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Evolution of highly pathogenic avian influenza (H5N1) virus populations in Vietnam between 2007 and 2010

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ABSTRACT

We report on the genetic analysis of 213 highly pathogenic avian influenza (HPAI) H5N1 viruses isolated from poultry in Vietnam between 2007 and 2010. Phylogenetic analyses of the viral genomes revealed 38 distinct viral genotypes, 29 were novel and 9 were reported in Vietnam or neighboring countries in recent years. Viruses from only six genotypes persisted beyond one season or year. Thus, most reassortant viruses were transient, suggesting that such genotypes lacked significant fitness advantages. Viruses with clade 2.3.2.1 HA were re-introduced into Vietnam in 2009 and their prevalence rose steeply towards the end of 2010. Clade 2.3.4-like viruses (genotype V) were predominant in northern Vietnam and caused the majority of zoonotic infections, whereas clade 1.1 (genotype Z) viruses were only detected in the Mekong delta region, in southern Vietnam. Antigenic analysis of representative viruses from the four clades indicated substantial drift.

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Introduction

The highly pathogenic avian influenza (HPAI) virus, subtype H5N1, was first detected in 1996 in Southern China. Since then, the virus has spread to birds in more than 60 countries across Asia, the Middle East, Europe, and Africa (FAO, 2011) and resulted in more than 330 human fatalities in 15 countries. The potential for sustained human-to-human transmission of H5N1 and the high fatality/case ratio (approximately 60%) cause major concern for public health. Besides posing a threat to human health, H5N1 has caused major losses in poultry flocks in affected areas through either direct infection or preventive culling. Despite the implementation of biosecurity practices and poultry vaccination campaigns, H5N1 now appears to be enzootic in parts of Asia, the Indian Subcontinent, Indonesia, and Africa (Sims, 2010).

H5N1 viruses have spread regionally and over long distances by migratory waterfowl as well as poultry production and trade practices. Rapid viral evolution by accumulation of mutations has resulted in at least 32 clades, as defined by the WHO/OIE/FAO H5N1 Evolution Working Group, descending from the ancestral A/goose/Guangdong/96-like H5 HA gene, imparting distinct antigenic characteristics to some members of specific clades or potentially novel phenotypic properties (Arafa et al., 2010; Balish et al., 2010; Takano et al., 2009; Watanabe et al., 2011; WHO/OIE/FAO H5N1 Evolution Working Group, 2011). Virus evolution by genetic reassortment results in the emergence of new genotypes which may possess additional novel characteristics (Boltz et al., 2010; Chaichoune et al., 2009; Chen et al., 2009; Duan et al., 2008).

H5N1 virus was first detected in Vietnam in 2001 and poultry outbreaks have been regularly reported since the end of 2003 (Minh et al., 2009; Nguyen et al., 2009). Vietnam is among the countries with the highest number of human fatalities (59 deaths between January 2003 and September, 2011) due to zoonotic H5N1 infections, although no human cases have been reported in 2011 (WHO, 2011a)). In order to monitor the incidence of the virus in domestic poultry and inform control programs, the Vietnamese Department of Animal Health (DAH) launched a nationwide surveillance program in 2004, which has been implemented through the National Centre for Veterinary Diagnostics (NCVD) and the DAH Regional Animal Health offices. Since the start of this program, NCVD has detected more than 2500 H5N1 positive samples, many of which have been characterized by virus culture, full genome sequencing and antigenic testing.

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Previous studies have analyzed viruses collected in Vietnam between 2001 and 2007 and identified at least 9 genotypes, including 7 distinct HA gene clades (Davis et al., 2010; Nguyen et al., 2009; Wan et al., 2008; WHO/OIE/FAO H5N1 Evolution Working Group, 2008). As of 2007, genotype Z remained the predominant virus circulating in Vietnamese poultry (Nguyen et al., 2009). However, viral population "sweeps" can occur in which a previously persistent H5N1 genotype is rapidly displaced; e.g., in China, genotype B was replaced by genotype Z in 2002 and genotype Z was replaced by genotype V in 2005 (Duan et al., 2008). In order to gain a better understanding of the recent evolution of HPAI H5N1 in Vietnam, we analyzed the genomes of H5N1 viruses isolated from domestic poultry and other birds between 2007 and 2010. Results from this study demonstrate the introduction and reintroduction of several H5N1 clades into Vietnam and the sweeping expansion of genotype V viruses. In addition, molecular analyses show extensive genetic variation among endemic strains and numerous reassortment events both within the H5N1 gene pool (resident and introduced) and with low pathogenic avian influenza viruses. Finally, we describe the identification of several genotypes that were previously reported in other countries of the region, suggesting possible transboundary viral traffic.

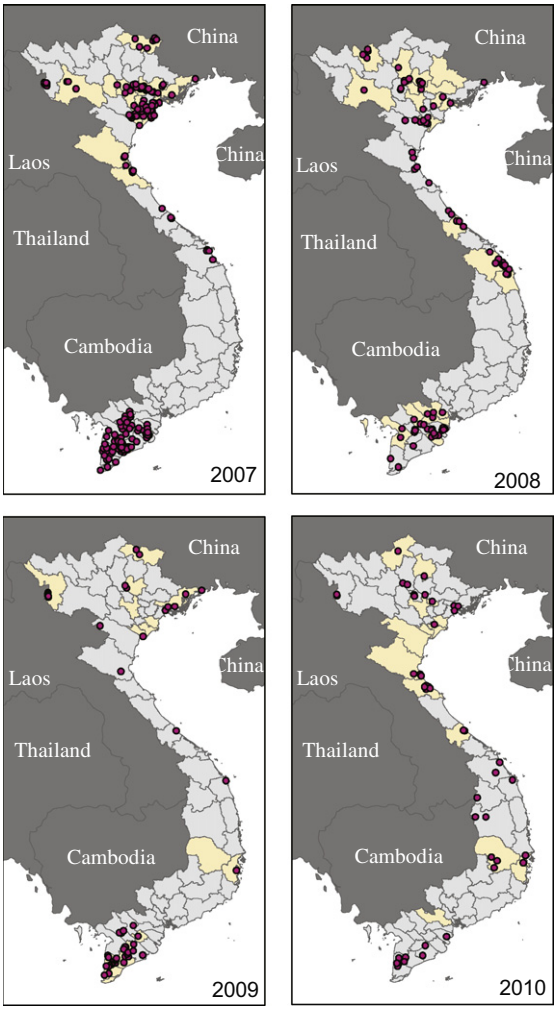

Fig. 1. Annual geographic distribution of H5N1 animal outbreaks and virus detection between 2007 and 2010 in Vietnam. H5N1 animal outbreaks are shown with pink circles. Outbreak information was based on http://empres-i.fao.org. Provinces where H5N1 virus was isolated are shown in beige. For a complete list of provinces, see Supplementary Table 3.

Results

H5N1 surveillance in Vietnamese poultry from 2007 to 2010

A total of 444 HPAI poultry outbreaks were detected between Jan. 1, 2007 and Dec. 31, 2010 and reported by international animal health agencies (FAO, 2011) (Supplementary Table 2). Although the number of outbreaks reported annually decreased from 258 in 2007 to 48 in 2010, the number of provinces reporting outbreaks remained high; between 17 and 29 each year (Supplementary Table 2). Outbreaks were reported from 42 of the 63 provinces (66%) since 2007, indicating the spread of the virus into the 8 geographic regions of the country (Supplementary Table 3) (Fig. 1). Since 2007, 3.1% of nearly 20,000 diagnostic samples collected from poultry in 39 provinces (including 5 major metropolitan municipalities, i.e., Hanoi, Hai Phong, Da Nang, Can Tho and Ho Chi Minh) tested positive for H5N1 by PCR (Table 1, Supplementary Tables 2 and 3, and Fig. 1). A total of 213 viruses were isolated from 283 representative clinical specimens previously confirmed as H5N1-positive by PCR. An average of 58 viruses was isolated each year between 2007 and 2009, while only 39 viruses were isolated in 2010. Overall, 76% of the provinces reporting H5N1 outbreaks ($n=42$) submitted samples that tested H5N1-positive ($n=32$). At least one virus was isolated from approximately 50% of the provinces reporting H5N1 outbreaks between 2007 and 2009; this frequency was reduced to 38% of the provinces in 2010, also suggesting decreased virus incidence (Fig. 1, Supplementary Tables 2 and 3). The correspondence between geo-temporal distribution of positive H5N1 virologic test results and HPAI outbreak reports indicates that the 213 viruses isolated since 2007 are likely to represent the predominant H5N1 viruses circulating in poultry in Vietnam during this period. The majority of viruses were isolated from domestic chickens and ducks with slight predominance of the latter (60%) (Supplementary Table 2).

Evolution and geo-temporal distribution of the H5 hemagglutinin gene of HPAI viruses

Phylogenetic analysis of the HA gene of viruses circulating in Vietnam since 2007 indicated extensive genetic diversity; 4 major clades were identified with the nomenclature defined in 2008 (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). These corresponded to clade 2.3.4 (75% of isolates), clade 1 (15%), clade 2.3.2 (7%) and clade 7 (3%) (Supplementary Fig. 1, Figs. 2 and 3). As recently reported by the WHO/OIE/FAO H5N1 Evolution Working

Table 1
Surveillance for H5N1 in Vietnamese poultry since 2004.

NCVD surveillance of HPAI H5N1 in birds in Vietnam from 2007 to 2010					
	2007	2008	2009	2010	Total
Total samples	**4,342**	**6,179**	**6,336**	**2,916**	**19,773**
Positive samples [a]	**277 (6.4%)** [b]	**152 (2.5%)**	**160 (2.5%)**	**31 (1.1%)**	**620 (3.1%)**
Number of provinces	**35**	**32**	**23**	**9**	**39**

[a] Results from real-time RT-PCR.
[b] Percentage of samples positive out of total collected.

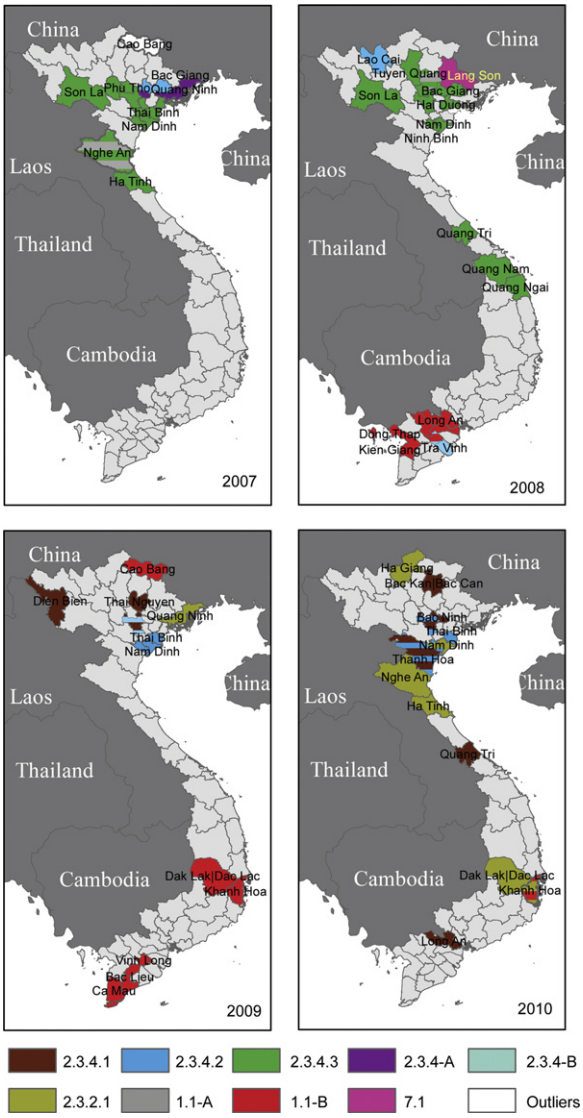

Fig. 2. Geographic distribution of H5 HA clades between 2007 and 2010 in Vietnam. Provinces where H5N1 virus has been collected are shown in color based on the HA clade of the viruses: 2.3.4.1 in brown; 2.3.4.2 in dark blue; 2.3.4.3 in green; 2.3.4-A in purple; 2.3.4-B in light blue; 2.3.2.1 in olive green; 1.1-A in dark grey; 1.1-B in red and clade 7.1 in pink; outliers are left blank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Group, four new fourth order clades emerged between 2008 and 2010; 2.3.2.1, 2.3.4.1, 2.3.4.2, 2.3.4.3, as well as three new second order clades 1.1, 7.1 and 7.2 (WHO/OIE/FAO H5N1 Evolution Working Group, 2011).

Most of the clade 2.3.4 viruses belonged to clade 2.3.4.3. The 2.3.4.3 HA sequences appeared to be closely related to viruses collected in Hong Kong in 2007 (A/avian/Hong Kong/0719/2007) (Supplementary Fig. 1). After their first detection in the North of Vietnam in 2007, the 2.3.4.3 viruses spread to the center of the country (Fig. 2). In 2008, the 2.3.4.3 viruses were still collected in the north but also in central provinces (Quang Tri, Quang Nam and Quang Ngai) indicating a geographical spread of these viruses. Interestingly, no 2.3.4.3 viruses were collected after 2008 in Vietnam. A second group (~20%) of viruses had the clade 2.3.4.2 HA (Supplementary Fig. 1). These viruses were closely related to A/duck/Yunnan/6490/2006 and were collected every year since 2007 from poultry in northern provinces (Fig. 2). The last major group of viruses (~15%) to emerge had clade 2.3.4.1 HA (Supplementary Fig. 1). Even though these viruses appeared to be closely related to A/chicken/Sichuan/81/2005, the 2.3.4.1 viruses were first detected in Vietnam in 2009 in northern provinces. It is not known whether these viruses have escaped detection before 2009 or were introduced into Vietnam in 2009. The 2.3.4.1 viruses have spread in Vietnam in 2010 reaching central and southern provinces (Quang Tri, Long An) (Fig. 2) and are likely to be circulating in 2011. Two additional 2.3.4 subclades, called here 2.3.4-A and 2.3.4-B, were identified in this study using lineage criteria described above (Material and methods) (Supplementary Fig. 2D). 2.3.4-A was collected in the northern provinces of Quang Ninh and Bac Ninh in 2007 and were closely related to A/goose/Guangxi/1458/2006 (Fig. 2, Supplementary Fig. 1). 2.3.4-B viruses were collected in 2008 in the southern provinces of Tra Vinh and Vinh Long and in 2009 and the northern province of Hanoi (Fig. 2). Finally, two 2.3.4 viruses did not fit the lineage criteria set in this study and were considered outliers. A/dk/VN/NCVD-013/08 was closely related to A/Jiangsu/1/2007 and collected in the coastal province of Hai phong. A/ck/VN/NCVD-109/07 closely related to A/goose/Guangxi/1633/2006 was collected in the Cao Bang province, at the border with China. (Fig. 2, Supplementary Fig. 1).

All the viruses formerly designated as clade 2.3.2 collected in Vietnam since 2009 belong to the recently designated clade 2.3.2.1 (Supplementary Fig. 1) (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). These viruses appeared to be closely related to A/little egret/Hong Kong/8863/2007. They were detected in northern and north-central coast provinces in 2009 and 2010 (Fig. 2). In addition, a few clade 2.3.2.1 viruses were also detected in the central highlands and south central coast provinces (Dak Lak, Khan Hoa) in late 2010. Although clade 2.3.2 viruses had been detected in Vietnam in 2005 and 2006, the 2.3.2.1 viruses detected in 2009/2010 were very divergent from those earlier isolates ($>1.5\%$ average p distance) and were part of the group of viruses that emerged in very distant locations from Eastern Europe to South East and North Asia since 2008. (Supplementary Fig. 1) (Boltz et al., 2010; Kang et al., 2011; Kim et al., 2010; Manin et al., 2010; Reid et al., 2010; Sakoda et al., 2010; Smith et al., 2009).

All analyzed viruses within the former clade 1 belong to the novel clade 1.1 (Supplementary Fig. 1) (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). However, the high divergence between two distinct subclades led to their designation as 1.1-A and 1.1-B, following the lineage criteria set in this study (Material and methods). 1.1-A viruses were collected in the northern provinces of Nghe An in 2007 and Thai Nguyen in 2008 whereas the 1.1B viruses were mainly collected in the Mekong River delta and southeast provinces of the country each year since 2008 (Fig. 2). In addition, one 1.1-B virus was detected in the northern province of Cao Bang in 2009, likely to be the result of trading of poultry or its products.

Six viruses analyzed in this study have a clade 7 HA gene. Four of them belong to the novel clade 7.1 (Supplementary Fig. 1) (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). These viruses were closely related to A/chicken/Shanxi/10/2006 and were collected exclusively in 2008 from imported birds seized at the northern border of Vietnam (Fig. 2) (Nguyen et al., 2009). The two clade 7 HA genes from 2008 (A/ck/VN/NCVD-093/08; A/ck/VN/NCVD-016/08) were highly divergent from the most similar known H5 genes, most likely due to extensively under-sampled evolution (Solovyov et al., 2010) and were considered outliers in the present study.

Antigenic characteristics of H5N1 viruses circulating in Vietnam between 2007 and 2010

Ferret antiserum raised against clade 1.1 reference virus, A/duck/Vietnam/NCVD-16/2007, inhibited hemagglutination of 2007–2010 viruses from the same clade (Table 2). In contrast, this antiserum showed >8 fold decreased HI titers in reactions with viruses from different clades: 2.3.2.1, 2.3.4, 7.1, and 7.2 (Table 2; data not shown).

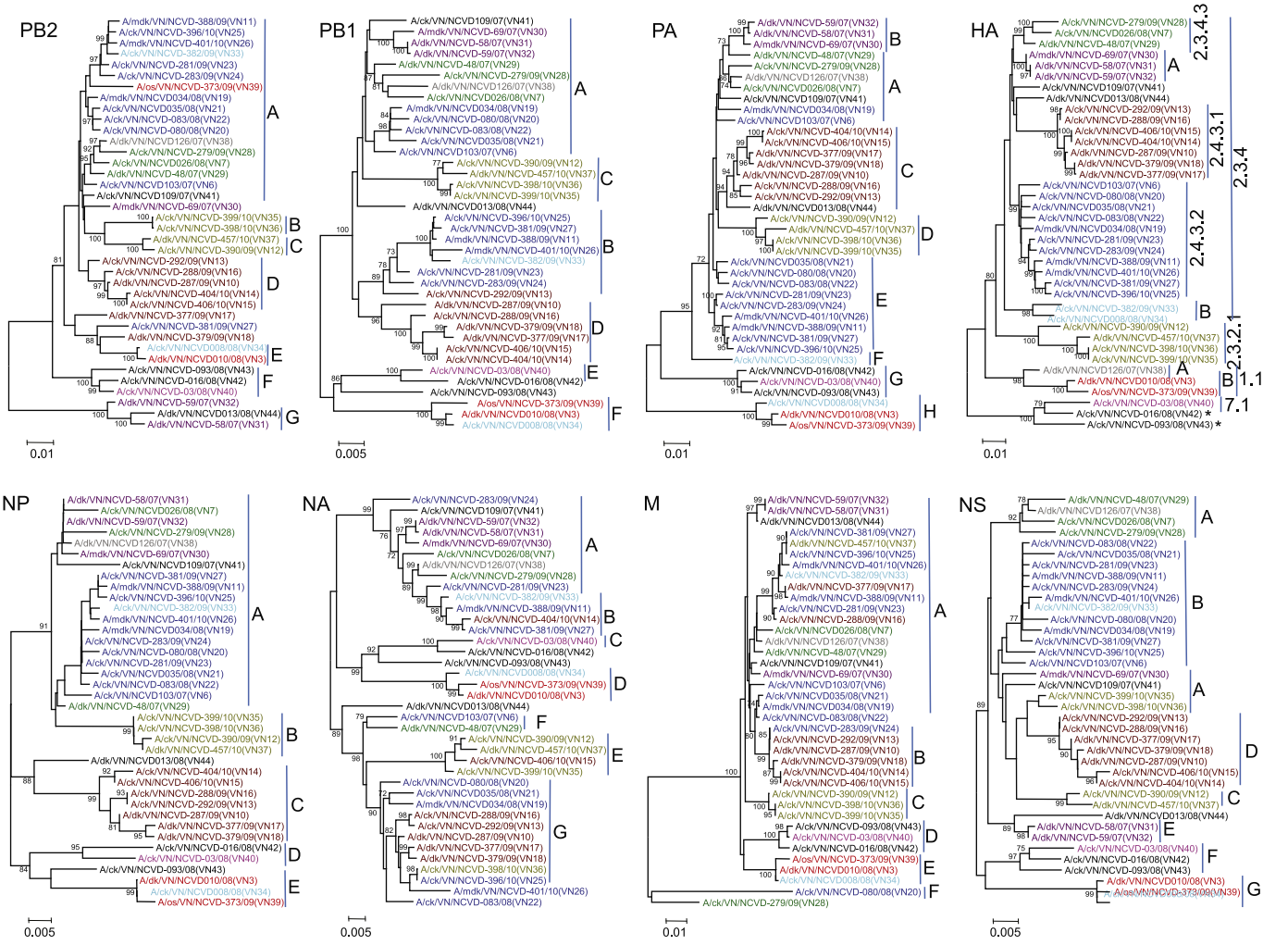

Fig. 3. Phylogeny of representative strains from the analyzed dataset. Phylogenetic trees were generated by the MEGA software package (version 4) using the neighbor-joining method with a maximum composite likelihood model (Kumar et al., 2004; Saitou and Nei, 1986). The reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications, using the MEGA package (Efron and Tibshirani, 1994). Viruses are colored based on their HA lineage: 2.3.4.1 in maroon; 2.3.4.2 in dark blue; 2.3.4.3 in green; 2.3.4-A in purple; 2.3.4-B in light blue; 2.3.2.1 in olive green; 1.1-A in grey; 1.1-B in red and clade 7.1 in pink (see HA trees in Supplementary Figs. 1 and 2 for the full dataset). Gene lineages are based on the analysis of 213 analyzed genomes (see trees in Supplementary Fig. 2). Genotype is indicated in parenthesis. Bootstrap values $>70\%$ are shown. Two clade 7 viruses (shown with *) are considered outliers in our study based on bootstrap and p distances as indicated in the text. The clade 7 outlier A/chicken/VN/NCVD-016/2008 is of clade 7.1 and the clade 7 outlier, A/chicken/VN/NCVD-093/2008, is of clade 7.2. dk: duck; mdk: muscovy duck; ck: chicken; av: avian; os: ostrich; VN: Vietnam.

In general, ferret antisera tended to yield HI titers with less than 8-fold difference compared to the homologous HI titer if they were raised against viruses from the same clade, indicating a strong correlation between genetic and antigenic characteristics. The HI titers of antisera to clade 2.3.2.1 viruses showed more than 16-fold reduction in reactions with viruses from clade 2.3.4, although there were fewer amino acid differences in HA1 between viruses from 2.3.2.1-like and 2.3.4-like viruses than between HA1s of 2.3.2.1 viruses and viruses from clade 1.1 or clade 7.1 (Table 2; data not shown). Thus, taken together, HI titers of viruses from heterologous clades were >8-fold lower than viruses from homologous clades. However, clade 2.3.2.1 and clade 7.1 comprised several examples of >8-fold reduction in HI titers of some viruses from the same clade relative to the homologous clade HI titers indicating significant antigenic variation within clades 2.3.2.1 and 7.1 of H5N1 viruses circulating in Vietnam (i.e., A/duck/Vietnam/NCVD-449/2010 versus A/chicken/Vietnam/NCVD-459/2010 from clade 2.3.2.1 or A/chicken/Vietnam/NCVD-3/2008 versus A/chicken/Vietnam/NCVD-016/2008 from clade 7.1) (Table 2; data not shown).

Interestingly, ferret antisera against candidate vaccine viruses, A/Vietnam/1203/2004 (clade 1) and A/Anhui/1/2005 (clade 2.3.4), selected from the WHO pandemic preparedness library demonstrated HI titers within 8-fold difference to the circulating clade 1.1 and 2.3.4-like viruses, indicating that despite the increasing number of amino acid substitutions between recent viruses and these H5N1 vaccine candidates, much of the antigenic surface of the HA is still recognized by antibodies in convalescent ferret antisera. HI titers of ferret antiserum to the clade 2.3.4 vaccine candidate virus showed slightly reduced inhibition of newly emerged clades 2.3.4.1 and 2.3.4.2 as compared to the homologous virus. In contrast, antisera to viruses from 2.3.4.1 and 2.3.4.2 showed significant cross-reactivity with each other. Studies on evaluation of cross-reactivity between the newly proposed clade 2.3.2.1 WHO pandemic vaccine candidates and viruses circulating in Vietnam are ongoing (WHO, 2011b).

Evolution of the NA gene of HPAI viruses from Vietnam

Like the H5 gene, the N1 NA has evolved rapidly in recent years (Duan et al., 2008). The gene lineage nomenclature system established by Duan et al. based on viruses collected in China before 2006 (Duan et al., 2008) demonstrated that all the Vietnam

Table 2
Hemagglutination-inhibition assay of H5N1 viruses circulating in Vietnam, 2007–2010.

		Reference ferret antisera											
Reference antigens	**CLADE**	**1** VN/1203	1.1 DK/VN/16	**2.1.3.2** IND/5	**2.2.1** EG/321	**2.3.2.1** BHG/X53	**2.3.2.1** CM/5052	**2.3.4.1** DK/VN/293	**2.3.4** DK/LO/3295	**2.3.4** ANH/1	**2.3.4.2** CK/VN/35	**7.1** CK/VN/03	**7.1** CK/VN/016
A/VIETNAM/1203/2004	**1**	**160**	320	40	20	10	10	40	10	10	160	5	5
A/CAMBODIA/R0405050/07	**1.1**	5	10	5	10	5	5	5	5	5	10	5	5
A/DUCK/VIETNAM/NCVD-016/2007	**1.1**	20	**40**	10	5	5	5	5	5	5	40	5	5
A/INDONESIA/5/2005	**2.1.3.2**	5	20	**640**	40	80	80	40	40	20	40	10	20
A/EGYPT/321-NAMRU3/2007	**2.2.1**	10	20	320	**320**	80	40	40	40	20	40	5	10
A/BARHEADED GOOSE/MG/X53/2009	**2.3.2.1**	40	10	160	20	**320**	320	5	5	5	40	5	5
A/COMMON MAGPIE/HK/5052/07	**2.3.2.1**	5	10	320	10	320	**640**	5	10	5	40	5	5
A/DUCK/VIETNAM/NCVD-293/09	**2.3.4.1**	5	10	10	10	5	5	**80**	80	40	80	10	5
A/DUCK/LAOS/3295/06	**2.3.4**	5	20	80	10	5	10	80	**80**	80	80	5	5
A/ANHUI/1/05	**2.3.4**	5	80	80	20	5	20	160	320	**320**	320	20	10
A/CHICKEN/VIETNAM/NCVD-35/08	**2.3.4.2**	10	40	80	5	10	10	80	80	80	**320**	5	5
A/CHICKEN/VIETNAM/NCVD-3/08	**7.1**	5	5	5	10	10	5	5	5	5	40	**320**	40
A/CHICKEN/VIETNAM/NCVD-016/08	**7.1**	5	5	20	10	5	5	10	10	10	10	80	**1280**
Test antigens													
A/CHICKEN/VIETNAM/NCVD-424/2009	**1.1**	20	80	5	5	5	5	5	5	5	40	5	5
A/CHICKEN/VIETNAM/NCVD-425/2009	**1.1**	40	80	5	5	10	5	5	5	5	40	5	5
A/M.DUCK/VIETNAM/NCVD-426/2009	**1.1**	80	160	10	10	5	10	5	5	5	80	5	5
A/CHICKEN/VIETNAM/NCVD-427/2010	**1.1**	80	80	10	5	5	5	5	5	5	40	5	5
A/CHICKEN/VIETNAM/NCVD-428/2010	**1.1**	20	80	5	5	5	5	5	5	5	40	5	5
A/CHICKEN/VIETNAM/NCVD-429/2010	**1.1**	80	160	5	10	5	5	5	5	5	80	5	5
A/DUCK/VIETNAM/NCVD-430/2010	**1.1**	80	160	10	10	10	5	10	5	5	80	5	5
A/DUCK/VIETNAM/NCVD-431/2010	**2.3.2.1**	10	20	160	20	80	80	5	5	5	80	5	5
A/DUCK/VIETNAM/NCVD-432/2010	**2.3.2.1**	5	20	80	10	20	20	5	5	5	20	5	5
A/DUCK/VIETNAM/NCVD-449/2010	**2.3.2.1**	40	80	20	10	10	10	5	5	5	80	5	5
A/DUCK/VIETNAM/NCVD-456/2010	**2.3.2.1**	5	10	40	5	80	10	5	10	5	40	5	5
A/DUCK/VIETNAM/NCVD-457/2010	**2.3.2.1**	10	10	40	5	80	10	5	10	5	80	5	5
A/DUCK/VIETNAM/NCVD-458/2010	**2.3.2.1**	5	10	40	10	80	10	10	20	5	80	5	5
A/CHICKEN/VIETNAM/NCVD-459/2010	**2.3.2.1**	10	20	80	10	160	20	10	20	10	80	5	5
A/DUCK/VIETNAM/NCVD-460/2010	**2.3.2.1**	10	10	40	5	80	10	5	10	5	80	5	5
A/DUCK/VIETNAM/NCVD-461/2010	**2.3.2.1**	5	5	20	5	80	10	5	10	5	80	5	5
A/DUCK/VIETNAM/NCVD-462/2010	**2.3.4.1**	5	20	40	10	5	5	160	160	80	80	10	10
A/DUCK/VIETNAM/NCVD-471/2010	**2.3.4.1**	5	10	10	5	5	5	40	80	80	80	5	10
A/DUCK/VIETNAM/NCVD-472/2010	**2.3.4.2**	10	40	20	10	5	10	40	80	40	160	5	10
A/DUCK/VIETNAM/NCVD-433/2010	**2.3.4.2**	20	80	40	10	10	10	80	160	80	320	5	5

viruses had an NA gene of the G, V, Z lineage (Supplementary Fig. 3-E). This is the most frequently detected NA lineage since 2002 and shares a 20-codon deletion with the A/goose/Guangdong/1996 (Gs/GD) NA lineage. In order to analyze the microevolution of the NA gene in Vietnam, lineages were identified using genetic distance and tree topology criteria (Material and methods). Briefly, a gene lineage is defined as such if it is monophyletic, supported by a bootstrap value ≥ 60 and with $<1.5\%$ average pairwise distances within the lineage and $>1.5\%$ average distance between lineages (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). A total of seven NA genetic lineages were identified (designated 6A thru 6G). Five NA were identified as outliers, two of these were from viruses that also contained outlier HA genes; i.e., A/chicken/Vietnam/NCVD-093/2008 and A/duck/Vietnam/NCVD-013/2008. Comparison of the HA and NA tree topologies indicated that most of the viruses that shared the same HA lineage shared also a common NA lineage indicating a co-evolution of the HA and NA genes (Fig. 3). However, 23 viruses ($\sim$10%) had different HA and NA lineages. For example, the 2.3.4.1 viruses could have an NA of three different lineages, 6B, 6E and 6G, the latter being the most common (Fig. 3). In addition, viruses sharing the 6G lineage can have a clade 2.3.4.2 and 2.3.2.1 HA. Database searches for the most closely related sequences for each lineage indicated that a majority of genes were most closely related to previously reported H5N1 viruses collected in Vietnam (Supplementary Table 4). Lineage 6C was relatively divergent. The closest sequence from Vietnam was A/chicken/Vietnam/8/2003 with 95% nucleotide identity. In contrast, all the other NA sequences were closely related to NA sequences of viruses collected in Vietnam and/or neighboring countries such as China, Cambodia and Laos.

Evolution of the internal genes of HPAI from Vietnam

All viruses in this study shared PB1, NP and NS genes from single lineages, termed B,G,W,V,Z,Z+ for PB1, G,V,X-series, Z,Z+ for NP and B,C,G,W,V,Z,Z+ for NS, using the nomenclature of Duan et al. (Supplementary Fig. 3B, 3D and 3G) (Duan et al., 2008). The rest of the genes (PB2, PA and M) were divided into two or more lineages. Most of the PB2 genes belonged to the Aquatic, B,C,V,Z,Z+ lineage (Supplementary Fig. 3A) with the exception of two PB2 sequences that belonged to the Aquatic, W,G lineage and one sequence (A/duck/Vietnam/NCVD-59/2007) that did not fit into any previously identified groups and was most closely related to the PB2 of A/China/GD01/2006 (H5N1) isolate (Supplementary Table 4). The majority of the PA genes ($n=180$, 84%) belonged to the Aquatic V lineage (associated with genotype V viruses), whereas the rest belonged to the B,C,G,W,Z,Z+ lineage (associated with genotype Z viruses) (Supplementary Fig. 3C). Finally, the majority of the M genes belong to the B,C,G,W,V,Z,Z+ lineage, whereas two genes belonged to the G1 lineage and one clustered with the Early lineage (Supplementary Fig. 3F) (Duan et al., 2008).

Using the criteria established to analyze the microevolution of the viruses from Vietnam (Material and methods), we identified between 5 (NP) and 8 (PA) new internal gene lineages, totaling 39 newly defined internal gene clusters (Fig. 3, Supplementary Fig. 2). Database searches indicated that the vast majority of the internal genes analyzed matched those from previously reported H5N1 viruses from Vietnam (Supplementary Table 4). However, many lineages ($n=17$) showed relatively low levels of identity to the most closely related gene from Vietnam and greater similarity to genes from viruses detected in neighboring countries (China, Mongolia, Laos and Cambodia) i.e., the nucleotide identity of the exotic virus was at least 2% higher than that of the homolog from Vietnam) (Supplementary Table 4, color coded). While the majority of genes shared highest nucleotide identities ($>97\%$) with other H5N1 viruses from the region, the M genes of lineage 7F were most closely related to sequences of low pathogenic avian influenza (LPAI) viruses, subtype H6N1, collected in 2004 from China (Cheung et al., 2007; Hoffmann et al., 2000). In addition, the M gene of the outlier A/ck/VN/NCVD-279/09 was closely related (97% identity) to LPAI H6N1 from Hong Kong, SAR China, collected in 1976 (Obenauer et al., 2006).

Genomic evolution of HPAI from Vietnam

The majority (81%) of the viruses collected in Vietnam since 2007 were classified as genotype V, according to the system of

Table 3
Genotypes identified in H5N1 viruses from Vietnam, 2007–2010.

		Gene lineages identified in this stud										
Genotype	**Prototype strain-name**	**PB2**	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	**NS**	**Circulation status in VN**	**Number of isolates**	**Genotype[a]**
VN10	A/duck/VN/NCVD-287/2009	1D	2D	3C	2.3.4.1	5C	6G	7B	8D	Persistent (09–10), new[b]	17	V
VN11	A/muscovy duck/VN/NCVD-388/2009	1A	2B	3E	2.3.4.2	5A	6B	7A	8B	Persistent (09–10), new	12	V
VN7[c]	A/chicken/VN/NCVD026/2008	1A	2A	3A	2.3.4.3	5A	6 A	7A	8A	Persistent (07–08)	91	V
VN12	A/chicken/VN/NCVD-390/2009	1C	2C	3D	2.3.2.1	5B	6E	7C	8C	Persistent (09–10), new	12	V
VN3	A/duck/VN/NCVD010/2008	1E	2F	3H	1.1-B	5E	6D	7E	8G	Persistent (08–10)	30	Z
VN13	A/chicken/VN/NCVD-292/2009	1D	2B	3C	2.3.4.1	5C	6 G	7B	8D	Transient, new	1	V
VN14	A/chicken/VN/NCVD404/2010	1D	2D	3C	2.3.4.1	5C	6B	7B	8D	Transient, new	3	V
VN15	A/chicken/VN/NCVD406/2010	1D	2D	3C	2.3.4.1	5C	6E	7B	8D	Transient, new	2	V
VN16	A/chicken/VN/NCVD-288/2009	1D	2D	3C	2.3.4.1	5C	6 G	7A	8D	Transient, new	1	V
VN17	A/duck/VN/NCVD-377/2009	O	2D	3C	2.3.4.1	5C	6 G	7A	8D	Transient, new	1	V
VN18	A/duck/VN/NCVD-379/2009	O	2D	3C	2.3.4.1	5C	6G	7B	8D	Transient, new	1	V
VN19	A/muscovy duck/VN/NCVD034/2008	1A	2A	3A	2.3.4.2	5A	6G	7A	8B	Transient, new	1	V
VN20[d]	A/chicken/VN/NCVD-080/08	1A	2A	3E	2.3.4.2	5A	6G	7F	8B	Transient, new	2	V-like (M)
VN21	A/chicken/VN/NCVD035/2008	1A	2A	3E	2.3.4.2	5A	6G	7A	8B	Transient, new	4	V
VN22	A/chicken/VN/NCVD-083/08	1A	2A	3E	2.3.4.2	5A	O	7A	8B	Transient, new	1	V
VN23	A/chicken/VN/NCVD-281/2009	1A	2B	3E	2.3.4.2	5A	6A	7A	8B	Transient, new	4	V
VN24	A/chicken/VN/NCVD-283/2009	1A	2B	3E	2.3.4.2	5A	6A	7B	8B	Transient, new	1	V
VN25	A/chicken/VN/NCVD396/2010	1A	2B	3E	2.3.4.2	5A	6G	7A	8A	Transient, new	2	V
VN26	A/muscovy duck/VN/NCVD401/2010	1A	2B	3E	2.3.4.2	5A	O	7A	8B	Transient, new	1	V
VN6	A/chicken/VN/NCVD103/2007	1A	O	3A	2.3.4.2	5A	O	7A	8A	Transient	1	V
VN27	A/chicken/VN/NCVD-381/2009	O	2B	3E	2.3.4.2	5A	6B	7A	8B	Transient, new	1	V
VN28	A/chicken/VN/NCVD-279/2009	1A	2A	3A	2.3.4.3	5A	6A	O	8A	Transient, new	1	V-like (M)
VN29	A/duck/VN/NCVD-48/07	1A	2A	3A	2.3.4.3	5A	6F	7A	8A	Transient, new	2	V
VN30	A/muscovy duck/VN/NCVD-69/07	1A	2A	3B	2.3.4-A	5A	6A	7A	8A	Transient, new	1	V
VN31	A/duck/VN/NCVD-58/2007	1G	2A	3B	2.3.4-A	5A	6A	7A	8E	Transient, new	1	V-like (PB2)
VN32	A/duck/VN/NCVD-59/2007	O	2A	3B	2.3.4-A	5A	6A	7A	8E	Transient, new	1	V-like (PB2)
VN33	A/chicken/VN/NCVD-382/2009	1A	2B	3F	2.3.4-B	5A	6B	7A	8B	Transient, new	2	V
VN34	A/chicken/VN/NCVD008/2008	1E	2F	3H	2.3.4-B	5E	6D	7E	8G	Transient, new	2	Z
VN35	A/chicken/VN/NCVD399/2010	1B	2C	3D	2.3.2.1	5B	6E	7C	8A	Transient, new	1	V
VN36	A/chicken/VN/NCVD398/2010	1B	2C	3D	2.3.2.1	5B	6G	7C	8A	Transient, new	1	V
VN37	A/duck/VN/NCVD-457/2010	1C	2C	3D	2.3.2.1	5B	6E	7A	8C	Transient, new	1	V
VN38[e]	A/duck/VN/NCVD126/2007	1A	2A	3A	1.1-A	5A	6A	7A	8A	Persistent (07–08), new	2	V
VN39	A/ostrich/VN/NCVD-373/2009	O	2F	3H	1.1-B	5E	6D	7E	8G	Transient, new	1	Z
VN40	A/chicken/VN/NCVD-03/08	1F	2E	3G	7.1	5D	6C	7D	8F	Transient, new	4	V
VN41	A/chicken/VN/NCVD109/2007	1A	O	3A	2.3.4-O	5A	6A	7A	8A	Transient, new	1	V
VN42	A/chicken/VN/NCVD-016/2008	1F	2E	3G	7-O	5D	6C	7D	8F	Transient, new	1	V
VN43	A/chicken/VN/NCVD-093/08	1F	O	3G	7-O	O	O	7D	8F	Transient, new	1	V
VN44	A/duck/VN/NCVD013/2008	1G	O	O	2.3.4-O	O	O	7A	O	Transient, new	1	V-like (PB2)

O: outlier; VN: Vietnam.
[a] Genotype using nomenclature from Duan et al. (2008); reassorted lineage is indicated in parenthesis.
[b] Persistent genotype likely to be currently circulating are shown in green.
[c] Genotype previously described are shown in grey.
[d] Genotype characterized with an M gene of a low path virus are shown in purple.
[e] Persitent genotype not currently ciruculating are shown in blue.

Duan et al. (2008) (Table 3). A small proportion (16%) was genotype Z. The genotype of six viruses (3%) did not correspond to any other characterized previously; it was designated as "V-like" since only one gene (PB2 or M) differed from those of genotype V. In contrast, 38 distinct genotypes were identified using the updated lineages proposed herein (Table 3). Each genotype was defined based on criteria which accounted for gene lineage assignment based on tree topology, bootstrap support, pairwise distance comparison within and between lineages and the number of non-redundant sequences belonging to a monophyletic group (see Material and methods for additional details). Three of these genotypes were previously described as VN3, VN6 and VN7 among Vietnamese H5N1 viruses circulating before 2007 (Table 3) (Wan et al., 2008). To be consistent with previous analyses, we expanded the previous nomenclature of Wan et al. (2008) and named the 35 new genotypes reported in this study VN10 through VN44.

Only six of the 38 genotypes, namely VN7, VN10, VN11, VN12, VN3 and VN38 were observed in at least two consecutive years within this four-year study in Vietnam. These genotypes were observed in different seasons; i.e., for one year or more, and therefore could be designated as persistent. Interestingly, the persistent genotypes corresponded to distinct HA clades: 2.3.4.3, 2.3.4.1, 2.3.4.2, 2.3.2.1, 1.1-B and 1.1-A, respectively. Two of these six genotypes were described previously: VN7 (with 2.3.4.3 HA) and VN3 (with 1.1-B HA). VN7 viruses (derived from an A/chicken/Fujian/584/06-like virus) (Wan et al., 2008) was the most abundant genotype identified in this study (91 isolates 42%; Table 3). Three of the 4 novel persistent genotypes [VN10 (2.3.4.1), VN11 (2.3.4.2), and VN12 (2.3.2.1); in green in Table 3] have emerged recently in Vietnam (in 2009 and 2010) and represent the dominant viral genotypes circulating by the end of the study period (Supplementary Table 1). VN10 (2.3.4.1) was found mainly in northern provinces but also in the north-central coast (Quang Tri) and in Mekong River delta regions (Long An) whereas VN11 (2.3.4.2) was observed exclusively in northern provinces. VN12 (with clade 2.3.2.1 HA) was previously found in Hong Kong SAR (A/grey-heron/HK/1046/08) and found in Vietnam since 2009 mainly in the north (Ha Giang, Quang Ninh, Nam Dinh) but also in north central (Nghe An, Ha Tinh) and south central (Dac Lac, Khanh Hoa) provinces (Supplementary Table 1). Several related genotypes containing clade 2.3.2.1 HA genes have also been isolated from birds in Mongolia, Korea, Russia, China, and Japan (Boltz et al., 2010; Kang et al., 2011; Kim et al., 2010; Manin et al., 2010; Reid et al., 2010; Sakoda et al., 2010; Smith et al., 2009). Genotype VN38 (blue in Table 3) is the only genotype that was

persistent in 2007/2008 but was not seen since then and therefore might be considered extinguished.

Most genotypes (32/38) were detected only sporadically in the course of this study and were designated as transient (i.e., detected in only a single year and identified in four or fewer viruses). The previously described genotype (VN6) was only observed in 2007. The newly described genotype, VN40, with a clade 7.1 HA gene, was observed only in 2008. Genotype VN40 was transient as no clade 7 viruses were detected in 2009 or 2010 and this genotype has not been associated with any poultry outbreaks (Davis et al., 2010). Finally, five genotypes (VN30, VN41, VN42, VN43 and VN44) were observed sporadically in Vietnam but found previously in other Asian countries (Supplementary Table 5). These 32 transient genotypes differed from the persistent genotypes described above by at least one gene lineage (shown in pink in Supplementary Table 5) and probably arose by gene reassortment. A comparison of the gene constellations using a prototype virus for each genotype (Supplementary Table 5) showed that NA and HA genes were the most frequently involved in reassortment ($n=12$ and 9, respectively), while the NP gene was least prone to detectable reassortment ($n=2$). Although slightly fewer viruses were isolated from chickens (40%) as compared to ducks, a majority (64%) of the viruses with newly detected reassortant genomes was identified in chickens. The odds ratio showed that viruses isolated from chickens were 4.44 times more likely to have a reassorted genome than their duck counterparts.

Molecular characterization of protein sequences

To understand the potential functional significance of the recent evolution of the H5N1 viral population in Vietnam, we analyzed the distribution of amino acid changes in viral proteomes. To this end, we identified signature residues defined as amino acids found in at least 70% of sequences in a given lineage and found in less than 3% of sequences in all other lineages (Supplementary Tables 6and 7). Stricter criteria (100% of sequences in a lineage) were used for lineages containing ≤ 5 sequences. Some substitutions were strictly restricted to one lineage; i.e., they were not found in any other sequences from our dataset, and were considered as putative lineage signatures (Supplementary Table 6, pink shading). A subset of these positions was identified in 21 lineages containing at least 10 sequences (in green in Supplementary Table 6) and is likely to be significant. Lineage 8G had 9 signature substitutions, the largest number among all lineages.

The multibasic cleavage site of the HA that imparts high pathogenicity to avian influenza viruses was present in all the viruses analyzed. The cleavage site motif PLRERRRKR↓G (↓ denotes cleavage site) was found in all 2.3.4.1 sequences as well as most (96%) of the 2.3.4.3 sequences). A slight variation (underlined in PLREKRRKR↓G) was seen in 91% of 2.3.4.2 sequences. The PQRERRRKR↓G motif was found in all but one 2.3.2.1 HA, whereas the motif PQRE**G**RR*K*KR↓G with a glycine insertion (in **boldface**) was shared between all the 2.3.4-B sequences and 29 of the 31 clade 1.1 B sequences. The 4 clade 7 viruses are characterized by the cleavage motif PQRE**REGG**RRRKR↓G, with four additional amino acids inserted (boldface). Several other substitutions/insertions were observed but the biological significance of these mutations within the cleavage site remains unclear.

The deduced amino acid sequences of HA1 revealed numerous substitutions in putative antigenic sites. However, only two positions within the antigenic sites (140A in 2.3.4 B and 189Q in clade 7) were lineage-specific signatures (Supplementary Table 6). Residues at ten other positions throughout the whole HA protein were linked to a certain HA lineage (Supplementary Table 6). Five positions (2H, 120D, 162K, 226I, 240H) were found in all 15 2.3.2.1 sequences (Supplementary Table 6, green shading). The biological significance of these substitutions would require further investigation.

On average, the clade 2.3.4 HA had 5 substitutions in HA1 relative to the A/Anhui/2005 vaccine candidate The 2.3.4.3 HAs were the most related to that of the candidate vaccine virus with only 3 substitutions on average. The other 2.3.4-like HAs were more divergent with 6–11 substitutions compared to the vaccine candidate. V265M/I is found in all 2.3.4 viruses analyzed in this study. The clade 2.3.2.1 HAs had on average 7 substitutions compared to the A/common-magpie/Hong Kong/5052/2008 vaccine candidate, while the clade 1.1 viruses had approximately 10 and 6 substitutions compared to the A/Vietnam1203/2004 and A/Cambodia/R0405050/2007 vaccine candidates, respectively. Finally, despite the high divergence of clade 7 HA1 protein sequences (Davis et al., 2010), the four viruses analyzed in this study had on average 2 amino acid substitutions relative to the A/chicken/Vietnam/NCVD-03/08 vaccine candidate virus, also included in our dataset. The outlier viruses A/chicken/Vietnam/NCVD-093/08 and A/chicken/Vietnam/NCVD016/2008 featured 30 and 18 substitutions, respectively, compared to the clade 7 vaccine candidate HA1 sequence.

Among the 19 positions previously shown to be involved in neuraminidase activity, (Colman et al., 1993), only 3 sites (positions 203, 273, 275) were variable in the NA sequences analyzed in this study (Supplementary Table 6). At position 273, one of the three arginine residues of the catalytic site was substituted to a methionine in A/chicken/Vietnam/NCVD-081/2008. Five viruses had an NA with I203T and one virus had an NA with N275Y. Several NA gene sequences from clade 2.3.4.3 viruses (VN7, $n=8$) possessed the substitution I97V (I117V in Gs/GD numbering), a known marker for reduced susceptibility to neuraminidase inhibitors. However, no isolates possessed the mutations H255Y (H275Y in Gs/GD numbering) or N275S (N295S in Gs/GD numbering), suggesting a lack of markers of neuraminidase inhibitor resistance (Le et al., 2005). Thirteen additional positions were found to be restricted to a particular NA lineage (Supplementary Table 6). Seven of these positions (Supplementary Table 6, green shading) characterized lineages of more than 15 sequences (6B, 6D and 6E) may be biologically relevant. However, the functional significance of these substitutions remains unclear.

As had been previously shown, the M2 protein of all viruses with HA of clade 1.1-B (genotype VN3, lineage 7E) possessed conserved changes at positions L26I and S31N (Hurt et al., 2007). The combination of L26I and S31N has been previously associated with reduced susceptibility to the adamantane drugs. L26I and S31N mutations were also shared by two 2.3.4-B viruses (A/chicken/Vietnam/NCVD008/2008 and A/duck/Vietnam/NCVD009/2008). Whereas L26I was only found in lineage 7E (Supplementary Table 6), S31N was also shared by lineage 7D and 7F and therefore did not meet the criteria of a lineage specific residue. In addition, three positions (58, 64 and 68) were found in all sequences of lineage 7E (Supplementary Table 6, green shading); the biological relevance of these substitutions remains to be established.

Amino acid substitutions were also noted at positions predicted to modulate virulence/pathogenicity in animal models. Of particular interest was the truncation at the C-terminus of the NS1 protein of lineage 8B viruses which resulted in the elimination of the PDZ domain and may impact virulence (Golebiewski et al., 2011) (Supplementary Table 6). Likewise, a truncation of the PB1-F2 protein (resulting in a 24 or 25 amino acid protein) found among 92% of lineage 2D viruses may modulate their virulence (Schmolke et al., 2011).

Mutations in the four proteins that constitute the RNP complex (PB2, PB1, PA and NP) could have an impact on the replication efficiency of the virus in different host. PB2 protein from most viruses did not contain mutations predicted to enhance virus replication in mammalian cells (209/213 isolates); e.g., glutamic acid

(E in single letter notation) at position 627 and D at position 701 (all the isolates). The amino acid signature analysis revealed positions that are specific to one particular lineage (Supplementary Table 6). Of particular interest were the positions characterizing large lineages, with more than 10 sequences (Supplementary Table 6, green shading). Some of these positions were within functional domains and an amino acid substitution at these positions could have a biological effect. In PB1, five substitutions were found in lineages 2B, 2C, 2D and 2F within binding sites for cRNA, vRNa and PB2. In PA, three substitutions were found in all sequences of lineages 3D and 3H at sites involved in proteolysis and nuclear localization. Three positions were identified in NP lineages 5C and 5E. These positions were not known to be linked to functional properties but the significance of amino acid substitutions at these positions would be worth pursuing. Finally, M1, NS1 and NS2 featured amino acid substitutions that could modulate virus replication and pathogenesis (Supplementary Table 6). For example, NS1 lineage 8G was characterized by specific substitutions within binding site for eIF4G (81V) and CPSF (190T, 199G and 214N). The same lineage in NS2 was characterized by residue 62T involved in M1 binding site.

Discussion

In spite of sustained control efforts, H5N1 viruses continue to circulate in Vietnam. Previous reports have demonstrated the introduction of several new H5N1 genotypes into Vietnam as well as local generation of novel genotypes through reassortment before 2008 (Wan et al., 2008). This study reports results obtained from surveillance activities from July 2007 to December 2010. During this time period, close to 20,000 samples were collected by the Vietnamese Department of Animal Health (DAH) and the National Centre for Veterinary Diagnostics (NCVD, Hanoi, Vietnam) in 39 provinces throughout the country. Although the majority of samples were collected by passive surveillance following poultry outbreaks (FAO, 2011), in 18 instances, isolates were collected from provinces where no outbreak was reported indicating H5N1 circulation without mortality (possibly due to vaccination), or alternatively, a lack of consistent outbreak reporting. In addition, 59% of the total samples collected came from the north of the country suggesting a potential sampling bias, especially during 2007 (88% of viruses collected were from northern provinces). However, the distribution of sample collection versus reported outbreaks appears to be consistent throughout 2008, 2009 and 2010. Interestingly, the number of outbreaks reported to FAO has steadily decreased since 2007 (FAO, 2011), despite the steady number of human infections detected (5–8 annually) detected between 2007 and 2010) (WHO, 2011a). Taken together, these data highlight the need for sustained surveillance throughout the country, with well-defined data collection/management/reporting, in order to maintain an understanding of the many factors influencing both the ecology of the virus and the complexities of surveillance.

Genetic characterization, beginning with HA phylogeny, showed that viruses collected in Vietnam since 2007 were from six different HA clades (2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.2.1, 1.1, and 7.1). Nearly 75% percent of viruses analyzed in this study were from clade 2.3.4 (representing mostly isolates from northern Vietnam). The 2.3.4.3 viruses were dominant until 2008 and appeared to be replaced by clades 2.3.4.1 and 2.3.4.2 during 2008. Both 2.3.4.1 and 2.3.4.2 viruses appear to have emerged independently in Vietnam either by introduction from elsewhere or by disparate evolution of the HA gene. Although the exact ancestor of these two groups cannot be clearly deciphered by the current analysis, Vietnamese viruses from both groups were found at tips of branches with apparent ancestral viruses from other countries. Interestingly, the 2.3.4.1 viruses were located in a basal position of the overall 2.3.4 clade (Supplementary Fig. 1) and likely represent surveillance/sequencing gaps that were previously undetected. As such, these viruses may represent the progeny of an earlier group that was an intermediate lineage between the now extinct clade 2.3.3 and contemporary clade 2.3.4 viruses. Overall, the expansion of genetic diversity within clade 2.3.4 implies that this clade has been either under extensive pressure or evolving in genetically isolated populations leading to the accumulation and fixation of mutations. It remains to be tested whether or not this diversification could be driven by vaccination of poultry and selection of neutralization escape mutant viruses, high mutation rates due to sustained transmission among Vietnamese poultry, or other factors.

Fifteen viruses from our dataset belonged to a newly emerging group of clade 2.3.2 recently identified as clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). To our knowledge, this was the first time since 2006 that clade 2.3.2 viruses have been detected in Vietnam suggesting a new introduction into the country. HA sequences of these viruses were closely related to viruses isolated from several neighboring countries, including Mongolia, China, Japan, Russia, Hong Kong SAR, Korea and Laos, as well as European countries, Romania and Bulgaria (Boltz et al., 2010; Kang et al., 2011; Kim et al., 2010; Manin et al., 2010; Reid et al., 2010; Sakoda et al., 2010; Smith et al., 2009). A recent report of this genetic group specifically being detected in migratory birds returning to northern Mongolia/Siberian nesting territory from southern Asia (Sakoda et al., 2010), together with its detection in numerous waterfowl species from many countries, suggests that clade 2.3.2.1 may have spread into Vietnam via bird migration. Despite this potential mechanism of introduction, the identification of the virus in domestic poultry in Vietnam and elsewhere and its association with recent human cases in Hong Kong SAR and China indicates some degree of endemicity of this clade in domestic poultry as well.

Clade 1 viruses have been detected in Vietnam since 2003 (Wan et al., 2008). Thirty-three clade 1 viruses were characterized in this study and belong to the recently identified clade 1.1. The clade 1.1 viruses were isolated since 2007, mainly in southern provinces, although at least one example was found in northern provinces during both 2008 and 2009 (Fig. 2). The genetic diversity amongst clade 1 HA genes resulted in the characterization of two different lineages termed 1.1-A and 1.1-B for this report. 1.1-A lineage viruses corresponded to isolates collected only in 2007–2008, which represented genetic outliers within the Vietnamese clade 1.1. Lineage 1.1-B samples corresponded to those collected since 2007, including samples from this report from 2008–2010. The detection of two clade 1.1 variants suggests either multiple introductions of clade 1.1 viruses into Vietnam in recent years or sustained genetic divergence of clade 1.1 in the Mekong Delta region since the virus first appeared. It is likely that both factors have influenced clade 1.1 evolution as a result of the constant transnational movement of poultry (and circulation of virus) throughout the delta regions of Vietnam and Cambodia where the majority of clade 1 viruses are now found (Naughtin et al., 2011).

Clade 7 viruses were detected in 2008 in the north of the country (Davis et al., 2010; Nguyen et al., 2009). Of the six clade 7 viruses characterized for this study, four belonged to the recently identified clade 7.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). Due to the high genetic diversity of the clade 7 viruses, the criteria chosen to identify the genetic lineages in this study led us to exclude two clade 7 viruses that were otherwise known as clade 7.1 (A/chicken/NCVD-016/2008) and 7.2 (A/chicken/NCVD-093/2008). As previously reported, clade 7 viruses have not been detected since 2008 in Vietnam and were likely detected as a consequence of illegal trade with neighboring countries. Nonetheless, clade 7 viruses continue to be sporadically reported outside of Vietnam and may

still represent a source of novel genetic variants capable of re-introduction into the country (Jiang et al., 2010).

Overall, these data show that despite the continued circulation of clade 1 in Vietnam, clade 2.3.4 continues to spread into southern provinces (Tra Vinh and Long An). In addition, samples collected in late 2010 showed that 2.3.2.1 had spread towards the south. Two southern provinces (Dac Lac and Khanh Hoa) were found with viruses of both clade 1.1 and 2.3.2.1 (Fig. 2). Since the re-introduction of clade 2.3.2 viruses in 2009, H5N1 viruses from four distinct clades have co-circulated in Vietnamese poultry (clades 1.1, 2.3.4.1, 2.3.4.2, and 2.3.2.1) making differentiation of the virus surface proteins complex using traditional molecular or serologic diagnostic methods. Antigenic characterization of select viruses from each of these clades confirmed, however, that using an appropriate panel of ferret antisera allowed for precise differentiation of clades by two-way hemagglutination inhibition (HI) assay. Additionally, HI assays suggest that while viruses within various clades continue to evolve genetically, there continues to be some level of cross-reactivity among viruses within each clade. Furthermore, results from antigenic analysis provide some evidence that pre-pandemic vaccine seed viruses developed for potential use in human vaccines remain cross-reactive against contemporary viruses in Vietnam. The exception may be the clade 2.3.2.1 viruses, which displayed on average higher reductions in titers compared to homologous antisera. Additional testing of newly proposed clade 2.3.2.1 WHO vaccine candidates against circulating clade 2.3.2.1 viruses are needed. Finally, how these antigenic data correlate to immunologic reactivity in poultry in the field remains to be tested but may be critical to inform poultry vaccination decisions in Vietnam in the future.

Analysis of amino acid residue signatures in each gene lineage revealed that Vietnamese H5N1 viruses retain avian consensus sequences with very few variations from protein sequences typical of H5N1 viruses previously described in Vietnam or other countries. One exception was the identification of a PB1-F2 truncation that resulted in a 25 or 26 amino acid protein in viruses with the lineage 2D PB1 gene (clade 2.3.4.1). The precise contribution of PB1-F2 to virulence and its function in the life cycle of influenza A viruses in mammalian hosts remain unclear, but expression of full length PB1-F2 has been associated with mitochondrial targeting and apoptosis (Chen et al., 2001; Gibbs et al., 2003). It has been suggested that mitochondrial disruption with subsequent cell death could contribute to virulence. Another possible function of the PB1-F2 protein, causing immunopathology by enhancing the inflammatory response, has been demonstrated in animal models of influenza disease (Conenello et al., 2007; McAuley et al., 2007). Thus, the exact role of PB1-F2 in H5N1 viruses remains to be elucidated and further studies will be necessary to assess whether or not this particular PB1-F2 truncation could have an effect on viral phenotype. Another potential virulence determinant previously recognized in H5N1 viruses is the presence of the PDZ ligand motif of the NS1 protein. 184 NS1 protein sequences of our dataset featured an E/G/K-S-E-V/I motif at the C terminus that fits the universal PDZ domain ligand motif X-S/T-X-V previously described (Obenauer et al., 2006). Animal studies showed that the PDZ domain ligand motif was associated with increased virulence and pathogenesis (Jackson et al., 2008). 29 viruses with lineage 8B NS genes were characterized as having a stop codon introduced prior to the start of the NS1 PDZ domain ligand motif. The implications of the loss of the ability to bind protein containing PDZ domain would need further investigation. Finally, all viruses of genotype VN3 (clade 1.1-B) possess an M2 gene with fixed antiviral resistance mutations (L26I and S31N). Such characteristics remain clinically relevant when considering the use of adamantane drugs to treat patients suspected or confirmed to have H5N1 infection.

Because of the continuous mixing of genes among HPAI H5N1 viruses and occasionally other LPAI viruses circulating in wild birds or poultry, reassortment remains a major hurdle in understanding how the genome influences viral phenotype. Duan et al. developed a nomenclature for both gene lineages and genotypes in order to better understand gene reassortment in H5N1 viruses collected in China from 1996 to 2006 (Duan et al., 2008). The authors identified 44 genotypes including 10 that were considered to be persistent and 34 that were transient. They showed that genotype B was replaced by genotype Z in 2002 and that genotype Z was subsequently replaced by genotype V in 2005. Unsurprisingly, our dataset showed the same trend with a predominance of genotype V in Vietnam since at least early 2007. The exception remains clade 1 viruses, which are still classified as genotype Z (due to the PA gene lineage). However, because this nomenclature did not convey the full extent of gene reassortment at the microevolutionary level within viruses collected in Vietnam, we identified gene lineages based on criteria similar to that used for the HPAI H5 HA nomenclature. Using the criteria stated herein, we identified 5 to 8 gene lineages depending on the gene and 38 different gene constellations. Although some of these were previously identified in Vietnam and/or China, 29 genotypes appeared to be newly identified using this methodology. As demonstrated by the identification of 38 genotypes within the 213 isolates analyzed here, reassortment between H5N1 viruses in Vietnam appears to be extensive. While the majority of reassortants contained genes from within the Vietnamese gene pool, several genes derived from viruses found in neighboring countries also indicate gene flow between countries. In addition, two genotypes contained genes from H6N1 LPAI viruses underscoring the potential for introduction of novel genes into the H5N1 gene pool.

Most of the genotypes reported here were considered transient (i.e., detected in only a single year) and were only seen in one or two viruses, suggesting perhaps reduced fitness or environmental conditions, which prevented further spread of the virus. Six genotypes were considered persistent as they were detected over different years and at different seasons. Three of the persistent genotypes were circulating in both 2009 and 2010 (VN10, VN11 and VN12) and are likely to continue circulating. However, VN7 (clade 2.3.4.3), which was originally reported in 2007, made up the highest percentage of virus genotypes in this study and may still be in circulation even though it was not found in 2009 or 2010. Additionally, VN3 (clade 1.1) viruses are likely to be detected in larger numbers than described here, especially as more samples are collected in southern Vietnam. The persistent genotypes corresponded to distinct HA lineages 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.2.1, 1.1 A and 1.1B, suggesting that the HA is a potential factor contributing to persistence. Genotype analysis also revealed that the majority of reassortants were isolated from chickens despite the majority of total isolates characterized coming from domestic ducks. Even though the size of our dataset is small, we speculate that this might be linked to different husbandry practices between chickens and ducks. Chickens, whether in large commercial holdings or smaller backyard flocks are raised in environments where they are likely to transmit more virus to one another in confined spaces where environmental loads may be high. Ducks, on the other hand, especially grazing ducks (which represent 75% of the duck population in Vietnam) tend to be raised in free-ranging, smaller flocks where contact with other birds is likely to be less (Minh et al., 2009). Thus, we surmise that the frequency of virus transmission, and consequently opportunities for co-infection, may be higher for chickens than ducks leading to more frequent reassortment events.

The present study demonstrates that H5N1 viruses remain widespread throughout Vietnam and represent as many as six distinct HA clades and numerous genotypes, several of which have recently emerged to become persistently circulating in the last two years.

These viruses remain a threat to poultry and wild bird populations as well as other animals and humans. Analysis of virus evolution and gene reassortment within endemic H5N1 countries, especially in regions like Vietnam where the genomic diversity is one of the highest in the world, is critical to monitor how the virus changes over time and under varying environmental and human induced pressures. That as many as 38 genotypes were identified in Vietnam consisting of gene segments derived from H5N1 viruses both within and outside the country, as well as from low pathogenic avian influenza subtypes, underscores the role of poultry as a melting pot for this continually evolving virus.

Materials and methods

Virologic surveillance in domestic birds

The Vietnam Department of Animal Health (DAH) and the National Centre for Veterinary Diagnostics (NCVD, Hanoi, Vietnam) has conducted surveillance to detect H5N1 infections in poultry since 2004. This study reports results obtained from both active and passive surveillance activities from July 2007 to December 2010. Collection sites included poultry farms, backyard flocks, and live bird markets in 39 provinces representing regions in Northern, Central, and Southern Vietnam. The provinces where samples were collected were mapped using ArcView (ArcGIS 9.3.1, ESRI). The maps included locations of reported poultry outbreaks (retrieved from http://empres-i.fao.org). The samples were obtained from cloacal or oropharyngeal swabs from chickens, ducks, quail, ostrich and an Owston's civet by DAH Regional Animal Health Offices. Swab specimens were placed in containers with 1.0 ml of virus transport medium prior to submission to NCVD. Following RNA extraction from 100 μl of the swab/transport medium supernatant, samples were screened for influenza A matrix gene RNA by real-time RT-PCR. Matrix positive samples were then tested for H5N1 virus using an influenza subtype H5N1 specific real-time RT-PCR assay (WHO, 2007). A subset of positive samples ($n=283$) was processed and the resulting isolates ($n=213$) fully characterized.

Virus isolation and sequencing

Highly pathogenic avian influenza (HPAI) H5N1 viruses were isolated from positive samples by inoculation into the allantoic sac of 9–11 day old embryonated chicken eggs (ECEs). Twenty-four hours post-inoculation allantoic fluid was harvested from eggs and screened for the presence of virus by a hemagglutination assay using chicken red blood cells. RNA was extracted from virus-infected allantoic fluid using the RNeasy extraction kit (Qiagen), and the Access Quick one-step RT-PCR kit (Promega) was used to amplify each of the individual influenza gene segments as overlapping fragments. The amplicons were sequenced on an automated Applied Biosystems 3730 system using cycle sequencing dye terminator chemistry and contigs of full length open reading frames were generated for each gene (Sequencher 4.8, Gene Codes). Sequences were submitted to the GISAID sequence database (accession numbers are included in Supplementary Table 1). Handling of infectious materials at CDC was performed in compliance with biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agents program (http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm).

Antigenic analysis

Antigenic characterization of HPAI H5N1 isolated in Vietnam during 2007–2010 was performed using the hemagglutination inhibition (HI) assay with ferret antisera to a panel of representative H5N1 viruses. The HI assay was performed using turkey red blood cells, as previously described (Balish et al., 2010). In addition to antisera generated against Vietnamese H5N1 viruses analyzed in this study, the antigenic analysis included antisera to viruses selected as WHO pre-pandemic H5N1 vaccine candidates (WHO, 2011b). Additional testing of Vietnamese H5N1 viruses representing each clade was conducted but data is not shown due to similarities to Table 2.

Phylogenetic analysis

This study is based on 3 datasets: (1) individual gene sequences of H5N1 avian influenza viruses collected only in Vietnam from 2007 to 2010 that were aligned in order to identify Vietnam-specific gene lineages; (2) HA H5 sequences of avian influenza viruses collected in Vietnam from 2007 to 2010 that were aligned in the context of representative H5 sequences of viruses collected in other countries in order to identify the relationship to designated HA clades; (3) the 8 individual gene sequences of H5N1 viruses collected in Vietnam from 2007 to 2010 that were aligned in the context of whole viral genome H5N1 sequences from other Asian countries. Phylogenies were inferred with MEGA software (version 4) using the neighbor-joining method and a maximum composite likelihood model (Kumar et al., 2004; Saitou and Nei, 1986). The reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications, using MEGA (Efron and Tibshirani, 1994). The HA tree used to determine clade designation was rooted with A/goose/Guangdong/1/1996. The other trees generated for gene lineage and genotype analysis were not rooted.

Gene lineage and genotype identification

Virus genotypes were determined using two levels of stringency. For the initial analysis, we used the approach established by Duan et al. (2008), whereby gene lineages were identified based on tree topology and bootstrap support values and named as suggested in this publication (Duan et al., 2008). To understand the microevolution of H5N1 in Vietnam, gene lineages reported in the current study were identified according to the following criteria: (i) the average *p*-distance between viruses within a gene lineage should be $<1.5\%$ and the average p-distance between lineages should be $>1.5\%$. These criteria were used previously to define H5N1 HA clade designations (WHO/OIE/FAO H5N1 Evolution Working Group, 2008, 2011); (ii) lineages should be supported by monophyletic tree topology with a bootstrap value higher than 60 at the lineage-defining node. In order to limit the number of lineages, additional criteria concerning outliers and sequence diversity were established; (iii) the number of outlier sequences should be less than 3% of all sequences analyzed; (iv) a lineage should contain at least two gene sequences that are not identical. As a consequence, the tree topology and bootstrap values were considered secondary compared to the genetic distance criteria.

For all genes except HA, we utilize a lineage nomenclature in which the first digit is a number assigned to each segment (PB2: 1, PB1: 2, PA: 3, NP: 5, NA: 6, M: 7 and NS: 8) followed by a letter indicating the group within a given lineage (i.e., 1A for PB2, lineage A). For the HA gene, the clade nomenclature system recently updated by the WHO/OIE/FAO H5N1 Evolution Working Group (WHO/OIE/FAO H5N1 Evolution Working Group, 2011) was used in combination with a letter, when necessary, to indicate smaller groups that meet the above criteria (for example, 2.3.4 A). The genotype nomenclature established for viruses isolated in Vietnam

from 2001 to 2007 (Wan et al., 2008) was used when appropriate and expanded to include new genotypes from this study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.06.021.

References

Arafa, A., Suarez, D.L., Hassan, M.K., Aly, M.M., 2010. Phylogenetic analysis of hemagglutinin and neuraminidase genes of highly pathogenic avian influenza H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct sublineages. Avian Dis. 54, 345–349.

Balish, A.L., Davis, C.T., Saad, M.D., El-Sayed, N., Esmat, H., Tjaden, J.A., Earhart, K.C., Ahmed, L.E., Abd El-Halem, M., Ali, A.H., Nassif, S.A., El-Ebiary, E.A., Taha, M., Aly, M.M., Arafa, A., O'Neill, E., Xiyan, X., Cox, N.J., Donis, R.O., Klimov, A.I., 2010. Antigenic and genetic diversity of highly pathogenic avian influenza a (H5N1) viruses isolated in Egypt. Avian Dis. 54, 329–334.

Boltz, D.A., Douangngeun, B., Phommachanh, P., Sinthasak, S., Mondry, R., Obert, C., Seiler, P., Keating, R., Suzuki, Y., Hiramatsu, H., Govorkova, E.A., Webster, R.G., 2010. Emergence of H5N1 avian influenza viruses with reduced sensitivity to neuraminidase inhibitors and novel reassortants in Lao People's Democratic Republic. J. Gen. Virol. 91, 949–959.

Chaichoune, K., Wiriyarat, W., Thitithanyanont, A., Phonarknguen, R., Sariya, L., Suwanpakdee, S., Noimor, T., Chatsurachai, S., Suriyaphol, P., Ungchusak, K., Ratanakorn, P., Webster, R.G., Thompson, M., Auewarakul, P., Puthavathana, P., 2009. Indigenous sources of 2007–2008 H5N1 avian influenza outbreaks in Thailand. J. Gen. Virol. 90, 216–222.

Chen, J., Fang, F., Yang, Z., Liu, X., Zhang, H., Zhang, Z., Zhang, X., Chen, Z., 2009. Characterization of highly pathogenic H5N1 avian influenza viruses isolated from poultry markets in central China. Virus Res. 146, 19–28.

Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R., Yewdell, J.W., 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat. Med. 7, 1306–1312.

Cheung, C.L., Vijaykrishna, D., Smith, G.J., Fan, X.H., Zhang, J.X., Bahl, J., Duan, L., Huang, K., Tai, H., Wang, J., Poon, L.L., Peiris, J.S., Chen, H., Guan, Y., 2007. Establishment of influenza A virus (H6N1) in minor poultry species in southern China. J. Virol. 81, 10402–10412.

Colman, P.M., Hoyne, P.A., Lawrence, M.C., 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J. Virol. 67, 2972–2980.

Conenello, G.M., Zamarin, D., Perrone, L.A., Tumpey, T., Palese, P., 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog. 3, 1414–1421.

Davis, C.T., Balish, A.L., O'Neill, E., Nguyen, C.V., Cox, N.J., Xiyan, X., Klimov, A., Nguyen, T., Donis, R.O., 2010. Detection and characterization of clade 7 high pathogenicity avian influenza H5N1 viruses in chickens seized at ports of entry and live poultry markets in Vietnam. Avian Dis. 54, 307–312.

Duan, L., Bahl, J., Smith, G.J., Wang, J., Vijaykrishna, D., Zhang, L.J., Zhang, J.X., Li, K.S., Fan, X.H., Cheung, C.L., Huang, K., Poon, L.L., Shortridge, K.F., Webster, R.G., Peiris, J.S., Chen, H., Guan, Y., 2008. The development and genetic diversity of H5N1 influenza virus in China, 1996–2006. Virology 380, 243–254.

Efron, B., Tibshirani, R.J., 1994. An Introduction to the Bootstrap. CRC Press, Boca Raton, FL.

FAO, 2011. Food and Agricultural Organization of the United Nations. 〈http://empres-i.fao.org〉.

Gibbs, J.S., Malide, D., Hornung, F., Bennink, J.R., Yewdell, J.W., 2003. The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. J. Virol. 77, 7214–7224.

Golebiewski, L., Liu, H., Javier, R.T., Rice, A.P., 2011. The avian influenza NS1 ESEV PDZ binding motif associates with Dlg1 and scribble to disrupt cellular tight junctions. J. Virol.

Hoffmann, E., Stech, J., Leneva, I., Krauss, S., Scholtissek, C., Chin, P.S., Peiris, M., Shortridge, K.F., Webster, R.G., 2000. Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1? J. Virol. 74, 6309–6315.

Hurt, A.C., Selleck, P., Komadina, N., Shaw, R., Brown, L., Barr, I.G., 2007. Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. Antiviral Res. 73, 228–231.

Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R., Lamb, R.A., 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc. Nat. Acad. Sci. U.S.A. 105, 4381–4386.

Jiang, W.M., Liu, S., Chen, J., Hou, G.Y., Li, J.P., Cao, Y.F., Zhuang, Q.Y., Li, Y., Huang, B.X., Chen, J.M., 2010. Molecular epidemiological surveys of H5 subtype highly pathogenic avian influenza viruses in poultry in China during 2007–2009. J. Gen. Virol. 91, 2491–2496.

Kang, H.M., Batchuluun, D., Kim, M.C., Choi, J.G., Erdene-Ochir, T.O., Paek, M.R., Sugir, T., Sodnomdarjaa, R., Kwon, J.H., Lee, Y.J., 2011. Genetic analyses of H5N1 avian influenza virus in Mongolia, 2009 and its relationship with those of eastern Asia. Vet. Microbiol. 147, 170–175.

Kim, H.R., Park, C.K., Lee, Y.J., Woo, G.H., Lee, K.K., Oem, J.K., Kim, S.H., Jean, Y.H., Bae, Y.C., Yoon, S.S., Roh, I.S., Jeong, O.M., Kim, H.Y., Choi, J.S., Byun, J.W., Song, Y.K., Kwon, J.H., Joo, Y.S., 2010. An outbreak of highly pathogenic H5N1 avian influenza in Korea, 2008. Vet. Microbiol. 141, 362–366.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Manin, T.B., Chvala, I.A., Kolosov, S.N., Pchelkina, I.P., Irza, V.N., Drygin, V.V., 2010. H5N1 avian influenza outbreak in the Far East of Russia in 2008: new introduction. Avian Dis. 54, 509–512.

McAuley, J.L., Hornung, F., Boyd, K.L., Smith, A.M., McKeon, R., Bennink, J., Yewdell, J.W., McCullers, J.A., 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe 2, 240–249.

Minh, P.Q., Schauer, B., Stevenson, M., Jones, G., Morris, R.S., Noble, A., 2009. Association between human cases and poultry outbreaks of highly pathogenic avian influenza in Vietnam from 2003 to 2007: a nationwide study. Transbound. Emerg. Dis. 56, 311–320.

Naughtin, M., Dyason, J.C., Mardy, S., Sorn, S., von Itzstein, M., Buchy, P., 2011. Neuraminidase inhibitor sensitivity and receptor-binding specificity of Cambodian clade 1 highly pathogenic H5N1 influenza virus. Antimicrob. Agents Chemother. 55, 2004–2010.

Nguyen, T., Davis, C.T., Stembridge, W., Shu, B., Balish, A., Inui, K., Do, H.T., Ngo, H.T., Wan, X.F., McCarron, M., Lindstrom, S.E., Cox, N.J., Nguyen, C.V., Klimov, A.I., Donis, R.O., 2009. Characterization of a highly pathogenic avian influenza H5N1 virus sublineage in poultry seized at ports of entry into Vietnam. Virology 387, 250–256.

Obenauer, J.C., Denson, J., Mehta, P.K., Su, X., Mukatira, S., Finkelstein, D.B., Xu, X., Wang, J., Ma, J., Fan, Y., Rakestraw, K.M., Webster, R.G., Hoffmann, E., Krauss, S., Zheng, J., Zhang, Z., Naeve, C.W., 2006. Large-scale sequence analysis of avian influenza isolates. Science 311, 1576–1580.

Reid, S.M., Shell, W.M., Barboi, G., Onita, I., Turcitu, M., Cioranu, R., Marinova-Petkova, A., Goujgoulova, G., Webby, R.J., Webster, R.G., Russell, C., Slomka, M.J., Hanna, A., Banks, J., Alton, B., Barrass, L., Irvine, R.M., Brown, I.H., 2010. First reported incursion of highly pathogenic notifiable avian influenza A H5N1 viruses from clade 2.3.2 into European poultry. Transbound. Emerg. Dis.

Saitou, N., Nei, M., 1986. Polymorphism and evolution of influenza A virus genes. Mol. Biol. Evol. 3, 57–74.

Sakoda, Y., Sugar, S., Batchluun, D., Erdene-Ochir, T.O., Okamatsu, M., Isoda, N., Soda, K., Takakuwa, H., Tsuda, Y., Yamamoto, N., Kishida, N., Matsuno, K., Nakayama, E., Kajihara, M., Yokoyama, A., Takada, A., Sodnomdarjaa, R., Kida, H., 2010. Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. Virology 406, 88–94.

Schmolke, M., Manicassamy, B., Pena, L., Sutton, T., Hai, R., Varga, Z.T., Hale, B.G., Steel, J., Perez, D.R., Garcia-Sastre, A., 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. PLoS Pathog. 7, e1002186.

Sims, 2010. Strategies for Controlling Animal Flu and Its Implications for Human Health, Hong Kong SAR, Options Meeting 2010.

Smith, G.J., Vijaykrishna, D., Ellis, T.M., Dyrting, K.C., Leung, Y.H., Bahl, J., Wong, C.W., Kai, H., Chow, M.K., Duan, L., Chan, A.S., Zhang, L.J., Chen, H., Luk, G.S., Peiris, J.S., Guan, Y., 2009. Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong, 2004–2008. Emerg. Infect. Dis. 15, 402–407.

Solovyov, A., Greenbaum, B., Palacios, G., Lipkin, W.I., Rabadan, R., 2010. Host Dependent Evolutionary Patterns and the Origin of 2009 H1N1 Pandemic Influenza: Alexander Solovyov*, Benjamin Greenbaum*, Gustavo Palacios, W. Ian Lipkin and Raul Rabadan (* Joint First Authors). PLoS Curr 2, RRN1147.

Takano, R., Nidom, C.A., Kiso, M., Muramoto, Y., Yamada, S., Sakai-Tagawa, Y., Macken, C., Kawaoka, Y., 2009. Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003–2007. Virology 390, 13–21.

Wan, X.F., Nguyen, T., Davis, C.T., Smith, C.B., Zhao, Z.M., Carrel, M., Inui, K., Do, H.T., Mai, D.T., Jadhao, S., Balish, A., Shu, B., Luo, F., Emch, M., Matsuoka, Y., Lindstrom, S.E., Cox, N.J., Nguyen, C.V., Klimov, A., Donis, R.O., 2008. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. PLoS One 3, e3462.

Watanabe, Y., Ibrahim, M.S., Ellakany, H.F., Kawashita, N., Mizuike, R., Hiramatsu, H., Sriwilaijaroen, N., Takagi, T., Suzuki, Y., Ikuta, K., 2011. Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. PLoS Pathog. 7, e1002068.

WHO, 2007. Recommendations and Laboratory Procedures for Detection of Avian Influenza A(H5N1) Virus in Specimens from Suspected Human Cases. 〈http://www.who.int/influenza/resources/documents/RecAllabtestsAug07.pdf〉.

WHO, 2011a. World Health Organization. 〈http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_08_19/en/index.html〉.

WHO, 2011b. Antigenic and Genetic Characteristics of Influenza A(H5N1) and Influenza A(H9N2) Viruses and Candidate Vaccine Viruses Developed for Potential use in Human Vaccines. September 2011. 〈http://www.who.int/influenza/resources/documents/characteristics_virus_vaccines/en/index.html〉.

WHO/OIE/FAO H5N1 Evolution Working Group, 2008. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg. Infect. Dis. 14, e1.

WHO/OIE/FAO H5N1 Evolution Working Group, 2011. Continued Evolution of Highly Pathogenic Avian Influenza A (H5N1): Updated Nomenclature, Influenza Other Respi Viruses, 2011/11/01 ed.