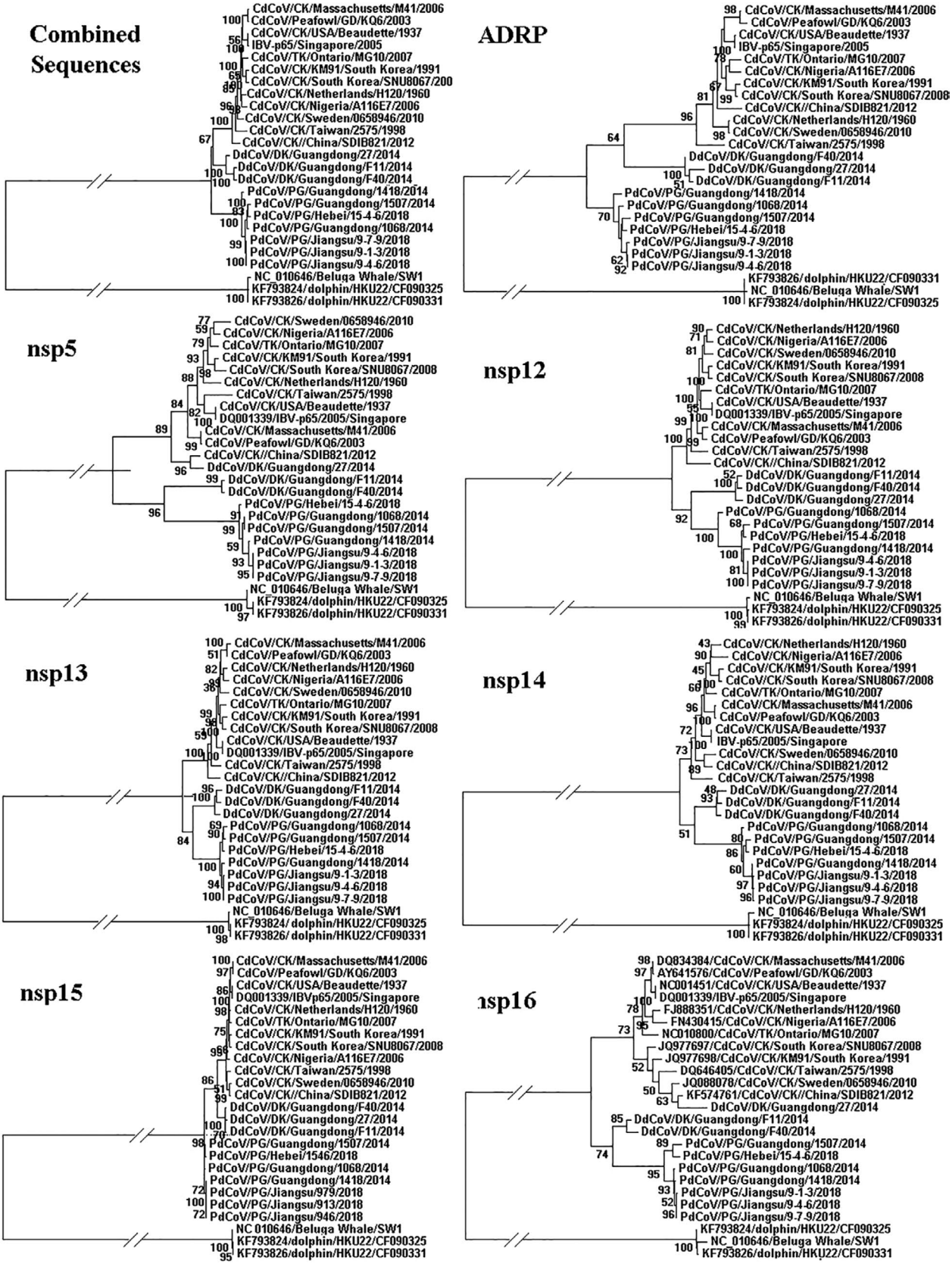

FIGURE 4 Phylogenetic relationships of 22 CoVs based on the sequences of seven conserved domains. The first phylogenetic tree was based on the sequences of the seven domains combined together, and the remaining trees were based on the sequences of each domain. The longest branches were shortened for space saving

TABLE 4 Genetic distances between CdCoVs, DdCoVs and PdCoVs in seven conserved genomic domains

	Genetic distance ($\bar{x} \pm s$)		
	CdCoVs	DdCoVs	PdCoVs
CdCoVs	96.1% ± 1.0%	86.7% ± 1.0%	84.3% ± 0.3%
DdCoVs		96.0% ± 2.5%	88.7% ± 0.9%
PdCoVs			98.2% ± 0.8%

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CONFLICT OF INTEREST

All the authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Jiming Chen and Qingye Zhuang conceived and designed the study; Qingye Zhuang, Shuo Liu, Guangyu Hou, Suchun Wang, Jinping Li, Xiaochun Zhang and Jingjing Wang performed the experiment; Qingye Zhuang, Jiming Chen, Hualei Liu, Wenming Jiang, Kaicheng Wang, Cheng Peng, Xiaohui Yu, Liping Yuan and Yang Li conducted data analysis; Qingye Zhuang and Jiming Chen wrote and revised the paper.

ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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