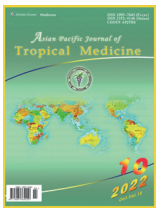

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Diversity of coronaviruses in wild and domestic birds in Vietnam

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ABSTRACT

Objective: To investigate the genetic diversity of coronaviruses in wild and domestic birds in the bird park and live poultry markets of Ca Mau province in Vietnam in 2020.

Methods: A total of 228 samples (121 from wild birds and 107 from poultry) were collected in the territory of Ca Mau province of Vietnam in 2020. The avian samples were tested for the presence of the four genera of coronaviruses using reverse transcription and nested PCR. Molecular genetic analysis using targeted NGS sequencing of *RdRp* gene was performed for 29 representative samples (12 samples from wild birds and 17 from poultry). Phylogenetic analysis was performed using the neighbour joininig method with 1 000 bootstrap replications using MEGA X software.

Results: Among wild birds, 21.5% samples were positive for the genetic material of coronaviruses and 38.3% samples were positive for coronaviruses among poultry. Genetic analysis of the partial sequence of *RdRp* gene from representative samples revealed that wild birds were carriers of gammacoronaviruses and deltacoronaviruses. Among poultry, three genotypes of infectious bronchitis virus (QX, Mass and Q1) were detected in chickens, with QX genotypes being predominant, and viruses of DCoV group were detected in domestic ducks. There was no detection of alphacoronaviruses or betacoronaviruses in the studied birds.

Conclusions: Coronaviruses of genera *Gammacoronavirus* and *Deltacoronavirus* were detected in wild birds. A high percentage of infectious bronchitis virus was detected in poultry. Thus, there is a need for broader surveillance of coronaviruses in birds, which can be used for evaluation of diversity, evolution and distribution of coronaviruses in Vietnam. Continuous surveillance of coronaviruses circulation in wild and domestic animals is necessary for implementing strategic measures for poultry and domestic animal protection and for evaluation of possible risk of circulating coronaviruses to human health.

KEYWORDS: Avian coronavirus; *Gammacoronavirus*; *Deltacoronavirus*; Surveillance; Wild birds; Poultry; Vietnam

1. Introduction

Coronaviruses (CoVs) are a large group of diverse viruses, representatives of which can infect humans and a wide variety of animals from birds to mammals. Pandemic caused by SARS-CoV-2 and previous outbreaks caused by the CoVs such as SARS-CoV, MERS-CoV of the genus *Betacoronavirus* draw close attention to the importance of the viruses and the need for worldwide broad surveillance of CoVs circulating in animals for better understanding of the evolution and ecology of the viruses and for strategic preparedness to outbreaks and pandemics[1]. In addition, CoVs are among the most widely spread pathogens that affect poultry and cause serious economic burden for agriculture worldwide[2].

Coronaviruses are common name of representatives of family Coronaviridae, suborder Cornidovirineae, order Nidovirales.

Significance

Previous coronavirus studies demonstrated circulation of alphacoronaviruses and betacoronaviruses in different species of mammals; while gammacoronaviruses and deltacoronaviruses are commonly found in birds and sporadically in mammals. Previously infectious bronchitis virus has been shown to circulate in poultry in Vietnam. In this study, the circulation of gammacoronaviruses and deltacoronaviruses among wild birds and the simultaneous detection of gammacoronaviruses and deltacoronaviruses in one bird was shown for the first time in Vietnam.

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According to the International Committee on Taxonomy of Viruses (ICTV), the Coronaviridae family includes four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus* and *Deltacoronavirus* and are represented by a large number of species. The role of individual species and the mechanism of transmission of the viruses in various populations is largely under investigation. Of the four genera of CoVs, alphacoronaviruses (alphaCoVs) and betacoronaviruses (betaCoVs) are believed to be common among mammals; while birds are the main hosts of gammacoronaviruses (gammaCoVs) and deltacoronaviruses (deltaCoVs)[3–5]. CoVs can be transmitted from wild animals to humans causing zoonotic infections (*e.g.* betaCoVs MERS-CoV, SARS-CoV and SARS-CoV-2). AlphaCoVs spread predominantly among bats. There are human CoVs designated as HCoV group that cause mild respiratory infection in humans and are responsible for a substantial number of seasonal common cold in adults. These viruses include betaCoVs (HCoV- HKU 1 and -OC43) and alphaCoVs (HCoV-229E, -NL63) and were likely zoonotically transmitted to human host in the distant past[6]. Two other genera *Gammacoronavirus* and *Deltacoronavirus* circulate primarily among wild and domestic birds, with gammaCoVs, in particular infectious bronchitis virus (IBV), being infectious agent causing outbreaks in poultry. Among gammaCoVs and deltaCoVs, there are viruses that were isolated from infected mammals such as beluga whale (gammaCoV, subgenus *Cegacovirus*) and swine (porcine deltaCoV, PDCoV group)[7]. There was a recent report of human infections with PDCoV group virus[8]. Wild birds play a special role in the spread of CoVs, which are able to migrate over long distances and may be involved in spreading potentially dangerous variants of the viruses to domestic animals and to humans[7]. BetaCoVs have not been detected in birds, however, they demonstrated ability of various CoVs for crossing interspecies barrier and rapid evolution *via* recombination points to importance of continuous monitoring and risk assessment of CoV circulation in various wild animal reservoirs. At live animal markets, which are common in Asia, humans interact with wild and domestic animals and poultry, which are known carriers of various CoVs that may transmit to humans (potentially from mammalian species) or spread from infected wild birds and live poultry from markets to large and small poultry farms. Currently, improvements of strategy for the control of the spread of CoVs and other infectious agents through live animal markets in Asia are being considered in collaboration with international One Health community[9]. Certain measures are being implemented for virus control and improving sanitary conditions. Internationally, One Health approach, which includes surveillance and risk assessment based on data from human health, wild animal disease and environmental considerations is now widely being advocated and applied as effective approach for addressing spread of zoonotic infections and animal health control[9,10]. Sustainable and systematic surveillance of infectious agents, including CoVs, is suggested internationally and by researchers in Vietnam as one of the key activities for infection prevention and strategic protection of human and animal health[9].

Previous research in Vietnam detected alphaCoVs, betaCoVs and gammaCoVs in places of wild-life human interface such as wildlife farms[11]. Recently, due to SARS-CoV-2 pandemic, surveillance of CoVs was performed in wild life animals that are likely to be hosts for similar viruses such as bats and pangolins in the territory of Asia, including Vietnam[12,13]. Importance of avian CoVs, in particular IBV, which is endemic in poultry in Asia, has long been recognized in the countries of Asia. The first study of genetic characterization of IBV in Vietnam published in 2019 reported three IBV genotypes similar to the ones previously reported in China but different from the commonly used IBV vaccines for poultry in Vietnam[14]. Another study of poultry pathogens in Mekong Delta region of Vietnam published in 2020 revealed relatively high percentage of infected birds with IBV in small poultry farms[15]. These studies suggested the need for extended surveillance of genetic variants of IBV circulating in Vietnam and implementing measures of effective control of poultry infections by improving small-scale poultry farms biosafety and developing and using regionally appropriated and more effective IBV vaccines.

The aim of this work was to contribute to the understanding of diversity and ecology of avian CoVs in Vietnam and to study the genetic diversity of CoVs in wild and domestic birds in bird park and live poultry markets of Ca Mau province in Vietnam in 2020.

2. Materials and methods

The samples from birds were collected in accordance with Vietnam legislation, for which all relevant licenses and permits from the relevant departments were obtained. Capture and sampling of birds were carried out under bioethics protocol No. Vector/04-04.2018 issued by BioEthics Committee at FBRI SRC VB Vector Rosbotrebnadzor. The samples were presented by cloacal swabs, droppings and internal organ fragments which were collected into individual tubes for each sample. The Copan Universal Transport Medium (UTM-RT) System (COPAN Diagnostics, USA) was used for sample collection. Swabs, droppings and organ homogenates were prepared as described[16].

Viral RNA was isolated from the primary material using the RIBO-sorb or RIBO-prep kit (Interlabservice, Russia) according to the manufacturer's instructions. To identify the genetic material of CoVs, we used the published modified pan-coronavirus test system and the test system developed in our laboratory using nested PCR[17–19]. Degenerate primers for the modified pan-coronavirus

test system (AC-CoV-F: GGTTGGGATTATCCWAARTGTG, AC-CoV-R: TGYTGTGARCAAAAYTCRTG) target the conserved region of the avian CoV polymerase gene (*RdRp*), producing a 602 bp amplicon. This test system allows for the detection of CoVs of all four genera (alphaCoVs, betaCoVs, gammaCoVs and deltaCoVs). Synthesis of cDNA and the amplicon was carried out in one reaction using BioMaster RT-PCR SYBR Blue (2×) and BioMaster RT-PCR-Extra (2×) kits manufactured by Biolabmix LLC (Russia). The reaction profile was as described in Chamings *et al*[17]. A test system was developed using nested PCR, which allows for detection with significantly increased sensitivity. DNA obtained in conventional PCR using a modified pan-coronavirus test system was used to perform nested PCR (conventional PCR).

The nested PCR test system included degenerate nested PCR primers for the detection of gammaCoVs and deltaCoVs (Vec_CoVgd1 F2: CWAARTGTGAYAGRKCHATGCC, Vec_CoVgd1 R2: CCRTCRTCAGAMARDATCATNAR). The size of the amplicon with primers is 422 bp. For nested PCR, the BioMaster LR HS-PCR (2×) kit manufactured by Biolabmix LLC (Russia) was used according to the manufacturer instructions. The strain of chicken infectious bronchitis virus IBV D274 (GenBank ID: MH021175.1) was used as a positive control in all PCR performances. Purified water was used as a negative control in all PCR reactions. Sequencing of CoV amplicons was performed by Illumina MiSeq using a v3 reagent kit. Obtained sequences were deposited in the GenBank database (NCBI) under accession numbers: OP355469-OP355507. Phylogenetic analysis of the obtained partial *RdRp* gene sequences of avian CoVs was performed in comparison to reference sequences of avian CoVs from NCBI GenBank representing all relevant groups. Multiple sequence alignment was performed using Muscle alignment. Phylogenetic analysis was performed using the neighbour joininig method with 1 000 bootstrap replications using MEGA X software (http://www.megasoftware.net/).

3. Results

3.1. Detection of coronaviruses in poultry and wild birds

A total of 228 samples (121 from wild birds and 107 from poultry) were collected during monitoring in the territory of Ca Mau province of Vietnam in 2020. Samples were collected from Ca Mau bird park and from three live poultry markets in Ca Mau. The examined samples from wild birds belonged to 13 species of 7 orders (Table 1). The examined samples from poultry were collected from chickens and domestic ducks. The PCR analysis showed that 67 samples were positive for CoV: 26 samples among wild birds (21.5% among wild birds) and 41 samples from poultry (38.3% among poultry). The diversity of birds (including two orders of domestic birds), from which the samples for CoV analysis were collected, was represented by 8 orders and 16 species. Of these, the *Anseriformes* order accounted for 8 cases of infection, and the *Pelecaniformes* order 9 cases. The diversity of species involved in the circulation of CoVs was represented by 2 species from the order *Anseriformes* and 2 species from the order *Pelecaniformes*. The *Suliformes* order was represented by one species. Molecular genetic analysis using targeted NGS sequencing of *RdRp* gene was performed for 29 representative samples (12 samples from wild birds and 17 samples from poultry). In total, 40 CoVs were identified, including 27 gammaCoVs (3 in wild bird, 24 in poultry) and 13 deltaCoVs (all detected in wild birds) (Table 1). The simultaneous presence of genetically similar (various SNP variants) virus variants of gammaCoVs or deltaCoVs in one sample was detected by NGS for 8 cases (Table 1).

3.2. Phylogenetic analysis of gammacoronaviruses in poultry and wild birds

Phylogenetic analysis of partially sequenced *RdRp* gene revealed that the gammaCoVs identified in this study belonged to the *Igacovirus* subgenus and grouped into two groups: the AvCov/AvCov9203 group and *Duck coronavirus* 2714 (DCoV 2714) group (Figure 1).

The viruses that belonged to AvCov/AvCov9203 group represented the QX (GI-19), Q1 (GI-16), and Mass (GI-1) genotypes. Most of the viruses from Vietnam belonged to the QX genotype and were grouped with viruses identified in China and Laos (Figure 1). Phylogenetic analysis showed that each of the other two genotypes was represented by a single virus detected in this study. The AvCoV/chicken/Vietnam/M2365/2020 virus belonged to the Mass (GI-1) genotype and was similar to the IBV viruses identified in China and other countries. The AvCoV/chicken/Vietnam/M2352/2020 virus belonged to the Q1 genotype, and it is in the same clade as the IBV from China and Europe. There was one case of detection of IBV grouped with viruses of Mass genotype in a wild duck (Figure 1).

The viruses identified during the study belonged to the DCoV 2714 group with close similarities to the ratified species *Duck coronavirus* 2714. Most of the DCoV 2714 viruses were identified in domestic ducks, and one virus was detected in the black-crowned night heron (*Nycticorax nycticorax*). All identified viruses were grouped with the viruses of Southeast Asia. The black-crowned night heron virus AvCoV/black-crowned night-heron/Vietnam/M2346/2020 was genetically slightly different from those found in domestic ducks and showed the greatest similarity to the J1451/Anas acuta/091230 virus from China Hong Kong and two other viruses from Bangladesh and China.

Table 1. The species of birds among which coronaviruses were detected.

No.	Order	Species	No. of samples	No. of positive samples	Sequenced (MiSeq)	No. of viruses detected	Detected virus genera	Detected mixed variants in one sample
Wild birds								
1	*Anseriformes*	Lesser whistling duck (*Dendrocygna javanica*)	6	1	1	2	*Gammacoronavirus*	2 gammaCoVs
2	*Pelecaniformes*	Little egret (*Egretta garzetta*)	7	2	2	2	*Deltacoronavirus*	deltaCoV; deltaCoV
3	*Pelecaniformes*	Black-crowned night-heron (*Nycticorax nycticorax*)	17	7	7	10	*Gammacoronavirus* *Deltacoronavirus*	2 deltaCoVs; 2 deltaCoVs; deltaCoV; deltaCoV; deltaCoV; 2 deltaCoVs; gammaCoV
4	*Suliformes*	Indian cormorant (*Phalacrocorax fuscicollis*)	2	2	2	2	*Deltacoronavirus*	deltaCoV; deltaCoV
5	*Passeriformes*	Streak-eared bulbul (*Pycnonotus conradi*)	3	0	0	0		
6	*Passeriformes*	Chinese pond heron (*Ardeola bacchus*)	1	0	0	0		
7	*Passeriformes*	Oriental magpie-robin (*Copsychus saularis*)	1	0	0	0		
8	*Passeriformes*	Common tailorbird (*Orthotomus sutorius*)	1	0	0	0		
9	*Passeriformes*	Yellow-rumped flycatcher (*Ficedula zanthopygia*)	1	0	0	0		
10	*Passeriformes*	Paddyfield warbler (*Acrocephalus agricola*)	3	0	0	0		
11	*Coraciiformes*	Blue-eared kingfisher (*Alcedo meninting*)	1	0	0	0		
12	*Columbiformes*	Common pigeon (*Columba livia*)	2	0	0	0		
13	*Cuculiformes*	Indian cuckoo (*Cuculus micropterus*)	1	0	0	0		
Poultry								
14	*Galliformes*	Chicken (*Gallus gallus domesticus*)	95	34	13	15	*Gammacoronavirus*	11 gammaCoVs from 11 birds; 2 gammaCoVs; 2 gammaCoVs
15	*Anseriformes*	Domestic duck (*Anas platyrhynchos domesticus*)	8	7	4	9	*Gammacoronavirus*	gammaCoV; gammaCoV; 3 gammaCoVs; 4 gammaCoVs
16	*Anseriformes*	Domestic goose (*Anser anser domesticus*)	4	0	0	0		
Untyped wild birds								
		Environment samples (droppings from untyped wild birds)	75	14	0	0		
	8 orders	**16 species**	**228**	**67**	**29**	**40**		

3.3. Phylogenetic analysis of deltacoronaviruses in wild birds

Phylogenetic analysis of partially sequenced *RdRp* gene revealed that the deltaCoVs identified in this study belonged to two subgenera *Buldecovirus* and *Hardecovirus* (Figure 2). Three viruses grouped with the ratified species *White–eye coronavirus* HKU 16 (subgenus *Buldecovirus*) and belonged to a subgroup that includes previously identified three viruses from falcon, pigeon and houbara from China Hong Kong, for which whole genome sequences have been obtained (Figure 2). These viruses are less than 90% similar in amino acid sequence to *White–eye coronavirus HKU16* ratified species and may be considered as a distinct subgroup and subsequently be identified as a new ratified species group[7].

Some of the viruses registered in the territory of Vietnam, circulating among two species of birds [little egret (*Egretta garzetta*) and black-crowned night heron (*Nycticorax nycticorax*)], belong to the clade of the established species of CoVs *Night–heron coronavirus HKU19*. This group also includes viruses identified in other countries in Asia, Europe, and in Australia (Figure 2).

Figure 1. The phylogenetic analysis of 400 bases region of the *ORF1ab* (*RdRp*) gene of select members of gammaCoVs by neighbor-joining method using 1 000 bootstrap. New viruses identified in Vietnam are marked with black triangles. Ratified species (ICTV) were used as references and are marked with black circles (the reference sequences were taken from GenBank, NCBI).

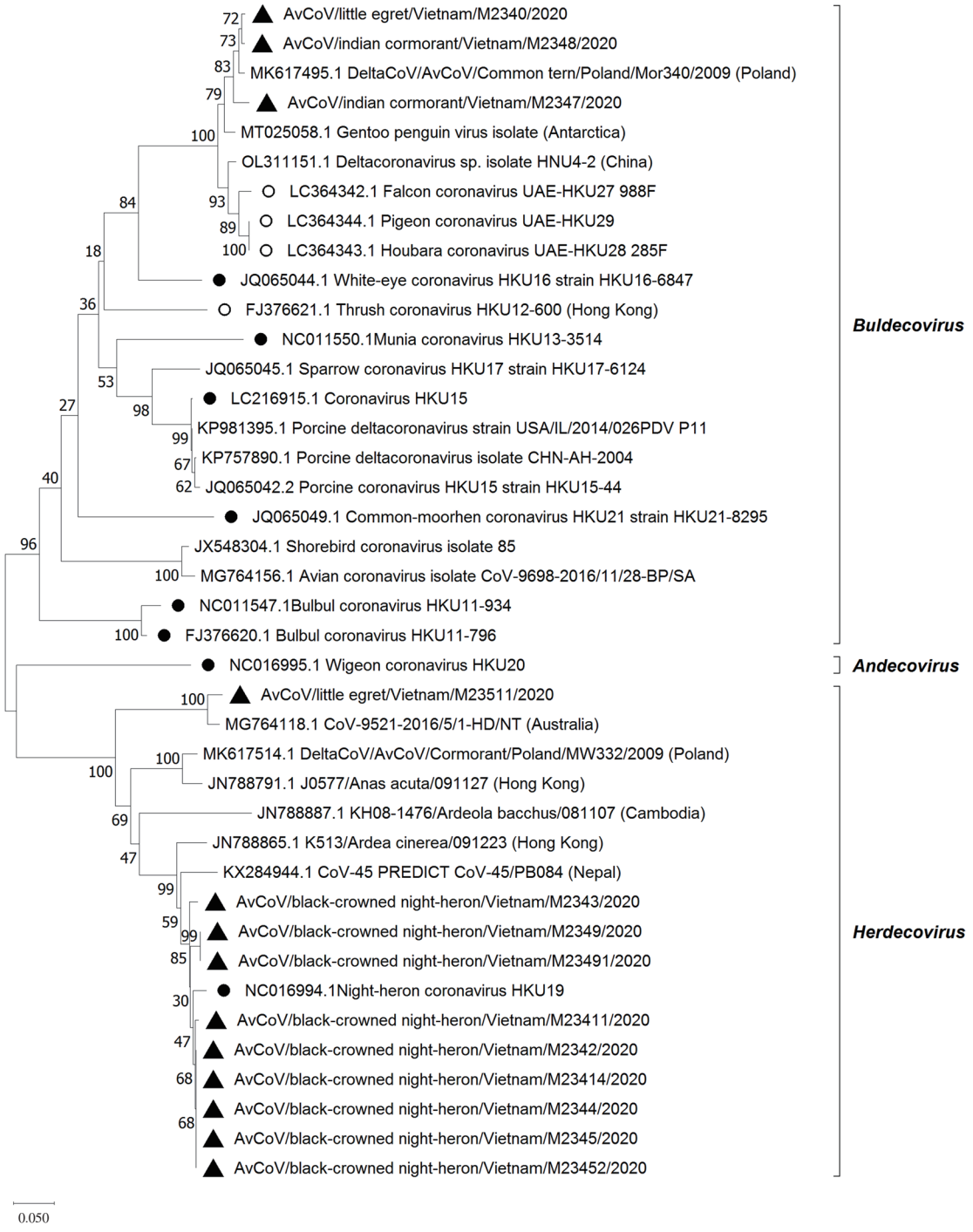

Figure 2. The phylogenetic analysis of 400 bases region of the *ORF1ab* (*RdRp*) gene of select members of deltaCoVs by neighbor-joining method using 1 000 bootstrap. New viruses identified in Vietnam are marked with black triangles. Ratified species (ICTV) were used as references and are marked with black circles; other reference viruses with obtained full genome sequence are marked with empty circles (reference sequences were taken from GenBank, NCBI).

4. Discussion

The surveillance of avian CoVs in bird park and markets in Ca Mau province in Vietnam showed substantial circulation of gammaCoVs and deltaCoVs in wild birds and poultry. The percentage of detection of CoVs in wild birds (21.5%) was greater than previously reported studies of avian CoVs in Sweden (19%)[20] and Australia (15%)[17]. High percentage of detection of CoVs in poultry (38.3%) correlated with previous studies in Vietnam that detected IBV in over 20% of studied chicken flocks[15] and high prevalence of IBV in Asia was confirmed by many studies in China, Thailand and other countries in close geographical proximity to Vietnam[21].

GammaCoVs identified in this study in poultry from AvCoV/AvCoV9203 group belonged to QX, Q1 and Mass IBV genotypes. Most of the detected viruses belonged to QX genotype, which is circulating in Asia and globally[2,22,23]. The data obtained are

consistent with the previous analysis of IBV genotypes in Vietnam, in which the studied strains were grouped with Q1-like, QX-like and TC07-2-like genotypes by the phylogenetic analysis of *S1* genes and were closely related to reference IBV strains from China[14]. Mass genotype has also been detected in Asia[21] and globally[2].

Several gammaCoVs identified in the study belonged to the group of recently established ratified species *Duck coronavirus 2714* in the subgenus *Igacovirus*, which includes a lot of gammaCoVs detected in wild birds[7]. Most of the viruses from this group in this study were identified in domestic ducks. This correlates well with previously reported studies that DCoV is common among birds of orders *Anseriformes* and *Charadriiformes*[7] and confirms that DCoV is frequently found in domestic ducks[24,25]. In this study, there was one case of detection of IBV viruses in a wild duck, which are not common hosts for IBV. Such cases of IBV viruses infecting wild birds are considered as spillovers from common hosts and they show that the CoVs can be transmitted from poultry to wild birds. Similarly, common detection of viruses from DCoV 2714 group in poultry, in particular in domestic ducks is also indicative of virus transfer between wild birds and domestic species[7].

DeltaCoVs were identified in this study only in wild birds. This is consistent with previously reported studies, which showed that deltaCoVs currently contain seven established species belonging to three subgenera (*Hardecovirus, Andecovirus, Buldecovirus*), all of which have been found in wild birds[26,27]. To our knowledge, this is the first report of detection of deltaCoVs in wild birds in Vietnam. Diversity of deltaCoVs in wild birds is still largely understudied. According to Genbank, as of October 10, 2022, only 671 deltaCoVs were detected in the world[7]. Several identified deltaCoVs belonged to group of ratified species *Night-heron coronavirus HKU 19* (subgenus *Herdecovirus*), which combines viruses identified in Brazil, Poland, Finland, China Hong Kong and Australia[7]. These data together with previously reported studies indicate that viruses of this group are common in circulation in Southeast Asia and are spread globally *via* migration of avian hosts. The rest of the identified viruses belonged to *Buldecovirus* subgenus to a subgroup of viruses HKU27, HKU28, HKU29 that were previously identified in Asia and Europe and Australia[7].

Overall, the study showed that in poultry sold on markets, there is high percentage of carriers of IBV that cause outbreaks and substantial economic damage to poultry farming in Asia and worldwide[2]. Two widely used vaccines in Vietnam are based on vaccine strains of Mass (Ma5) and 4/91 genotypes[14]. It was shown that when applied alone Mass vaccines (H120 and Ma5 strains) provided only partial protection against QX genotype that may not be sufficient for IBV control[28]. However, combined use of the two vaccines Mass and 4/91, especially when applied consecutively, was shown to be effective against the largest diversity of genotypes including substantial cross-protection against QX genotype[29,30]. In addition, combined use of viruses of Mass and 4/91 genotypes with viruses of QX genotype used in vaccines in China was shown to be highly effective against QX genotype but not against another genotype in circulation[31]. Studies of IBV in Asia showed a large number of IBV genotypes and groups of IBV viruses circulating in poultry[18]. The studies in Asia, including Vietnam and China showed the importance of surveillance of IBV for developing and implementing most effective strategies for IBV prevention and control for poultry farming.

Detection of deltaCoVs in wild birds in this study expanded the data for geographical spread of the deltaCoVs in Vietnam. The ecological significance and evolution of the detected viruses need to be further studied. Previous studies in China reported detection of viruses in swine from porcine CoVs group (genus *Deltacoronavirus*), which includes viruses that can infect mammals and humans[7,8]. The viruses of the group were not detected in this study but they are globally spread and may be detected with broader surveillance of CoVs in birds and mammals in Vietnam.

AlphaCoVs and betaCoVs, for which mammals are common hosts, have not been identified in this study in birds, which is consistent with the lack of detection of such cases in the world. However, alphaCoVs have been previously identified in bats and betaCoVs have been previously identified in bats and rats in Vietnam in the environment where the wild life CoV carries are in close contact with humans and poultry (such as farms)[11]. In addition, recent studies identified SARS-CoV-2 related viruses in pangolins in Vietnam, which were similar to viruses from pangolins in China[11]. Thus, CoVs from all four genera have been detected in Vietnam as a result of several surveillance studies.

Large diversity of CoVs in Vietnam, Asia and the whole world and their importance for human health and agriculture require broad surveillance of CoVs in birds and other animals, especially at the human animal interface. This surveillance is necessary in order to understand diversity, geographical spread and evolution of the CoVs. One Health approach, in which surveillance in animals and environment is coordinated with data from human health sector, helps to plan and organize surveillance and research projects in order to maximize capacity for prevention and response to CoV outbreaks threats. The obtained data can be used for evaluation of risk to humans and animals and for developing effective strategies for prevention and containment of CoVs outbreaks in agriculture and in human populations.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Authors' contributions

VYM supervised the project, formulated ideas, research goals and aims and prepared original draft. Both NPK and AVD conducted PCR analysis, NGS experiments, bioinformatics analysis and data interpretation. MKB conducted phylogenetic analysis. TTN conducted sample collection and preparation. ABR conceptualized the overall study and coordinated the investigation.

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