Bulgarian Journal of Veterinary Medicine, 2023, **26**, No 1, 40–52
ISSN 1311-1477; DOI: 10.15547/bjvm.2021-0022

Original article

MOLECULAR SURVEILLANCE OF AVIAN INFLUENZA A (H5N6) VIRUS IN QUANGBINH PROVINCE, VIETNAM, 2015–2018

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Summary

Pham, H. M., K. H. Pham & Kh. V. Nguyen, 2023. Molecular surveillance of avian influenza A (H5N6) virus in Quangbinh province, Vietnam, 2015–2018. *Bulg. J. Vet. Med.,* **26**, No 1, 40–52.

The haemagglutinin (HA) gene of highly pathogenic (HP) avian influenza H5Nx viruses (AIVs) has undergone continuous evolution, generating emerging reassorted subtypes and clades. Vietnam has been experiencing HPAI outbreaks since 2003, H5N1 and H5N6 virus infections resulted in culling millions of poultry. This paper presents the results of an active AIVs surveillance in domestic poultry, free-grazing domestic ducks and wild waterfowl in QuangBinh province, Vietnam from September 2015 through March 2018. A total of 452 pool cloacal swabs were analysed by real-time RT-PCR (rRT-PCR). The positive rates of the H5 subtype AIVs were 1.8% of chickens, 5.7% of ducks, and 11.1% of wild birds. Most of H5N6 AIVs were identified from apparently healthy domestic ducks and wild waterfowl (grey heron). Molecular analysis of ten H5N6 AIVs detected in 2016–2018 showed that these viruses have the basic amino acid motif at the HA1–HA2 cleavage site associated with HPAIV and belonged to the clade 2.3.4.4.c. H5N6 HPAI detected in wild waterfowl determined the possibility of virus transmission between wild and free-grazing domestic ducks in QuangBinh. This type of study is very novel in QuangBinh province. Surveillance in wild and domestic birds should be implemented for early detection of the virus and efficient control of its spread in this area.

Key words: avian influenza virus, free-grazing domestic ducks, molecular epidemiology, VietNam

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is one of the most concerning infectious diseases in the world. Among eighteen distinct subtypes of HA (H1–H18), the H5 subtype is found to switch to a higher pathogenicity level (Alexander, 2007) and has caused huge economic losses to poultry industry. In addition, several H5Nx influenza A virus (IAV) combinations have also been proven to be zoonotic,

thereby raising global concern for human health (Yu *et al.,* 2017). Since the first detection of A/Gs/Guangdong/1/1996, its H5 haemagglutinin (HA) gene has subsequently evolved into 10 genetically distinct virus clades (0–9) and multiple subclades (Smith & Donis, 2017).

HPAI viruses have been endemic in domestic poultry population of Vietnam since they were first reported in 2003, affecting southern as well as northern parts of the country (Li *et al.,* 2004). Numerous reassortant viruses have been generated resulting from cocirculation of multiple clades and genotypes during the last ten years. Clade 2.3.4.4 H5N6 were likely introduced in the country in April 2014 and appeared to gain dominance across northern and central regions (Nguyen *et al.,* 2017).

Free-grazing domestic ducks, which often allows exposure of ducks to the wild waterfowl populations, is a common management practice in Vietnam. Although the domestic-wild interface is key to the emergence and spread of potentially zoonotic AIV (Cappelle *et al.,* 2014), little is known about the presence of H5 viruses in wild waterfowl and their relationship with other viruses in domestic ducks and chickens in Vietnam. In order to better understand the situation of AIV H5N6, virological surveillance and molecular characterisation of viruses from wild waterfowl and domestic poultry in north-central district of Vietnam were conducted.

MATERIALS AND METHODS

Ethical statement

Swab sample collections were conducted by QuangBinh Sub Department of Animal Health under the guidelines of National Technical Regulation on Animal diseases − General requirements for sample collection, storage and shipment (QCVN01-83:2011/BNNPTNT).

Field sampling

Active avian influenza surveillance was carried out in QuangBinh province, North central Vietnam. One city (DongHoi) and four districts (BoTrach, LeThuy, QuangNinh, QuangTrach) were selected on the basis of their high poultry population density and presence of wild waterfowl, free-grazing duck flocks, and live bird markets (LBMs). From September 2015 through March 2018, 2260 cloacal swab samples were collected from domestic poultry and wild waterfowl. In farms, samples were collected from ducks and chickens. Along with the specimens collected from individual birds, the poultry owners were interviewed about the poultry farming system, types of birds reared, flock size, bird's age and health status during specimen collection. In LBMs, samples were focused on captured birds and free-grazing ducks. Cloacal swab was taken from each bird and placed separately in 1 mL of virus transport medium containing Dulbecco's modified Eagle Medium supplemented with HEPES (20 mM), L-glutamine (2 mM), gentamicin (250 µg/mL), sulfamethoxazole (200 µg/mL), ofloxacin (60 µg/mL), polymyxin B (2000 IU/mL), amphotericin B (2 µg/mL) and bovine serum albumin (2.5%), and kept in cool boxes until it arrived in the laboratory.

Virus identification, full length HA and NA gene amplification, and sequencing

Aliquots from samples of the same species were pooled (up to five invidual samples) and the remainder of each original sample was stored at −80 ^{o}C for future analysis as needed. Viral RNA was extracted from pool swab samples by using the QIAamp

Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. In the screening phase, all pooled samples were tested for type A influenza virus by real time RT-PCR for M gene (Heine *et al.,* 2007). The primers and probe for M gene were: M forward primer (5′-AGATGAGYCTT CTAACCGAGGTCG-3′); M reverse primer (5′-TGCAAANACATCYTCAAGT CT CTG-3′ and probe (FAM)5'-TCAG GCCCCCTCAAAGCCGA-3'(TAMRA). Pools of positive samples for the M gene were then subtyped for H5 (Heine *et al.,* 2007; 2015). The primers and probe for H5 gene were: H5 forward primer (5′-AAACAGAGAGGAAATAAGTGGAGT AAAATT-3'), H5 reverse primer (5′-AAAGATAGACCAGCTACCATGATT GC-3') and probe (FAM)5'TCAACAGT GGCGAGTTCCCTAGCA-3'(TAMRA). Briefly, the QuantiTech Probe RT-PCR Kit (Qiagen) was used with 12.5 µL of the Master Mix, 1.5 µL each of forward and reverse primers (10 µM), 0.5 µL of a probe (5 µM), 0.25 µL of the QuantiTect RT Mix, 3.75 µL of RNase-free water, and 5.0 µL of an RNA template in a 25-µL total volume and the one step real-time RT-PCR was performed. On an ABI 7500 real-time thermocycler (Applied Biosystems, Foster City, CA, USA), reverse transcription was carried out for 15 min at 50 °C, followed by polymerase activation for 2 min at 95 °C. Denaturation for 10 s at 94 °C and annealing with extension for 40 s at 60 °C were conducted for 40 cycles to obtain threshold cycle (Ct) values.

Each of the samples in H5 pool was inoculated individually into 9–11-day-old specific pathogen-free (SPF) chicken eggs (Woolcock, 2008). Following three days of growth the allantoic fluid was harvested. The virus in the allantoic fluid was then checked by real time RT-PCR for M gene and H5 gene and convenient RT-PCR for N1 and N6 gene using primers described previously (Tsukamoto *et al*., 2009): N1F forward primer: 5′- TCA RTCTGYATGRYAAYTGG-3'; N1 reverse primer: 5′-GGRCARAGAGAKGAA TTGCC-3'; N6 forward primer: 5'-AG GAATGACACTATCSGTAGTAAG-3' and N6 reverse primer: 5′-GAYAGR ATRTGCCATGAGTTYAC-3'. One step RT-PCR was conducted with 5 µL of RNA template in a final reaction volume of 50 µL using OneTaq® One-Step RT-PCR Kit (NEB). The PCR cycling was performed as follows: 48 °C for 30 min, 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 3.5 min with a final extension at 68 °C for 10 min.

Sequencing and phylogenetic analysis

The reverse-transcription reaction was carried out at 42 °C for 60 min with the Uni12 primer (5'AGCRAAAGCAGG-3') and M-MLV Reverse Transcriptase (Life Technologies, USA). HA and NA full-length gene segments of H5N6 strains from 2016–2018 were amplified as described previously (Hoffmann *et al.,* 2001). PCR products were separated on a 1.2% agarose gel (FMC, Rockland, ME, USA) and purified by means of a QIAquick PCR Purification Kit (Qiagen). The purified PCR products were sequenced in a commercial laboratory (1st Base, Malaysia) using PCR primers.

The raw sequence data were first checked for quality and then edited and assembled using BioEdit Sequence Alignment Editor Ver 7.2.0 (Hall, 1999). Homologous sequences were obtained by BLAST search at the GenBank (http://ncbi.nlm.nih.gov) along with other reference sequences. The sequences established in this study have been submitted to

Table 1. Accession numbers of nucleotide sequences for avian influenza viruses used in this study

Segment	Accession numbers				
H5	EPI1915870	KT762446	LC342717	KX121198	EPI859212
	MG891807	LC028331	LC342716	LC041319	EPI859650
	LC274942	KM496970	LC342715	KJ938658	EPI860239
	KY402073	KR063687	LC342714	KM251471	KP732646
	KP765796	KT245143	LC342713	AB979455	KU042769
	KU143266	MN566053	LC376796	JQ041401	MF166576
	LC279816	MN809352	LC376797	KU201832	KU646917
	LC164822	MN577280	LC376798	KX838899	KX297866
	KY171708	MN577334	LC376799	MG964840	KX297872
	MG891804	EPI1195331	LC376800	MG965728	KJ413842
	KY272997	EPI1195323	EPI533583	KJ413834	
	LC199868	EPI1327144	KJ754145	KM504101	
	LC198536	KM251463	KP732638	AB932556	
N6	KY273006	KT762448	KY415708	MN809354	LC383940
	KM496967	LC028333	KY415733	MN566063	LC387332
	KM251489	KT245145	LC208493	MN577336	LC387331
	KM251486	KR063689	KP765790	KM251486	LC388385
	EPI915872	KJ754147	EPI1195330	LC383944	LC388384
	KY402075	KP732679	EPI1195322	LC383943	
	KY273005	KX121200	EPI1327143	LC383942	
	LC199870	KY415714	MN566055	LC383941	

GenBank/EMBL/DDBJ under accession No: LC342713-LC342717; LC376796-LC376799; LC383940-LC383944; LC38 7331-LC387332; LC388384-LC388385 (Table 1). The full-length nucleotide sequences of the HA gene and NA gene were compared with those in selected representative isolates obtained from the National Center for Biotechnology Information (NCBI) and EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID). Multiple sequence comparison was performed by the FASTA program at the DDBJ web site (http://clustalw.ddbj.nig.ac.jp). ClustalW version 2.1 was used to produce multiple alignments. Phylogenetic analysis was inferred by the maximum likelihood (ML) method based on the Tamura-Nei model by using the MEGA 6.0 software. Stability of the branch topology in phylogenetic tree was tested using 1000 bootstrap replicates.

RESULTS

Prevalence of avian influenza virus

A total of 452 pooled samples (2260 samples) including 9 pooled wild bird swab samples, 163 pooled chicken swab samples, and 280 duck swab samples from five places were collected in QuangBinh province during September 2015–March 2018. Detection rates for avian influenza A were 25.5% of chickens, 27.9% of ducks and 33.3% of wild birds (Table 2). H5 was detected in 1.8% of chickens, 5.7% of ducks, and 11.1% of wild birds. N6 was detected in 1.2% of chickens, 5% of ducks, and 11.1% of wild birds (grey herons). N1 was detected in 0.6% of chickens and 0.7% of ducks. The numbers of AIV positive samples corresponding to the yearly detection rate ranged from

Table 2. Surveillance statistics for Influenza A virus in QuangBinh province, VietNam during 2015–2018

		Number of samples	Type/subtype, no. (%)*			
			Influenza A	H5	N1	N6
Bird species	Chickens	163	40 (24.5)	3 (1.8)	1 (0.6)	2 (1.2)
	Ducks	280	78 (27.9)	16 (5.7)	2 (0.7)	14 (5)
	Wild waterbirds	9	3 (33.3)	1 (11.1)	0 (0.0)	1 (11.1)
Year	2015	85	10 (11.8)	1 (1.2)	0 (0.0)	1 (1.2)
	2016	60	15 (25.2)	5 (8.3)	0 (0.0)	5 (8.3)
	2017	181	53 (29.3)	5 (2.8)	2 (1.1)	3 (1.7)
	2018	126	43 (34.1)	9 (7.1)	1 (0.8)	8 (6.4)
Total		452	121 (26.8)	20 (4.4)	3 (0.7)	17 (3.8)

*no. – number of positive samples; % –detection rate.

11.8% to 31.1%. In 2018, the highest rate was detected (31.1%), followed by 2017 (29.3%), 2016 (25.2%), and 2015 (11.8%). H5N6 virus was detected in all four years (2015–2018) and the detection rate in 2016 was higher than the rates in the other three years. H5N1 virus was detected only in 2017 and 2018. The higher detected rate (1.1%) was in 2017.

Phylogenetic analysis and molecular characterisation

In this study, the HA and NA genes of 10 H5N6 viruses detected in 2016–2018 from wild birds, chicken, and ducks were newly sequenced, annotated, and confirmed. Sequence comparison disclosed high nucleotide identity amongst HA genes of the H5N6 viruses isolated in 2016–2018 (up to 99%). HA genes of the H5N6 viruses isolated in 2016 shared highest and lowest sequence similarity with H5N6 viruses isolated in 2017–2018: 98% and 96%, respectively (Table 3). Higher nucleotide identity was observed amongst NA genes ranging from 98–99% (Table 3).

The phylogenetic tree based on the HA and NA gene segments showed that all H5N6 subtypes fell within genetic clade 2.3.4.4 group C, found in China, Taiwan, South Korea, and Japan during 2016–2019 (Fig. 1 and 2). These viruses showed a HA cleavage site with multiple basic amino acids, PLRERRRKR/GLF (Table 4), which is characteristic of HPAIV. The receptor-binding pocket of 10 H5N6 subtypes retained the amino acid residues Q226 and G228 (H3 numbering). None of them contained one amino acid deletion at position 133 of HA1 (Table 4). NA stalk of all these viruses had the 11 amino acid deletion at positions 59–69 (N6 numbering). No drug resistance-associated mutation (H274Y) was observed in the NA protein (Table 4).

DISCUSSION

AIVs are distributed worldwide, with a global impact on animal and human health. When a poultry bird, such as a duck, is infected with multiple subtypes of AIV, the eight gene segments from the different HA subtypes may exchange genetic information with each other, which

Table 3. Nucleotide sequence homology of HA and NA segments of H5N6 HPAI viruses isolated in QuangBinh

	Strain names	Nucleotide identity (%)									
		1	2	3	4	5	6	7	8	9	10
	HA segment similarities										
1	A/duck/Vietnam/QuangBinh/LBM0818/2016 (H5N6)	100									
2	A/duck/Vietnam/QuangBinh/LBM0908/2016 (H5N6)	99	100								
3	A/duck/Vietnam/QuangBinh/LBM0909/2016 (H5N6)	99	99	100							
4	A/Heron/Vietnam/QuangBinh/LBM0910/2016 (H5N6)	99	99	99	100						
5	A/duck/Vietnam/QuangBinh/LBM0911/2016 (H5N6)	99	99	99	99	100					
6	A/chicken/Vietnam/QuangBinh/BD1113/2017(H5N6)	98	97	97	97	98	100				
7	A/chicken/Vietnam/QuangBinh/BT1113/2017(H5N6)	96	96	96	96	96	98	100			
8	A/duck/Vietnam/QuangBinh/DH330718/2017(H5N6)	96	96	96	96	96	98	99	100		
9	A/duck/Vietnam/QuangBinh/DH130723/2017(H5N6)	97	97	97	97	97	98	98	98	100	
10	A/duck/Vietnam/QuangBinh/QN530206/2018(H5N6)	96	96	96	96	96	98	99	99	98	100
	NA segment similarities										
1	A/duck/Vietnam/QuangBinh/LBM0818/2016 (H5N6)	100									
2	A/duck/Vietnam/QuangBinh/LBM0908/2016 (H5N6)	98	100								
3	A/duck/Vietnam/QuangBinh/LBM0909/2016(H5N6)	99	99	100							
4	A/Heron/Vietnam/QuangBinh/LBM0910/2016(H5N6)	98	98	99	100						
5	A/duck/Vietnam/QuangBinh/LBM0911/2016(H5N6)	99	99	99	98	100					
6	A/duck/Vietnam/QuangBinh/DH130723/2017(H5N6)	99	99	99	99	99	100				
7	A/duck/Vietnam/QuangBinh/DH330718/2017(H5N6)	99	99	99	99	99	99	100			
8	A/chicken/Vietnam/QuangBinh/BD1113/2017(H5N6)	99	99	99	99	99	99	99	100		
9	A/chicken/Vietnam/QuangBinh/BT1113/2017(H5N6)	99	99	99	99	99	99	99	99	100	
10	A/duck/Vietnam/QuangBinh/QN530206/2018(H5N6)	98	98	98	98	98	98	99	99	98	100

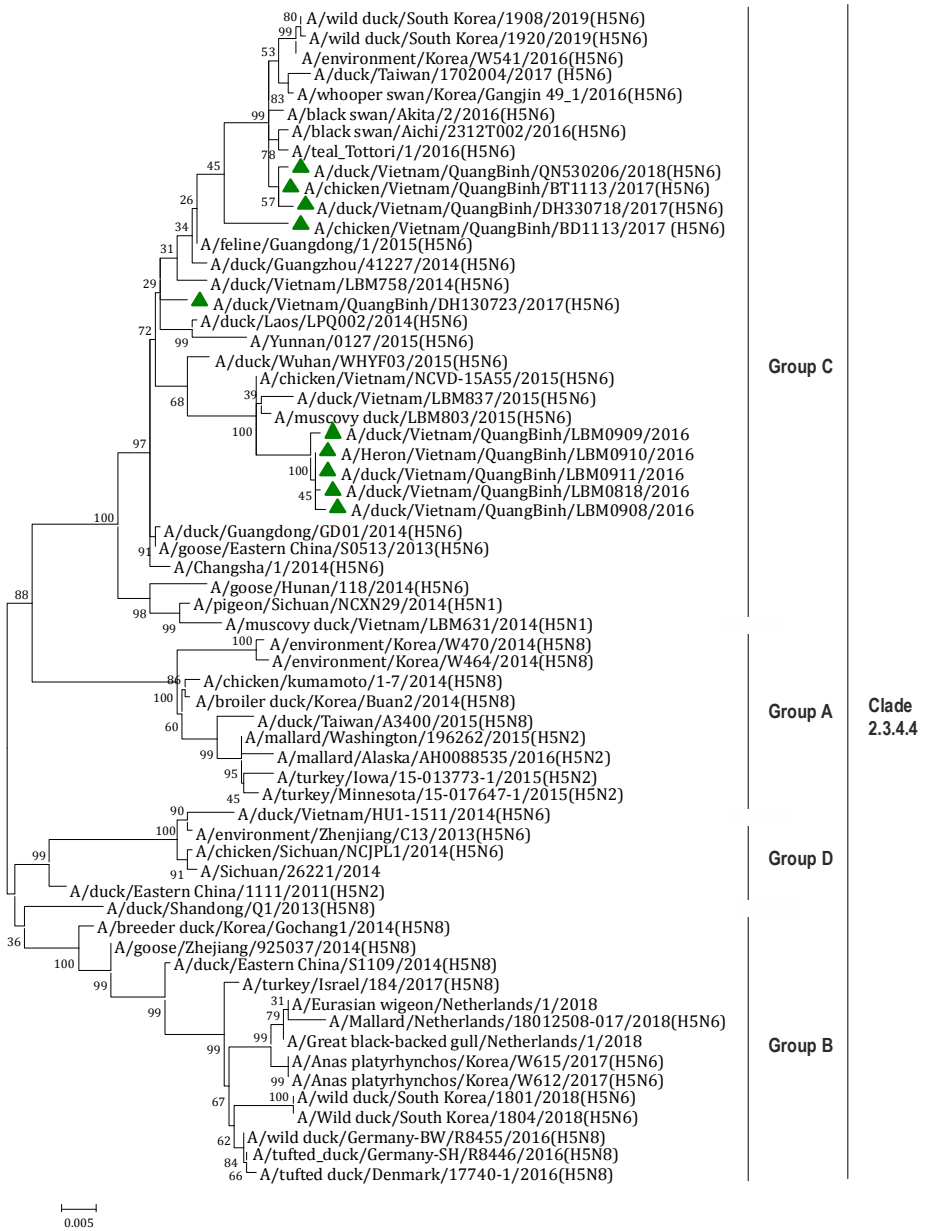

Fig. 1. Phylogenetic trees for HA gene of H5N6 influenza A viruses isolated in QuangBinh province, VietNam. The full-length HA genes of ten viruses were compared with H5Nx in selected representative isolates obtained from NCBI and GISAID. Maximum-likelihood (ML) phylogenetic trees were inferred with MEGA 6.0 software under the Tamura-Nei model with 1,000 bootstraps. The deposited GenBank accession numbers of HA genes are LC342713–LC342717 and LC376796–LC376800. Green filled triangle indicates viruses isolated in this study. The numbers at the branches show ML bootstrap support values. The bar at the bottom of the figure denotes the distance.

can lead to the emergence of a novel AIV and a potentially unpredictable pathogenic strain that could infect humans (Luo *et al.,* 2017). Wild waterfowl pose a potential risk to biosecurity because they can transfer pathogens into poultry farms. Therefore, disease surveillance in wild birds and domestic birds has become unavoidable in order to prevent outbreaks of avian diseases.

The results of the present study showed that the detected rates of AIV and A (H5) viruses were the highest in wild-birds and the lowest in chickens. Most of

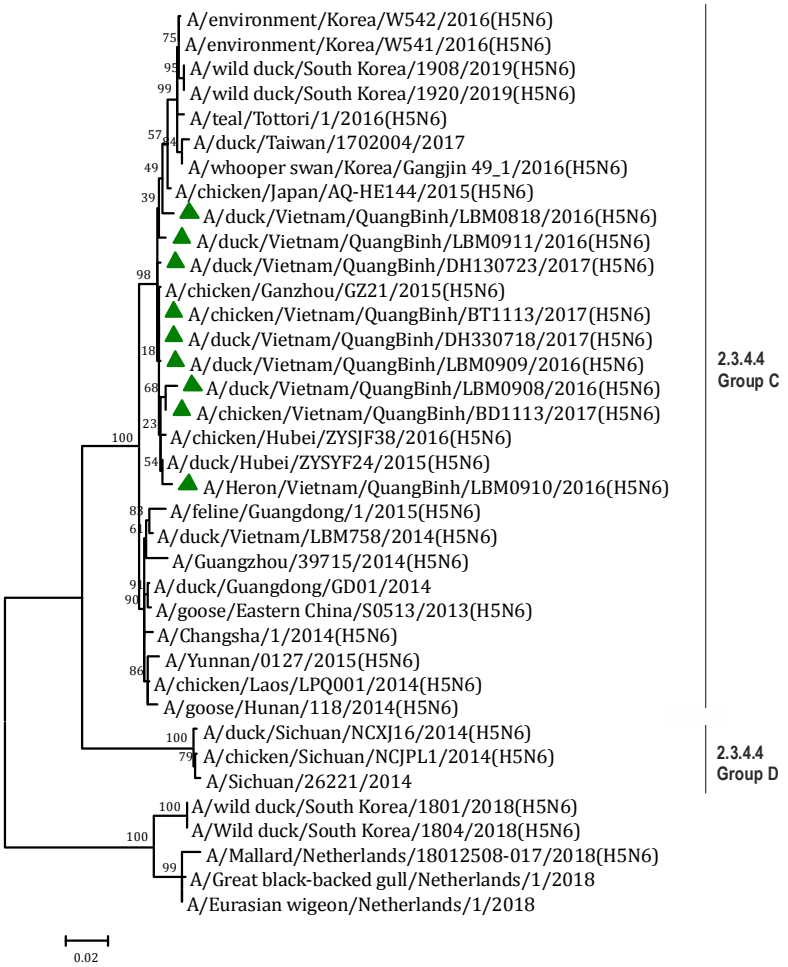

Fig. 2. Phylogenetic trees for NA gene of H5N6 influenza A viruses isolated in QuangBinh province, VietNam. The full-length nucleotide sequences of the NA gene were compared with those in selected representative isolates obtained from NCBI and GISAID. Maximum-likelihood (ML) phylogenetic trees were inferred with MEGA 6.0 software under the Tamura-Nei model with 1,000 bootstraps. The deposited GenBank accession numbers of NA genes are LC383940–LC383944, LC387331–LC387332, and LC388384–LC388385. Green filled triangle indicates viruses isolated in this study. The numbers at the branches show ML bootstrap support values. The bar at the bottom of the figure denotes the distance.

Table 4. Molecular characterisation of H5N6 viruses in QuangBinh compared with similar isolates

Viruses*	HA (H3 numbering)								NA	
	Cleavage site	158	160	224	226	228	318	HA deletion 133	274†	NA stalk deletion 59–69
A/duck/Vietnam/QuangBinh/LBM0909/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/LBM0908/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/Heron/Vietnam/QuangBinh/LBM0910/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/LBM0818/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/LBM0911/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/DH130723/2017	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/DH330718/2017	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/chicken/Vietnam/QuangBinh/BD1113/2017	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/chicken/Vietnam/QuangBinh/BT1113/2017	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/duck/Vietnam/QuangBinh/QN530206/2018	RERRRKR/GLF	N	A	N	Q	G	T	No	H‡	Yes‡
A/teal/Tottori/1/2016	RERRRKR/GLF	N	A	N	Q	G	T	No	H	Yes
A/whooper swan/Korea/Gangjin 49_1/2016	RERRRKR/GLF	N	A	N	Q	G	T	Yes	H	Yes
A/duck/Taiwan/1702004/2017	RERRRKR/GLF	N	A	N	Q	G	T	Yes	H	Yes
A/wild duck/South Korea/1801/2018	RERRRKR/GLF	N	A	N	Q	G	T	No	H	No
A/wild duck/South Korea/1920/2019	RERRRKR/GLF	N	A	N	Q	G	T	Yes	H	Yes

*The viruses in bold are the influenza A (H5N6) viruses isolated in this study; † N2 numbering; ‡ This NA gene sequence was not deposited into GenBank/ EMBL/DDBJ.

the H5 viruses were detected from apparently healthy domestic ducks and wildbirds. Ducks in QuangBinh raised in scavenging systems that roam in rivers and other water bodies such as ponds, ditches, other wetlands and rice fields. Raising ducks in this way is associated with a low level of biosecurity and these birds might acquire IAVs through ingestion of water contaminated by wildbirds and, therefore, is considered a high risk factor for HPAI in Vietnam (Gilbert *et al.,* 2006).

The intensive rice cultivation region in QuangBinh hosts a number of grey herons (*Ardea cinerea*), little egrets (*Egretta garzetta*), white-breasted waterhen (*Amaurornis phoenicurus*) or black-crowned night herons using rice field for foraging habitat. These birds having been caught from wild, are used as a source of food by local population. The detection of H5N6 AIV in a pooled grey heron swab sample which was taken from healthy captured birds raised the possibility that wild birds might carry H5 viruses to other areas and risk for human exposure to AIVs. On the other hand, capturing and selling migratory birds at live bird markets where domestic and resident wild bird species were sold enabled the exchange of genetic material between viruses infecting the different species (Hassan *et al.,* 2018). The detection of H5 viruses at different times indicated that these viruses circulate continuously in the province and might be dispersed either via poultry-to-poultry transmission or wild birds.

In previous phylogenetic analyses, the HA gene of clade 2.3.4.4 viruses was divided into 4 distinct subgroups. Group intercontinental A (icA) contains H5N8 subtype virus and its reassortant viruses identified in China, South Korea, Japan, Taiwan, Canada, the United States, and countries in Europe during 2013–2016. Group B contains H5N8 subtype viruses identified in China and South Korea during 2013–2014, and in Russia in late 2016. Group C contains H5N1 and H5N6 subtype viruses identified in China, Vietnam, Laos, and Hong Kong, including isolates from humans in Guangdong, Yunnan, and Hunnan Provinces, China. Group D contains H5N6 subtype viruses identified in China and Vietnam, including an isolate from a human in Sichuan Province, China (Lee *et al.,* 2016). Recently, novel reassortant Group C H5N6 was found in Japan, South Korea, and Taiwan during 2016–2017 and 2019 (Kwon *et al.,* 2017; Okamatsu *et al.,* 2017; Chen *et al.,* 2018; Shin *et al.,* 2020). This genotype had independently evolved and had been maintained in wild bird populations in the bird flyway of East Asia, highlighting the important role of wild waterfowl in the maintenance and dissemination of this HPAIV (Chen *et al.,* 2018). All 9 H5N6 viruses detected in 2016–2017 in QuangBinh belong to novel reassortant Group C H5N6 HPAIVs suggesting that these viruses originating from China might be introduced to Vietnam, Korea and Japan by wild birds during 2016–2017.

Although sequence analysis suggested that these viruses preferentially recognised the avian influenza virus receptor (α-2,3 galactose sialic acids) (Ha *et al.,* 2001), they had no deletion of HA1 133A, which was a mutation commonly found in the 2.3.4.4 HA genes of human infectious influenza A(H5N6) viruses (Yu *et al.,* 2017) and no H274Y NA mutation indicating that these H5 isolates might be sensitive to neuraminidase inhibitor (Suzuki *et al.,* 2003). However, they had the stalk domain deletion in the NA protein, which associated with the enhancement of the viral replication ability and pathogenicity

to mice, as well as with a better ability to be transmitted in poultry (Matsuoka *et al.,* 2009; Munier *et al.,* 2010; Li *et al.,* 2011; Yu *et al.,* 2017). Thus, it's possible that they could pose considerable threats to public health.

The endemic situation, continued evolution of various reassortant HPAIVs in domestic poultry and a new introduction mediated by migratory birds warrants poses concern about further reassortment. Enhanced active surveillance in domestic fowl and controlling the market of wild birds and live poultry are required to monitor the spread and onward reassortment and to design improved prevention and control strategies of HPAIV in Vietnam.

ACKNOWLEDGEMENTS

The authors wish to thank all participating farmers and officials in QuangBinh provinces for their cooperation and help. This work was supported by Ministry of Agriculture and Rural Development of Vietnam (No. 1264/ QĐ-BNN-KHCN), QuangBinh Department of Agriculture and Rural Development (No. 152/QĐ-SNN), and QuangBinh Department of Science and Technology (No. 09/QĐ-SKHCN-CN).

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Paper received 03.03.2021; accepted for publication 20.06.2021

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