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EVOLUTIONARY CHARACTERIZATION OF CLADES 2.3.4.4 H5N6 AND 2.3.2.1C H5N1 HPAI VIRUSES IN VIETNAM (2013–2019) REVEALED DISTINCT REASSORTANTS FROM DISTANT SPILLOVERS

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SUMMARY

Highly pathogenic avian influenza (HPAI) H5Nx viruses have continually undergone multiple evolutionary dynamics for the generation of various clades, subclades, and genotypes where 2.3.2.2c, and 2.3.4.4 become predominant and co-circulating in Vietnam from 2014 to date. In this study, fifteen H5 sequences in our study and 90 from others from different clades, 0, 1, 1.1, 2.3.2.1a, 2.3.2.1c, 2.3.4, 2.3.4.1, 2.3.4.2, 2.3.4.3 and 2.3.4.4 of H5N1, H5N2, H5N6, were characterized for hemagglutinin (HA) properties, genetic and phylogenetic analyses. Blast searching using the dataset of the full length of two H5N6 viruses revealed one strain, e.g., A/Duck/Vietnam/HT7/2014(H5N6) in May 2014, belonging to the Sichuan 2014-lineage of Group D (Minor). The other strain, A/Chicken/Vietnam/NT3/2017(H5N6)/or CkNT3-2017 in the Spring of 2017, belonged to the Japanese-Korean late 2016-cluster of Group C (Major). This cluster possessed 140NHETS-145^{del} stretch of Leucine/Serine deletion at position 145 in HA_1 ($^{S/L}145^{del}$), distinct from all the 2.3.4.4 H5N6 viruses known to date. There has been no report of the similar CkNT3-2017 of 2.3.4.4 reassortant in Vietnam prior to our study. The migration flyway might be the route for transportation of this novel H5N6 virus from Japan to Vietnam. In addition, the topology revealed another novel subclade of H5N6 (2018–2019) possibly, of the Vietnamese internal reassortments. The "H5Nx" viruses in Vietnam, in fact, have continually undergone multiple evolutionary processes in parallel with those lineages in China and East-Asia. Variations at the key sites in HA and altered genetic characteristics in novel HPAI H5Nx viruses in Vietnam present a caution for the vaccination program and the risk for human infection.

Keywords: Avian influenza, reassortment, 2.3.4.4 H5N6 viruses, 2.3.2.1c H5N1 viruses, phylogenetic analysis, Vietnam

INTRODUCTION

Since 1996, the H5 genes of highly pathogenic avian influenza (HPAI) viruses have continuously evolved to generate ten genetically distinct clades (0–9) of which clades 1 and 2 have continued undergoing diversification to form the second-, third-, and fourth-order subclades (Smith *et al*., 2015; Claes *et al*., 2016; Antigua *et al*., 2019). Among these reassortants, clades 2.3.2.1 and 2.3.4.4 seemed to have concurrent circulating in wild birds and domestic poultry in

Asia (Lee *et al*., 2017; Nguyen *et al*., 2019a; Suttie *et al*., 2019). As a such of evolutionary dynamics, H5N1 of clade 2.3.2.1 has further diversified into 2.3.2.1a, b, and c (Smith *et al*., 2015), and recently, into 2.3.4.4 generating reassortants A, B, and C H5N6 viruses by the sequential multiple-step reassortment of HA(H5) and NA between and within the 2.3.2.1c and 2.3.4.4HPAI and various subtype viruses (Claes *et al*., 2016; Yang *et al*., 2017; Zhang *et al*., 2019). Moreover, since 2012 the original Gs/GD/1996 lineage–rooted H5 clade 2.3.4.4 viruses have undergone reassortment of H5 and N1/N2/N3/ N5/N6/N8 genes to develop the unidentified, so-called 2.3.4.4 "H5Nx" viruses expanded to worldwide distribution threatening pandemic potential (Feng *et al*., 2016; Claes *et al*., 2016; Antigua *et al*., 2019). Migratory wild birds and waterfowls have played significant transmission routes and reservoirs for genesis and generation of novel reassortants with the threat to infect domestic poultry and humans (Bi *et al*., 2014; Feng *et al*., 2016; Lee *et al*., 2017; Tsunekuni *et al*., 2019).

Of much concern, 2.3.4.4 H5N8 and 2.3.4.4 H5N6 viruses of this "H5Nx" complex have become predominant and been diversifying into four distinct genetic groups, A, B, C, and D of worldwide dispersion (Bi *et al*., 2016; Lee *et al*., 2017; Si *et al*., 2017). Group A and B comprising H5N8 emerged in countries of North Asia and North America (Japan, Korea, Taiwan, China, Canada, the United States) in 2013–2015 are moving to Europe in recent years (Pohlmann *et al*., 2019; King *et al*., 2020); Group C and D of H5N1 and H5N6 viruses were identified in China, Laos, Vietnam in 2013–2014, recently in Vietnam, Japan, Korea, Taiwan, and Russia (Lee *et al*., 2017; Chen *et al*., 2017; Takemae *et al*., 2017; Nguyen *et al*., 2017; Nguyen *et al*., 2019a, b; Susloparov *et al*., 2019; Baek *et al*., 2020). According to the number of H5N6 viruses clustered in each group, Group C and Group D are designated as Major and Minor groups in the phylogenetic tree construction (Bi *et al*., 2016; Takemae *et al*., 2017). Becoming common, all "H5Nx" viruses possess multiple basic amino acids of PLRE/RRRKR/G, with one Lysine (K) being deleted compared to the ancestral GD1/1996 and historic H5N1 viruses of clades 0 and 1, at the cleavage site of the hemagglutinin (HA) between HA_1 and HA_2. A deglycosylation occurrence at site 158 in the HA_1 was noted due to mutation of amino acid T to A ($T^{160}A$) affecting the receptor-binding properties (Gao *et al*., 2018; Antigua *et al*., 2019).

In Vietnam, HPAI H5N1 viruses of clades 2.3.2.1 and H5N6 of 2.3.4.4 have been identified in wild and domestic ducks, chickens, and quails since 2012 (Creanga *et al*., 2013; Le, Nguyen, 2014; Thanh *et al*., 2018; Nguyen *et al*., 2019a). The emergence of subclades 2.3.2.1a, 2.3.2.1b, and 2.3.2.1c viruses were traced back to 2009 with those of genetic similarity of the real-time Chinese strain origins and the subclade 2.3.2.1c viruses soon became predominant, continuing to cause outbreaks in poultry and wild birds (Creanga *et al*., 2013; Le, Nguyen, 2014; Nguyen *et al*., 2017; Nguyen *et al*., 2019a; Suttie *et al*., 2019). The 2.3.4.4 H5N1 and the reassortant 2.3.4.4 H5N6 viruses were first reported in Vietnam in 2014 and likely introduced by a single source from China until 2017 (Nguyen *et al*., 2017; Tsunekuni *et al*., 2019). The 2.3.2.1c H5N1 HPAI Vietnamese viruses remain to have homologous HA(H5) segment derived from those introduced from China during 2012–2013, while the H5 genes of 2.3.4.4 H5N6 Vietnamese viruses were heterologous, aggregated from different reassortants of China and possibly, of spillovers of foreign strains (Nguyen *et al*., 2019a). However, many previous studies up to date showed that the predominant H5 2.3.2.1c and H5 2.3.4.4 Vietnamese viruses have multiple genetic linkages with Chinese H5Nx viruses, particularly been generated from those brought over by migratory birds (Nguyen *et al*., 2015; Nguyen *et al*., 2017; Nguyen *et al*., 2019a, b; Tsunekuni *et al*., 2019). No detection was reported from any other foreign spillovers rather than China which might play an initial source for the emergence of another imported novel reassortant(s) in Vietnam. We have sequenced the full length (8

segments) of the genome for two H5N6 isolates in Vietnam, including A/Duck/Vietnam/HT7/ 2014(H5N6) isolated on 14 May 2014 from a duck in Ha Tinh Province (abbreviated as DkHT7-2014) and A/Chicken/Vietnam/NT3/ 2017(H5N6) isolated on 15 March 2017 from a chicken in Nha Trang city (CkNT3-2017), respectively); and HA(H5) and NA(Nx) genes from a number of various H5N1 and H5N6 isolates, 2013–2017, collected in our study. The analysis of the complete H5 sequences obtained from our study and from other sources was conducted for clarification of the origin and the evolution of the multiple H5 linkages in Vietnam.

Given the possibility of the persistence of the current, or the emergence of new or novel genotypes/(sub)clades of H5N1 and H5N6 or any H5Nx viruses in Vietnam, where open live-bird markets, busy transboundary poultry trading, and unexpected stopovers of migratory birds are encountered (Chu *et al*., 2016; Zhang *et al*., 2018; Mellor *et al*., 2018; Vergne *et al*., 2019; Nguyen *et al*., 2019b), this study provides useful data for evaluating the evolutionary progress of avian influenza viruses and the risk of the next H5Nx infection in poultry and humans in Vietnam and the surrounding regions.

MATERIALS AND METHODS

Tissue and RNA samples and ethical statement

In this study, swabs or tissues of clinically infected or dead poultry including chickens, ducks, and quails were taken by the provincial veterinarians in 2013, 2014, 2016, and 2017 in Provinces/Cities of northern and central Vietnam, such as Ha Noi (21°1'39.95" N, 105°50'2.976" E), Ha Tinh (18°20'34.15" N, 105°54'20.48" E), Quang Tri (16°44'48.84" N, 107°11'38.40" E) and Khanh Hoa (12°15'30.636" N 109°3'9.389" E)/Nha Trang city (12°14'19.648" N, 109°11' 48.296" E).

Total viral RNA was extracted directly from the supernatant of the processed samples in the provincial or in our laboratories, using TRIzol Reagent (Invitrogen, San Diego, USA), or QIAamp Viral RNA Mini Kit (QIAGEN Inc., Hilden, Germany) following the manufacturer's instructions. The RNAs were first tested for the presence of avian influenza virus by RT-PCR according to the guidelines for evaluation and the assessment of the molecular criteria from the OIE Terrestrial Manual 2015/2018 (World Organisation for Animal Health, https://www.oie.int/). cDNA was synthesized using a Maxima Reverse Transcriptase kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) with random hexamer and universal primers for all influenza A viruses described in Hoffmann *et al*. (2001) and stored at –20°C. The bird sample collection was approved by the Department of Animal Health (DAH) of the Vietnamese Ministry of Agriculture and Rural Development (MARD) and carried out in accordance with licenses from the MARD. The laboratory work was approved by the Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST), number 1442014.

Sequencing and sequence analysis

Primers and the protocol described by Hoffmann *et al*. (2001) were used for amplification of HA and NA segments from all samples in this study and the full length of the genome of the DkHT7-2014 and CkNT3-2017 isolates. The PCR products were sequenced directly, or after cloning using the pCR2.1-TOPO TA-cloning vector (Invitrogen, USA) from both ends, by a commercial service, Macrogen Inc. (Seoul, South Korea). Additional internal primers were designed for sequencing of long products (ie., for PB2, PB1, PA, and HA). GenBank accession numbers: DkHT7-2014: MT297571–MT297578 (segments 1–8); CkNT3-2017: MT298096–MT298103 (segments 1–8).

Sequences obtained in this study were used to search for similarity by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and used with those of reference strains from

GenBank for molecular analysis. BLAST was also used for searching the viruses in GenBank matching the highest nucleotide identity (%) for 8 protein-coding genes of the DkHT7-2014 and CkNT3-2017 viruses, respectively.

Phylogenetic analyses

To construct a phylogenetic tree, we had collected and made an alignment of 105 complete H5 nucleotide sequences including 15 HA sequences in this study (2013–2017) and 90 available sequences from GenBank (those isolated during 2013–2019 in Vietnam). These strains represented clades and subclades 0, 1, 1.1, 2.3.2.1a, 2.3.2.1c, 2.3.4, 2.3.4.4 (a majority are listed in Table 2). The alignment was carried out using GENEDOC 2.7 (http://iubio.bio.indiana.edu/soft/ molbio/ibmpc/genedoc-readme.html), confirmed by MAFFT 7.122 (Katoh, Standley, 2013) and used for phylogenetic tree construction by MEGA 7.0 (www.megasoftware.net), with a maximum-likelihood method tested by bootstrapping with 1000 replications (Kumar *et al*., 2016). The substitution model with the best score according to the Bayesian information criterion was the Jones, Taylor & Thornton +F + G + I model, with residue frequencies estimated from the data(+F), rate variation along the length of the alignment (+G), and allowing for a proportion of invariant sites (+I).

RESULTS

Genetic characterization of two Vietnamese 2.3.4.4 H5N6 viruses (DkHT7-2014 and CkNT3-2017)

To investigate the genetic similarity of two Vietnamese H5N6 viruses of this study (A/Duck/Vietnam/HT7/2014(H5N6), abbreviated as DkHT7-2014, and A/Chicken/Vietnam/ NT3/2017(H5N6), as CkNT3-2017), full protein-coding nucleotide sequences of each segment were used for BLAST searching and the highest blast scoring virus sequences from GenBank were recorded (Table 1). As result, both were identified as 2.3.4.4 H5N6 reassortants.

The DkHT7-2014 belonged to the A/chicken/Sichuan/NCJPL1/2014(H5N6)-like virus lineage (clade 2.3.4.4), of reassortant C (Yang *et al*., 2017) or of Group D (Minor) (Tsunekuni *et al*., 2019) detected in chickens and ducks between April and June 2014 (Bi *et al*., 2015). A Blast-search indicated that there was over 99% (99.22–99.80%) nucleotide identity for the polymerase complex (PB2, PB1, PA), HA(H5), NA(N6), and NP genes to the reference A/chicken/Sichuan/NCJPL1/2014(H5N6) and near 100% for M and NS genes to the A/environment/Chang Sha/399/2014(H5N6) and A/mig.waterfowl/Hubei/Chenhu1347/2014 (H5N6) strains, respectively (Table 1). Sichuan of Southwestern China is one of the "epicenters" where a "gene pool" was likely pertained for the generation of new reassortant H5N6 viruses giving ways of northbound and southbound transmissions (Zhang *et al*., 2018). The actual detection in May 2014 in a northern province, Ha Tinh, Vietnam and the high hits of nucleotide identity of this 2.3.4.4 H5N6 Vietnamese strain may lead to our assumption of being concurrently introduced into Vietnam from the Sichuan territory of China by, possibly, migratory birds during the Spring of 2014.

The CkNT3-2017, on the Blast search in GenBank, hits very high nucleotide identity for HA(H5), NA(N6) and the polymerase complex (PB2, PB1, PA) to the cluster of the Japanese-Korean 2.3.4.4 H5N6 viruses (referred to as the Japanese-Korean late 2016-cluster), all were isolated from the wild birds and environment in November-December 2016, showing 99.59–99.78% identity to the highest matching A/n.gosHawk/Tochigi/0912A004/2016(H5N6) and A/tundra swan/Tottori/3111S001/ 2016(H5N6) strains (Okamatsu *et al*., 2017; Takemae *et al*., 2017; Baek *et al*., 2020). The other three genes (NP, M, NS) of CkNT3-2017 showed close identity (98.66– 99.90%) to a Chinese (A/goose/Guangdong/GS014/2015 (H5N6)) and two Vietnamese strains (A/muscovy duck/Viet Nam/HN-2506/2015; and A/duck/Viet

Nam/HN-2520/2015 (H5N6)), of isolation dated to late 2015 from domestic poultry (Table 1). The progenitor viruses of the Japanese-Korean late 2016-cluster were predicted to be transported into Japan by migratory birds from China then disseminated from Japan to Korea and possibly to Vietnam in the winter, Fall 2016, or Spring 2017 (Takemae *et al*., 2017) (Table 2).

Table 1. Strains giving the highest nucleotide sequence identity for eight protein-coding genes of A/Chicken/Vietnam/NT3/2017 (H5N6) and A/Duck/Vietnam/HT7/2014(H5N6).

Gene/ segment	Length (bp)	Viruses matching the highest nucleotide identity*	Accession No (GenBank)	Identity (%)	Date of collection	Reference
A/ Chicken/ Vietnam/ NT3/ 2017 (H5N6)						
PB2	2280	A/n.gosHawk/Tochigi/0912A004/2016(H5N6)	LC306914	99.78	2016-12-12	Okamatsu *et al*. (2017)
PB1	2274	A/tundra swan/Niigata/1/2016(H5N6)	LC318894	99.63	2016-12-08	Okamatsu *et al*. (2017)
PA and PA-X	2151	A/tundra swan/Tottori/3111S001/2016(H5N6	LC274917	99.63	2016-11-20	Okamatsu *et al*. (2017)
HA	1701	A/tundra swan/Tottori/3111S001/2016(H5N6)	LC274918	99.59	2016-11-20	Okamatsu *et al*. (2017)
NP	1497	A/goose/Guangdong/GS014/2015(H5N6)	MN128314	99.60	2015-12-16	GenBank
NA	1380	A/n.gosHawk/Tochigi/0912A004/2016(H5N6)	LC306916	99.78	2016-12-12	Okamatsu *et al*. (2017)
M (M1/M2)	982	A/muscovy duck/Viet Nam/HN-2506/2015	MK943423	99.90	2015-10-25	GenBank
NS (NS1/NS2)	823	A/duck/Viet Nam/HN-2520/2015(H5N6)	MK943269	98.66	2015-10-25	GenBank
A/Duck/Vietnam/HT7/2014(H5N6)						
PB2	2280	A/chicken/Sichuan/NCJPL1/2014(H5N6)	KM251533	99.74	2014-4-27	Bi *et al*., 2015
PB1	2274	A/chicken/Sichuan/NCJPL1/2014(H5N6)	KM251526	99.60	2014-4-27	Bi *et al*., 2015
PA and PA-X	2151	A/chicken/Sichuan/NCJPL1/2014(H5N6)	KM251513	99.58	2014-4-27	Bi *et al*., 2015
HA	1704	A/chicken/Sichuan/NCJPL1/2014(H5N6)	KM251493	99.71	2014-4-27	Bi *et al*., 2015
NP	1497	A/chicken/Sichuan/NCJPL1/2014(H5N6)	KM251493	99.80	2014-4-27	Bi *et al*., 2015
NA	1413	A/duck/Sichuan/NCXJ15/2014(H5N6)	KM251488	99.22	2014-4-27	Bi *et al*., 2015
M (M1/M2)	982	A/environment/Chang Sha/399/2014(H5N6)	MH156521	99.59	2014-9-18	GenBank
NS (NS1/NS2)	823	A/mig.waterfowl/Hubei/Chenhu1347/2014(H5N6)	KP083463	100%	2014-2-26	Bi *et al*., 2016

*For these two Vietnamese strains, for each dataset there are more than ten viruses matching over 99% nucleotide identity, but only one possessing the highest hit is presented in Table 1 (see Text for more description).

Characteristics of HA(H5) sequences 2013–2019

We have characterized properties of H5 hemagglutinin polypeptide for 15 HA(H5) obtained in our study and 54 other sequences representing clades 2.3.4.4 of H5N6, clades 2.3.4.3, 2.4.4.2, 2.4.4.1, 2.3.4, 2.3.2.1c, 2.3.2.1a, 1.1, 1, and 0 of H5N1 viruses (listed in Table 2). Molecular analysis demonstrated that all H5N6

viruses of 2.3.4.4 reassortant possess polybasic residues (PLRE/RRRKR/G) at the proteolytic cleavage site of HA(H5) (based on H5 numbering, 340/341–346/347) between HA_1 and HA_2 except for some Vietnamese 2.3.4.4 H5N6 strains of which this motif is PLRE/KRRKR/G including DkHT7-2014 of the genetic similarity to the early Sichuan-2014(H5N6)-like virus lineage.

The main receptor-binding domain (RBD) at position 238–240 in the Vietnamese NT3-2017 strain and the Japanese-Korean late 2016-cluster contained 238QQG240, distinct from other 2.3.4.4 H5N6 (QRG) and QSG of H5N1 viruses (Table 2). The potential N-link glycosylation at position 170–172 in HA(H5) has been changed to a completely non-glycosylated site in all the 2.3.2.1c H5N1 (NST to DNA) and 2.3.4.4 H5N6 viruses (N(N/D)T to NDA) induced by mutation of amino acid T (Threonine) to A (Alanine) ($T^{172}A$ in H5 numbering in our study or $T^{160}A$ in H3 numbering), facilitating the dual α-2,3 and α-2,6 receptor binding properties (Gao *et al*., 2018). One of the most remarkable distinctness for the CkNT3-2017 strain and the Japanese-Korean late 2016-cluster H5N6 viruses was the deletion of a codon for Leucine (L) or Serine (S) at position 145 resulting in a mutation of $^{L/S}145^{del}$ in the HA_1. The deletion $^{L/S}145^{del}$ has modified the antigenic epitope stretch to 140NHETS-145^{del}, completely different from 140NHETS(S/L)145 as seen common in 2.3.4.4 H5N6 viruses of other H5Nx lineages (Table 2).

Table 2. Properties of the H5 hemagglutinin polypeptide sequences and HA amino acid variations at the HA cleavage site, receptor-binding domain (RBD), antigenic epitope sites and the variable glycosylation sites (H5 numbering).

				HA amino acid variations								
	Strains and Accession No	**Country**	**Clade**	**RBD** (238-240)	145	69	99-102	140-145	152-157	**De/Glyco/** (170-172)	**HA cleavage site** (340/341–345/346)	**Accession No**
1	A/Chicken/Vietnam/NT3/2017(H5N6)	VN	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	This study
2	A/tunSwan/Tottori/3111s001/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC274918
3	A/tunSwan/Niigata/5112006/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC316699
4	A/Duck/Tochigi/0902C033/2017(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**LC309005**
5	A/Chicken/Hokkaido/002/2017(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC318472
6	A/bGoose/Ishikawa/1701A012/2017(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**LC275037**
7	A/ngosHawk/Tochigi/0912A004/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC306914
8	A/whSwan/Ibaraki/28309/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC314502
9	A/muSwan/Amori/4/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	LC318639
10	A/env/Gifu/21/2017(H5N6)	JP	2.3.4.4	QQG	-	K	ANPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**LC306922**
11	A/Wswan/Tochigi/1/2017(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	SYQGVP	NDA	PLRE/RRRKR/G	**LC318944**
12	A/Wigeon/Aichi/2301H025/2017(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**LC274997**
13	A/bswan/Aichi/2312T001/2016(H5N6)	JP	2.3.4.4	QQG	-	K	TNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**LC274934**
14	A/CTeal/KR/W559/2017(H5N6)	KR	2.3.4.4	QQG	-	K	NNPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**KY576117**
15	A/env/KR/W544/2016(H5N6)	KR	2.3.4.4	QQG	-	K	ANPA	NHETS-	PYQGVP	NDA	PLRE/RRRKR/G	**KY273000**
16	A/Duck/Vietnam/QB/QN530206/2018(H5N6)	VN	2.3.4.4	QQG	S	K	TNPA	NHETSS	PYQGVP	NDA	PLRE/RRRKR/G	**LC376800**
17	A/Chicken/Vietnam/QB/BT1113/2017(H5N6)	VN	2.3.4.4	QQG	S	K	TNPA	NHETSS	PYQGVP	NDA	PLRE/RRRKR/G	**LC376799**
18	A/Duck/Vietnam/QB/DH330718/2017(H5N6)	VN	2.3.4.4	QQG	S	K	TNPA	NHETSS	PYQGVP	NDA	PLRE/RRRKR/G	**LC376797**
19	A/Chicken/Vietnam/QB/BD1113/2017/H5N6	VN	2.3.4.4	QQG	S	K	TNPA	NHETSS	PYQGVP	NDA	PLRE/RRRKR/G	**LC376798**
20	A/cpHeron/Vietnam/WBT231/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGVP	NDA	PLRE/RRRKR/G	**KR135375**
21	A/Duck/Vietnam/LBM760/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGVP	NDA	PLRE/RRRKR/G	**LC028347**
22	**A/Guangzhou/39715/2014(H5N6)(B)**	CN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGVP	NDT	PLRE/RRRKR/G	**KP765788**
23	A/chicken/Vietnam/MT11/2016(H5N6)	VN	2.3.4.4	QRG	L	K	TNPA	NHETSL	PYQAVP	NDA	PLRE/RRRKR/G	This study
24	A/Chicken/Vietnam/15A59/2015(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGMP	NDA	PLRE/RRRKR/G	KY171732
25	A/Duck/Vietnam/QB/DH/2017(H5N6)	VN	2.3.4.4	QRG	L	K	ANPP	NHETSL	PYQGVP	NDA	PLRE/RRRKR/G	**LC376796**
26	A/mDuck/Vietnam/QN/4c111/2013(H5N6)	VN	2.3.4.4	QRG	L	K	ANPP	NHETSL	PYQAVT	NDA	PLRE/KRRKR/G	**LC050591**
27	A/Duck/Laos/LPQ002/2014(H5N6)	LA	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGMP	NDA	PLRE/RRRKR/G	KM496970
28	**A/CN/Yunnan/0127/2015(H5N6) (C)**	CN	2.3.4.4	QRG	L	K	ANPA	NH/TSS	PYQGVP	NDA	PLRE/RRRKR/G	**KT245143**
29	A/Chicken/Vietnam/HU9/842/2018(H5N6)	VN	2.3.4.4	QRG	S	K	ANPA	NH/TSS	PYTGVA	NDA	PLRE/RRRKR/G	LC497177
30	A/Chicken/Vietnam/HU9/847/2018(H5N6)	VN	2.3.4.4	QRG	S	K	ANPA	NH/TSS	PYTGVA	NDA	PLRE/RRRKR/G	LC497193
31	**A/Duck/Sichuan/NCXJ16/2014(H5N6) (A)**	CN*	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQAVT	NDA	PLRE/KRRKR/G	**KM251466**
32	A/Duck/Vietnam/NT/75c131/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPP	NHETSL	PYQAVT	NDA	PLRE/KRRKR/G	**LC050623**
33	A/mDuck/Vietnam/HU2/26/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQAVT	NDA	PLRE/KRRKR/G	**LC363972**
34	A/env/CN/Sichuan/NCLL1/2014(H5N6)	CN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQAVT	NDA	PLRE/KRRKR/G	**KM251468**
35	**A/Duck/Vietnam/HT7/2014(H5N6)**	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGTP	NDA	PLREKRRKR/G/	This study

36	A/Duck/Vietnam/HT12/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGTP	NDA	PLRE/KRRKR/G	This study
37	A/Duck/Vietnam/NT/75c131/2014(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	PYQGTP	NDA	PLRE/KRRKR/G	LC05062
38	A/mDuck/Vietnam/HU7/20/2017(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	QYQGVP	NDA	PLRE/RRRKR/G	**LC364036**
39	A/mDuck/Vietnam/HU7/23/2017(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	QYQGVP	NDA	PLRE/RRRKR/G	**LC364044**
40	A/Duck/Vietnam/HU13/71/2019(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	QYQGVP	NDA	PLRE/RRRKR/G	MT107042
41	A/Duck/Vietnam/HU12/970/2019(H5N6)	VN	2.3.4.4	QRG	L	K	ANPA	NHETSL	QYQGVP	NDA	PLRE/RRRKR/G	MT106954
42	A/Vietnam/HN31242/2007(H5N1)	VN	2.3.4.3	QSG	S	R	ANPA	DHEASS	PYQGPV	NNT	PLRE/RRRKR/G	EU294370
43	A/Duck/Vietnam/NA72/2007(H5N1) (2.3.4.3)	VN	2.3.4.3	QSG	S	R	ANPA	DHEASS	PYQGPV	NNT	PLRE/RRRKR/G	JX021305
44	A/Vietnam/HN31432M/2008(H5N1) (2.3.4.2)	VN	2.3.4.2	QSG	L	R	ANPA	DHEASL	PYQGTP	NNT	PLRE/RRRKR/G	HM114617
45	A/Duck/Yunnan/6490/2006(H5N1)	CN	2.3.4.2	QSG	S	R	ANPA	DHEASS	PYQGTP	NNT	PLRE/RRRKR/G	CY030897
46	A/Chicken/HaTay/44/2007(H5N1) (2.3.4.1)	VN	2.3.4.1	QSG	S	R	ANPA	DHEASS	PYQGTP	NNT	PLRE/RRRKR/G	JX420174
47	A/mDuck/Vietnam/NCVD/46/2007(H5N1)	VN	2.3.4.1	QSG	S	R	ANPA	DHEASS	PYQGTP	NNT	PLRE/RRRKR/G	CY030544
48	A/Anhui/1/2005(H5N1) (2.3.4)	CN	2.3.4	QSG	S	R	ANPA	DHEASS	PYQGTP	NNT	PLRE/RRRKR/G	HM172104
49	A/Duck/Vietnam/HT5/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	NHEVSL	SYQGNS	DNA	PLRE/RRRKR/G	This study
50	A/Duck/Vietnam/HT4/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	NHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
51	A/Duck/Vietnam/HT2/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	PNPA	NHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
52	A/Chicken/Vietnam/HN6/2013/H5N2/	VN	2.3.2.1c	QSG	L	K	ANPA	NHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
53	A/Chicken/Vietnam/QTCT5/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
54	A/Duck/Vietnam/KH15/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
55	A/Quail/Vietnam/KH21/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	HYQGNS	DNA	PQRE/RRRKR/G	This study
56	A/Chicken/Vietnam/KH18/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
57	A/Chicken/Vietnam/QTCT4/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNP	DNA	PQRE/RRRKR/G	This study
58	A/Chicken/Vietnam/KH17/2014(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
59	A/Duck/Vietnam/KH18/2013(H5N1)	VN	2.3.2.1c	QSG	L	K	TNPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	This study
60	A/Duck/Vietnam/OIE/2202/2012(H5N1)	VN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	AB769252
61	A/Duck/Tegal/BBVW/1727/2012(H5N1)	ID	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	KC417274
62	A/Duck/Laos/469/2010(H5N1)	LA	2.3.2.1c	QSG	L	K	ANPA	DHEASL	SYQGNS	DNA	PQRE/RRRKR/G	CY098344
63	A/bhGull/Qinghai/1/2009(H5N1)	CN	2.3.2.1c	QSG	L	K	ANPA	DHEASL	PYQGNS	DNA	PQRE/RRRKR/G	HQ020367
64	A/Hubei/1/2010(H5N1) (2.3.2.1a)	CN	2.3.2.1a	QSG	L	K	ANPA	DHEASL	PYQGKS	DNA	PQRE/RRRKR/G	CY098758
65	A/Cambodia/S1211394/2008(H5N1)	KH	1.1	QSG	L	R	ANPV	SHEASL	PYQGKS	NST	PQRE/GRRKKR/G	HQ200596
66	A/Chicken/Vietnam/HD1/2004(H5N1)	VN	1.1	QSG	L	R	ANPV	SHEASL	PYQGKS	NST	PQRE/RRRKKR/G	EF057807
67	A/Vietnam/1194/2004(H5N1)	VN	1	QSG	L	R	ANPA	SHEASL	PYQGKS	NST	PQRE/RRRKKR/G	EF541402
68	A/HK156/1997(H5N1)	CN	0	QSG	S	R	ASPA	NHDASS	PYLGRS	NSA	PQRE/RRRKKR/G	AF046088
69	A/Goose/GD/1/1996(H5N1)	CN	0	QSG	S	R	ASPA	NHDASS	PYHGRS	NSA	PQRE/RRRKKR/G	AF148678

Note: VN: Vietnam; JP: Japan; KR: South Korea; CN: China; ID: Indonesia; LA: Laos; KH: Cambodia; (A), (B), (C): Representative reassortants A, B, and C, respectively. RBD: receptor-binding domain; HA: hemagglutinin; De/Glyco-: A N-link glycosylation site where amino acids were changed to become deglycosylated; (-): sites where an amino acid (R) of the cleavage sites was deleted. Numbers 1–15: indicate the 2.3.4.4 H5N6 viruses of the Japanese-Korean cluster where the codon for Leucine (L) or Serine (S) was deleted ($^{L/S}145^{del}$).

Phylogenetic analysis of HA(H5) sequences

Phylogenetic analysis of 105 H5 nucleotide sequences including those from eleven 2.3.2.1c H5N1 and four 2.3.4.4 H5N6 Vietnamese viruses of our study and 90 of the representative clades (partly listed in Table 2). Fifty H5 sequences of Vietnamese H5Nx specimens were phylogenetically clustered into four subgroups **(**Figure 1). Only the A/Chicken/Vietnam/ NT3/2017(H5N6) isolates from Vietnam was grouped with the typical 2.3.4.4 H5N6 viruses of the distinct Japanese-Korean late 2016-cluster and this group was named "distinct Japanese-Korean-like cluster ($^{S/L}145^{del}$)" of Group C (Major), possessing neither L nor S at position 145. Interestingly, several Vietnamese H5N6 viruses of 2017-2018 isolation (A/Duck/Vietnam/QB/DH330718/ 2017(H5N6); A/Chicken/Vietnam/QB/BT1113/2017(H5N6); A/Duck/Vietnam/QB/QN530206/2018(H5N6)) joined the $^{S/L}145^{del}$ Japanese-Korean 2016–2017 group but in fact, they really do not have S145 deletion but with the full 140NHETSS145 stretch (Figure 1, Table 2). Another H5N6 isolated in 2016 (A/chicken/Vietnam/MT11/2016(H5N6)) was placed in a cluster with the A/CN/Yunnan/0127/2015(H5N6) reassortant C reference strain, and two others, A/Duck/Vietnam/HT7/2014(H5N6) and A/Duck/ Vietnam/HT12/2014(H5N6), were grouped with the reassortant A reference strains of the Sichuan 2014-lineage of Group D (Minor) (Fig. 1). The topology of the phylogenetic tree also clearly showed that eleven Vietnamese H5N1/N2 viruses of 2013–2014 isolation were placed in clade 2.3.2.1c with the A/Duck/Laos/469/2010(H5N1) reference strain (Fig. 1).

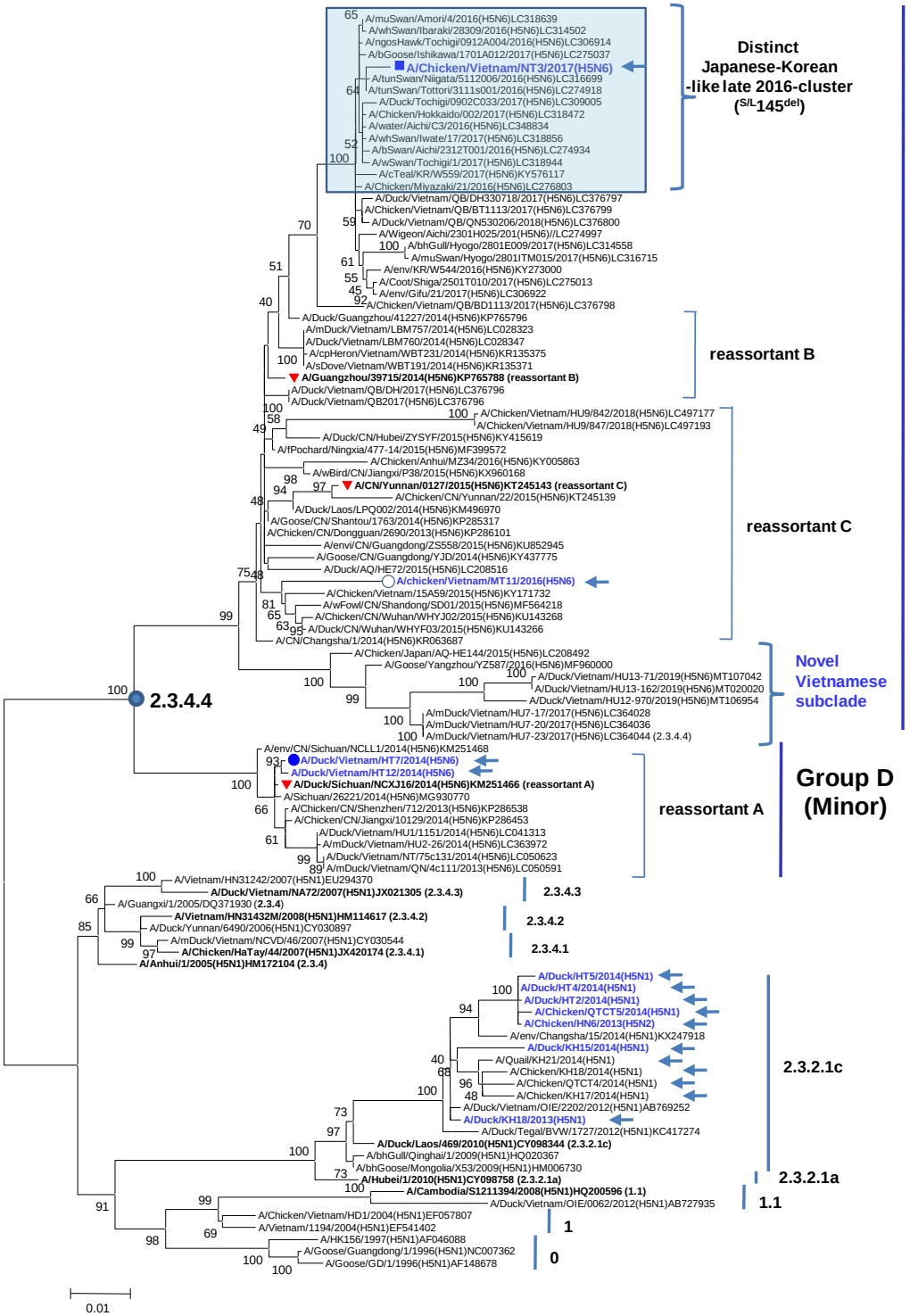

Figure 1. A maximum likelihood (ML) phylogenetic tree showing the topology of the sub/clade, reassortant, and group relationships of 15 Vietnamese isolates in this study and 90 others from Vietnam and global H5Nx viruses from GenBank, based on the analysis of the complete hemagglutinin sequences (1701 or 1704 nucleotides). Phylogenetic tree reconstruction was performed by MEGA 7.0 using an ML analysis based on the general time-

reversible model; supported for each node by 1000 bootstrap resamplings [Kumar *et al*., 2016]. Fifteen HPAI H5Nx isolates from Vietnam in this study are indicated by arrows, and those belonging to specialized reassortants or groups are marked by square or circle symbols (with the bold name of the representative strains and triangle symbol indication). The topology for clade 2.3.4.4 is marked with a solid circle at the root of branches. The late 2016 Japanese-Korean cluster and the A/Chicken/Vietnam/NT3/2017 (H5N6) Vietnamese strain are framed. The strain abbreviation is presented according to the nomenclature of avian influenza viruses by WHO (Smith *et al*., 2015), followed by the year of isolation and subtypes (in brackets). The accession numbers are given at the end of each sequence (if any). The scale bar represents the number of substitutions per site.

Additionally, the topology of the phylogenetic tree revealed a group of the Vietnamese H5N6 viruses recently isolated in 2017 and 2019 (collected from GenBank), which was placed in a cluster together with the A/Goose/Yangzhou/YZ587/2016(H5N6) of China and A/Chicken/Japan/AQ-HE144/2015(H5N6) of Japan origins (Figure 1), we named this group "Novel Vietnamese" subclade. H5 nucleotide-blast searching for strains in this cluster showed that the Vietnamese strains shared 98.50–99.50% identity among the Vietnamese 2.3.3.4 H5N6 (2018–2019), 97.50–98.50% to the H5N6 viruses of Vietnam (2017 isolation) and the above Chinese and Japanese strains (in GenBank: LC208492; MF960000; LC364028; LC364036; LC364044; MT107026; MT107042; MT020012; MT020020; MT107010; MT107018; MT107034; LC500374; MT106962; MT106954; MT106970; MT106978; MT106986; MT106994; MT107002; MT200035). It should be noted that all the Vietnamese viruses in this cluster were isolated from the swabs of poultry from live-bird markets which may really present a spatiotemporal pattern of distribution in Vietnam (Chu *et al*., 2016; Mellor *et al*., 2018; Vergne *et al*., 2019; Nguyen *et al*., 2019b). The availability of a novel/distinct subclade may give rise to a concern about the formation of a new lineage for the evolutionary and epidemiological direction in Vietnam.

DISCUSSION

The HPAI H5Nx viruses, since the first emergence in Guangdong, China in 1996, have undergone multiple evolutionary dynamics to generate various clades, subclades, and genotypes of which the most predominant reassortants are 2.3.2.1 and 2.3.4.4 H5 clades, co-existing in wild and domestic poultry and spreading over the world (Creanga *et al*., 2013; Bi *et al*., 2016; Lee *et al*., 2017; Nguyen *et al*., 2019a). Since 2014, clade 2.3.4.4 HPAI H5Nx has been continuously evolved through multi-steps of reassortments and, concurrently with 2.3.2.1 H5N1 viruses are responsible for outbreaks in poultry and infections in humans (Feng *et al*., 2016; Claes *et al*., 2016; Antigua *et al*., 2019). Novel reassortments have been continuously undergone for genetic constellations in wild waterfowls from main "gene pools" in China, including Sichuan, Qinghai, Hubei, Guangdong, and worldwide disseminated by migratory birds (Bi *et al*., 2016; Lee *et al*., 2017; Takemae *et al*., 2017; Zhang *et al*., 2018; Tsunekuni *et al*., 2019). Vietnam is a country located in a geographic connecting position of North and East Asia and Australia, along the East Asian-Australian migration flyway, where the infected wild birds have frequently stopped and disseminated the new or novel clade 2.3.2.1 and 2.3.4.4 H5Nx viruses (Le and Nguyen, 2014; Nguyen *et al*., 2017; Nguyen *et al*., 2019a,b; Tsunekuni *et al*., 2019).

Because CkNT3-2017 shared over 99.5% of the genetic similarity to A/n.gosHawk/Tochigi/0912A004/2016(H5N6) and members of the distinct Japanese-Korean late 2016-cluster of 2.3.4.4 H5N6 viruses, this Vietnamese strain might be transported into Vietnam in the Spring of 2017 by migratory birds from Japan. This Vietnamese isolate belonged to a novel 2.4.4.4 reassortant H5-lineage which was generated in 2016 and circulated in Japan and South Korea during 2016–2017. Okamatsu *et al*. (2017) and Takemae *et al*. (2017) have clearly defined in detail the genomic properties of the

clade 2.3.4.4 H5N6 HPAI viruses including the Japanese-Korean late 2016-cluster emerged in Japan during 2016–2017. Their studies indicated that genetic constellation has undergone to reassortment of segments originated from avian influenza viruses co-circulating in China and the viruses of novel reassortants were disseminated through the East-Asian flyway by migratory birds between China, Japan, Korea. There has been no report of the similar CkNT3-2017 of 2.3.4.4 reassortant closely related to the Japanese-like lineage prior to our study, therefore, it is the first time for the detection of this distinct H5N6 virus in Vietnam. In this case, its transportation from Japan to Central Vietnam directly by migratory birds is strongly conceivable.

The maximum likelihood-based phylogenetic tree presented in this study indicated the monophyletic topology between 2.3.4.4 H5N6 and the mixed clade-H5N1 strains. The precise placement of A/Chicken/Vietnam/NT3/2017(H5N6) in the Japanese-Korean late 2016-cluster of Group C, and A/Duck/Vietnam/HT7/2014(H5N6) in the Sichuan 2014-lineage of Group D, matched closely their genomic relationships described in each group (Fig. 1, Table 2).

We have identified similar properties of the H5 receptor-binding protein that the polybasic residues of PLRE/RRRKR/G motif between HA_1 and HA_2, QQG amino acids (position 238–240) for the main receptor binding domain and the $T^{172}A$ mutation (or $T^{160}A$, according to H3 numbering), are common for all H5N6 viruses of clade 2.3.4.4 newly formed in 2014 (Table 2). The mutation $T^{172}A$ has induced a deglycosylation at this site, shifting sialic acid (SA) receptors from α-2,3 to α-2,6 type to human respiratory epithelial cells facilitating infections in humans (Gao *et al.*, 2018). A remarkable finding was the determination of a distinct stretch, 140NHETS-145^{del} of the Leucine/Serine deletion at position 145 in HA_1 ($^{S/L}145^{del}$), that only the A/Chicken/Vietnam/NT3/2017(H5N6) strain and the 2.3.4.4 H5N6 members of the Japanese-Korean late 2016-cluster possessed. Whether the missing of L or S residues in a main antigenic epitope stretch of HA polypeptide affects the protective efficacy of the H5-vaccines currently used in Vietnam and other countries or not, it is reasonable for further investigation.

In addition, the phylogenetic analysis and H5 nucleotide-blast searching revealed a novel Vietnamese subclade of H5N6 viruses recently isolated in 2018–2019 together with A/Goose/Yangzhou/YZ587/2016(H5N6) and A/Chicken/Japan/AQ-HE144/2015(H5N6) (Fig. 1). This novel subclade may initiate a new lineage in terms of evolutionary evolution and give attention to the epidemiological monitoring of H5Nx viruses in Vietnam. Moreover, the detection of novel H5N6 subclade in the swabs of poultry from live-bird markets emphasizes a transboundary introduction from outside and the dissemination of new HPAI H5Nx viruses in Vietnam.

In conclusion, phylogenetic analysis, HA sequence characterization, and the well-defined topology of the Vietnamese H5Nx viruses of our study (2013–2017) and others in GenBank (2013–2019) confirmed the evolutionary dynamics of multiple lineages including those that originated from China and the spillovers from Japan and additionally, a distinct Vietnamese subclade of the recent reassortment. Variations at the key sites in hemagglutinin and altered genetic characteristics in novel HPAI H5Nx viruses in Vietnam may present a caution for proper vaccination and emphasize the risk of human infection.

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