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Genetic characterization of an H5N1 avian influenza virus from a vaccinated duck flock in Vietnam

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Abstract This study reports the genetic characterization of a highly pathogenic avian influenza virus subtype H5N1 isolated from a moribund domestic duck in central Vietnam during 2012. In the moribund duck's flock, within 6 days after vaccination with a commercial H5N1 vaccine (Re-5) to 59-day-old birds, 120 out of 2,000 ducks died. Genetic analysis revealed a substantial number of mutations in the HA gene of the isolate in comparison with the vaccine strains, Re-1 and Re-5. Similar mutations were also found in selected Vietnamese H5N1 strains isolated since 2009. Mutations in the HA gene involved positions at antigenic sites associated with antibody binding and also neutralizing epitopes, with some of the mutations resulting in the modification of *N*-linked glycosylation of the HA. Those mutations may be related to the escape of virus from antibody binding and the infection of poultry, interpretations which may be confirmed through a reverse genetics approach. The virus also carried an amino acid substitution in the M2, which conferred a reduced susceptibility to amantadine, but no neuraminidase inhibitor resistance markers were found in the viral NA gene. Additional information including vaccination history in the farm and the surrounding area is needed to fully understand the background of this outbreak. Such understanding and expanded monitoring of the H5N1 influenza viruses circulating in Vietnam is an urgent need to provide updated information to improve effective vaccine strain selection and vaccination protocols, aiding disease control, and biosecurity to prevent H5N1 infection in both poultry and humans.

Keywords HPAIV · H5N1 · Vietnam · Vaccination · Genetic

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Introduction

The first isolation of highly pathogenic avian influenza virus (HPAIV) of an H5N1 subtype was reported in the Chinese province of Guangdong province in 1996 [1]. Subsequently, the virus has been isolated from wild birds and domestic poultry in many countries in Asia, Europe, and Africa [2–4]. HPAIVs not only threaten animal health but also raise pandemic concern due to the evidence of zoonotic transmission to humans [5, 6]. Since its first detection, the H5N1 virus has undergone significant genetic diversification resulting in 10 viral clades originating from clade 0 to clade 9. Among them, clade 2 showed significant genetic variation into numerous subclades [7]. In addition, genetic reassortment has resulted in rapid viral evolution and created favorable circumstances for the emergence of new genotypes that may possess unique characteristics [8–11]. Thus, it is imperative to continue characterizing newly isolated HPAIVs to monitor for genetic changes that alter the viral phenotype.

In Vietnam, H5N1 virus was detected in poultry for the first time in 2001 [12]. The H5N1 HPAIVs frequently

isolated from outbreaks during 2001–2005 belonged to clade 1 [13]. In 2007, H5N1 clade 1 re-emerged and was followed by introduction of the new clade 2.3.4. From 2007 until 2010, while H5N1 clade 1 circulated in Southern Vietnam, H5N1 clade 2.3.4 was predominant in Northern Vietnam, although in 2008, H5N1 clade 7 was reported and from 2009, H5N1 clade 2.3.2.1 was sporadically detected [13, 14]. By 2012, viruses of clade 2.3.2.1 were dominantly circulating in Vietnam. The outbreaks in poultry have caused adverse health and economic effects in Vietnam [15–17]. In addition, Vietnam is one among the countries with a high rate of infection in humans with high mortality (125 infection cases with 62 fatalities as of Dec 10, 2013) [18].

In order to reduce the number of infections in poultry and in turn reduce the risk of human exposure and human cases caused by H5N1 virus as a step toward controlling the disease, the Vietnamese government has implemented a vaccination program for poultry throughout the country. This program has used inactivated Chinese vaccines (Harbin Weike Biotechnology Development Company, China) including Re-1 since 2005 and Re-5 since 2011 that respectively derive their HA and NA genes from A/goose/Guangdong/1/1996 (Gs/GD/1/96) and from A/duck/Anhui/1/2006 (Dk/Anhui/1/06). However, H5N1 outbreaks have still been sporadically reported in Vietnam. The FAO-OIE-WHO Technical Update (http://www.fao.org/docrep/014/al874e/ al874e00.pdf) reported on the evolution of HPAI H5N1 viruses causing outbreaks in Vietnam until September 2011, and stated that the vaccines were effective for clade 2.3.2.1 viruses except for one antigenic variant found in 2011. However, Creanga et al. [19], who conducted active virologic surveillance in Vietnam between September 2010 and September 2012, found the emergence of multiple clade 2.3.2.1 H5N1 virus subgroups, highlighting the continuous evolution of the circulating viruses. The findings raise concern that the current use of H5N1 poultry vaccines in Vietnam might not protect poultry against the recent antigenic variant H5N1 viruses in clade 2.3.2.1. Here we describe the molecular characterization of an HPAI H5N1 virus isolated in 2012 from a Re-5 vaccinated duck, which is one of the inactivated AIV vaccines most widely used in Vietnam in recent years.

Materials and methods

Virus isolation and identification

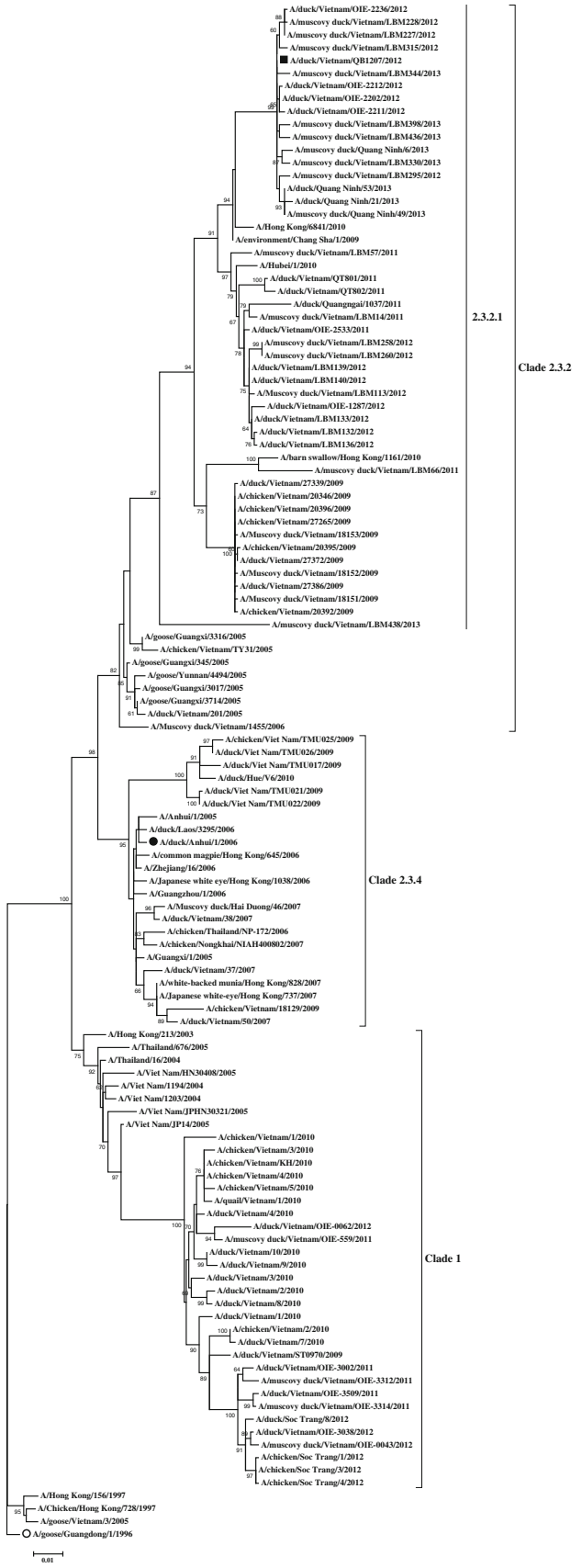
In July 2012, a farm in Quang Binh province in central Vietnam performed vaccination with Re-5 on 2,000 ducks at 59 days of age. Six days after the vaccination, 120 ducks were found dead. For AIV diagnosis in the flock, three ducks were sent to the national laboratory. Among those ducks, two were dead, and one was moribund. Viruses of subtype H5N1 were recovered from all three ducks. Partial sequences of the HA gene indicated that the three strains were almost identical. In this paper, the virus from the moribund duck is described. Since the duck was showing neurological signs, the brain was collected from the duck and prepared as a 10 % homogenate that was used for virus isolation and the detection of influenza A virus using RT-PCR according to the WHO manual [20]. Virus isolation was carried out in 10-day-old embryonated eggs via allantoic cavity inoculation. The virus titer was estimated by 50 % egg-infective dose (EID_{50}) according to the Spearman Kärber method. Virus subtyping using primers specific for the H5N1 subtype was conducted according to the WHO manual [20].

RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from the infectious allantoic fluids using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The viral RNA was transcribed into cDNA using the Uni12 primer (5′-agcraaagcagg-3′) and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) at 42 °C for 60 min followed by 70 °C for 10 min. Using the cDNAs as templates, PCR was conducted using primers specific for the H5 and N1 gene of AIV and the protocol recommended by WHO [20]. RT-PCR was also conducted to amplify the full length of the eight genomic segments by the method reported by Hoffmann et al. [21]. The PCR products obtained were separated by 1 % agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). The primer sets described above and walking primers designed by us (sequences available upon request) were used to obtain the full-length nucleotide sequence of the eight viral gene segments. The complete genome sequence of the isolate generated in this study was deposited in GenBank under the accession numbers KF182738 to KF182745.

Genetic analysis

The nucleotide sequences obtained were analyzed with the Genetyx Ver. 10 software (GENETYX Corp., Tokyo, Japan) and compared with other sequences available in GenBank identified by BLAST homology searches (http://www.ncbi.nlm.nih.gov/genomes/ FLU/FLU.html). Nucleotide sequences and deduced amino acid HA and NA



◀**Fig. 1** Phylogenetic tree of the full-length HA genes of HPAI H5N1 viruses. A/duck/Vietnam/QB1207/2012 (H5N1) (*black square*) together with other H5N1 Vietnamese strains isolated from 2004–2013, the vaccine strain of Re-1 derived from A/goose/Guangdong/1/96 (H5N1) (*white circle*), the vaccine strain of Re-5 derived from A/duck/Anhui/1/2006 (H5N1) (*black circle*) and other representative strains of H5N1 are shown in the tree. The evolutionary history was inferred using the maximum likelihood method. The evolutionary distances were calculated using the Tamura-Nei model. *Numbers* at each branch point indicate bootstrap values $\geq$60 % in the bootstrap interior branch test. All positions containing gap and missing data were eliminated. Phylogenetic analysis was conducted in MEGA6. The *scale bar* indicates 0.01 nucleotide substitutions per site

sequences of the isolate were further compared with representative Vietnamese strains and those of vaccine strains including Gs/GD/1/96 (Re-1) and Dk/Anhui/1/06 (Re-5) based on alignments by Clustal W, Ver. 1.4 using the BioEdit Package, Ver. 7.2 software. Phylogenetic analysis of the HA genes was conducted using a dataset containing 94 HA nucleotide sequences of Vietnamese H5N1 strains isolated from 2004 to 2013, which are available in GenBank, the sequence obtained in this study, reference strains and vaccine strains. The sequences were aligned by Clustal W [22] and evolutionary distances were calculated using Tamura-Nei model. The tree was constructed with Mega 6.06 software [23] using the maximum likelihood method supported by 500 bootstrap replications.

Results and discussion

Infection with influenza virus was confirmed by RT-PCR for detection of the Matrix gene of AIV in the brain homogenate sample of the vaccinated moribund duck. The virus was successfully recovered as a titer of $10^{7.5}$ EID_{50}/ml in eggs inoculated with the sample and the isolate was identified as AIV of the H5N1 subtype by RT-PCR. The virus isolated from the duck brain sample was designed as A/duck/Vietnam/QB1207/2012 (H5N1) (Dk/VN/QB1207/12).

Phylogenetic analysis of the HA gene clearly indicated that Dk/VN/QB1207/12 belonged to the Eurasian lineage together with other Vietnamese strains during 2012–2013, and those Vietnamese strains are closely related to A/environment/Chang Sha/1/2009 and A/Hong Kong/6841/2010, suggesting that they might share a common ancestor. The Dk/VN/QB1207/12 isolate fell into clade 2.3.2.1 by phylogenetic analysis using the HA gene as we recently reported [24]. On the other hand, Gs/GD/1/96 (Re-1) and Dk/Anhui/1/06 (Re-5) were grouped in clade 0 and clade 2.3.4, respectively. The HA gene sequence of Gs/GD/1/96 was close to those of Vietnamese strains isolated in 2005, and that of Dk/Anhui/1/06 was close to those of

Vietnamese strains isolated in 2007 and 2009. Phylogenetic analysis revealed that H5N1 viruses circulating in Vietnam are undergoing genetic change, possibly by antigenic drift and/or positive selection under immunological pressures (Fig. 1).

The complete genome sequence and the deduced amino acid sequences of Dk/VN/QB1207/12 were analyzed and compared with other strains available in GenBank as well as vaccine strains being used in Vietnam. Analysis of six internal genes indicated that the genetic sequences of Dk/VN/QB1207/12 were similar to those of other Vietnamese strains isolated in 2012, which belong to H5N1 clade 2.3.2.1 with identity levels ranging from 96.13 to 100 % (data not shown). The similarities of the HA and NA genes were further compared with representative Vietnamese strains of clade 2.3.2.1 isolated from 2009 to 2012 and vaccine strains that have been used for poultry in Vietnam. As shown in Table 1, the HA gene of Dk/VN/QB1207/12 shared high identity with those of Vietnamese strains isolated in 2012 including OIE2202 (99.82 %) and LMB315 (99.70 %). However, nucleotide sequence similarity less than 97.06, 97.18 and 95.84 % were observed between Dk/VN/QB1207/12 and other Vietnamese strains in 2012, 2011, and 2009, respectively. On the other hand, the HA gene of Dk/VN/QB1207/12 showed nucleotide identities of 91.79 and 93.83 % when it was compared with those of the vaccine strains Gs/GD/1/96 and Dk/Anhui/1/06, respectively. Comparison of amino acid sequence indicated that Dk/VN/QB1207/12 shared homology with selected Vietnamese strains ranging from 95.59 to 99.82 %. In contrast, amino acid identity of the HA between Dk/VN/QB1207/12 and Dk/Anhui/1/06 (Re-5) was 93.82 % and between Dk/VN/QB1207/12 and Gs/GD/1/96 (Re-1) was 91.19 %. Those identities were the lowest levels or close to the lowest level among comparisons between all Vietnamese H5N1 strains and the vaccines strains (Table 1). A similar result was obtained for the NA gene of Dk/VN/QB1207/12, which shares homology with Vietnamese strains ranging from 98.81 to 99.92 % at the nucleotide level and from 97.20 to 100 % at amino acid level. The NA gene of Dk/VN/QB1207/12 was almost closely related to the OIE2202 NA gene sequence. In contrast, Dk/VN/QB1207/12 NA showed nucleotide identities of 88.37 and 94.81 % and amino acid identities of 91.89 and 95.76 % when compared to Gs/GD/1/96 and Dk/Anhui/1/06, respectively (Table 2). Although all the Vietnamese strains compared in this study were H5N1 clade 2.3.2.1, substantial differences in the nucleotide and amino acid sequences of the HA and NA genes exist between these strains and between the H5N1 strains and the vaccine strains, including Re-5. These data highlight the ongoing generation of genetic diversity in H5N1 HPAIV. Thus, monitoring and molecular characterization of isolates would provide useful information for updating vaccine strain selection, especially in countries like Vietnam where endemic disease still occurs with circulating viruses of multiple clades and sub-clades.

The HA cleavage site sequence at position 320–331 of Dk/VN/QB1207/12 contained multiple basic amino acids (SPQRERRRKR/G), indicating an efficient cleavage by intracellular proteases [25]. The multiple basic cleavage site motif of Dk/VN/QB1207/12 was similar to the Dk/Anhui/1/06 (Re-5 vaccine) and to almost all of the representative Vietnamese strains except for the LBM66 and 27265 strains. It was also slightly different from Gs/GD/1/96 (Re-1 vaccine) with a deletion of K at position 329 (Table 3). In addition, mutations at the receptor-binding pocket of HA were not found in Dk/VN/QB1207/12 and all representative Vietnamese strains [26] and the HA gene of Vietnamese and vaccine strains maintained the avian receptor-binding specificity at positions Q-222 and G-224 (Table 3) [27]. However, the HA of Dk/VN/QB1207/12 contained amino acid substitutions of K/R140N and K189R when compared with the vaccine strains (Table 3). Positions 140 and 189 are reportedly involved in the major neutralizing epitopes of H5N1 viruses including both human and avian strains [28]. Additional mutations at positions 129, 263, and 277 sites that may also be important for antibody binding [29–31], were also found in the HA protein of Dk/VN/QB1207/12 (Table 3). More interestingly, mutation at HA position 140 of Dk/VN/QB1207/12 and all the representative Vietnamese strains has resulted in the generation of a *N*-linked glycosylation site ^{140}NSS^{143}, which is not carried by the vaccine strains Gs/GD/1/96 and Dk/Anhui/1/06. On the other hand, the ^{154}NNT^{156} glycosylation site found in the HA of Dk/Anhui/1/06 was not retained in Dk/VN/QB1207/12 and other representative Vietnamese strains nor in Gs/GD/1/96 (Table 3). The formation of a new site for potential glycosylation or loss of a glycosylation site possibly indicates the ability of the virus to evade the host response without negatively impacting survival and biological activity [32]. It was reported that *N*-linked glycosylation of the HA protein can mask the antigenic epitope on the HA, which can result in altered virus receptor binding and also inhibition of neutralizing antibody binding [33, 34]. Taken together, the mutations at the antigenic site and the differences in the glycosylation sites on the HA between Dk/VN/QB1207/12 and the vaccine strains, especially Dk/Anhui/1/06, may suggest that the current vaccine used in Vietnam cannot protect poultry from infection with genetic variants of clade 2.3.2.1 H5N1 viruses such as Dk/VN/QB1207/12. The typical peak season for H5N1 outbreak had been during January to February in Vietnam; however, multiple H5N1 outbreaks began in June of 2012, which was possibly related to a decline in protection by H5N1 poultry vaccines against H5N1 viruses, especially variants

Table 1 Percent identity of the HA sequence of A/duck/Vietnam/QB1207/2012 in relation to representative Vietnamese H5N1 viruses of clade 2.3.2.1 and vaccine strains

Virus strains	Representative Vietnamese strains in 2009–2012								Vaccine strains	
	2012					2011		2009		
	QB1207	OIE1287	OIE2202	LBM132	LBM315	LBM66	LBM57	27265	Anhui/06	GD/96
QB1207		96.94	99.82	97.06	99.70	94.49	97.18	95.84	93.83	91.44
OIE1287	98.41		96.72	99.54	96.72	94.61	98.44	96.30	94.36	92.03
OIE2202	99.82	98.23		96.84	99.48	94.20	96.84	95.78	93.66	91.27
LBM132	98.41	96.64	98.23		96.78	94.61	98.45	96.19	94.60	92.26
LBM315	99.64	98.05	99.47	98.05		94.15	96.78	95.60	93.66	91.27
LBM66	95.59	95.07	95.59	94.71	95.24		94.84	96.19	93.55	91.44
LBM57	98.23	99.11	98.05	99.11	97.88	94.54		96.60	94.71	92.26
27265	96.65	96.83	96.65	96.47	96.30	96.83	96.30		95.19	93.32
Anhui/06	93.82	94.35	93.82	94.35	93.82	93.48	93.82	95.07		95.43
GD/96	91.19	92.25	91.19	92.07	91.19	91.37	91.54	93.48	95.42	

Percent identity of nucleotide (above the diagonal) and amino acid (below the diagonal) is shown

QB1207 A/duck/Vietnam/QB1207/2012, *OIE1287* A/duck/Vietnam/OIE-1287/2012, *OIE2202* A/duck/Vietnam/OIE-2202/2012, *LBM132* A/duck/Vietnam/LBM132/2012, *LBM315* A/muscovy duck/Vietnam/LBM315/2012, *LBM66* A/muscovy duck/Vietnam/LBM66/2011, *LBM57* A/muscovy duck/Vietnam/LBM57/2011, *27265* A/chicken/Vietnam/27265/2009, *Anhui/06* A/duck/Anhui/1/2006, *GD/96* A/goose/Guangdong/1/1996

Table 2 Percent identity of the NA sequence of A/duck/Vietnam/QB1207/2012 in relation to representative Vietnamese H5N1 viruses of clade 2.3.2.1 and vaccine strains

Virus strains	Representative Vietnamese strains in 2009–2012								Vaccine strains	
	2012					2011		2009		
	QB1207	OIE1287	OIE2202	LBM132	LBM315	LBM66	LBM57	27265	Anhui/06	GD/96
QB1207		97.55	99.92	97.55	99.55	98.29	97.48	96.81	94.81	88.37
OIE1287	98.21		97.63	99.85	97.42	97.42	98.45	97.28	95.11	88.47
OIE2202	100.00	98.21		97.63	99.63	98.37	97.56	96.89	94.88	88.45
LBM132	98.44	99.77	98.44		97.43	97.43	98.45	97.28	95.11	88.43
LBM315	99.77	97.99	97.77	98.21		98.01	97.35	96.66	94.81	88.65
LBM66	99.77	97.10	99.77	97.10	97.77		97.65	96.89	94.81	88.29
LBM57	97.55	98.66	97.55	98.66	97.32	96.65		97.51	95.18	88.17
27265	97.20	97.67	97.20	97.90	96.97	96.27	97.67		96.27	90.29
Anhui/06	95.76	96.21	95.76	96.43	95.76	95.10	95.99	97.44		91.42
GD/96	91.89	91.68	93.75	91.89	93.75	91.04	91.04	92.22	93.75	

Percent identity of nucleotide (above the diagonal) and amino acid (below the diagonal) is shown

QB1207 A/duck/Vietnam/QB1207/2012, *OIE1287* A/duck/Vietnam/OIE-1287/2012, *OIE2202* A/duck/Vietnam/OIE-2202/2012, *LBM132* A/duck/Vietnam/LBM132/2012, *LBM315* A/muscovy duck/Vietnam/LBM315/2012, *LBM66* A/muscovy duck/Vietnam/LBM66/2011, *LBM57* A/muscovy duck/Vietnam/LBM57/2011, *27265* A/chicken/Vietnam/27265/2009, *Anhui/06* A/duck/Anhui/1/2006, *GD/96* A/goose/Guangdong/1/1996

of clade 2.3.2.1 viruses, as well as an overall decline in poultry vaccination coverage in Vietnam [19]. Dk/VN/QB1207/12 was isolated from an outbreak which occurred in July 2012, corresponding to the above-mentioned time frame. Thus, it should be important to assess the efficacy of the vaccines currently used against virus such as Dk/VN/QB1207/12 and to clarify the phenotypic importance of the changes found in the HA of Dk/VN/QB1207/12 using reverse genetics and site-directed mutagenesis.

In addition to monitoring mutation of H5N1 viral strains, optimizing the vaccination protocol is extremely important for the countries that have been using an AI vaccine for poultry. Current HPAI vaccine protocol in Vietnam involves an administration of the inactivated Re-5

Table 3 Comparison of amino acid sequence of the HA of A/duck/Vietnam/QB1207/2012 with that of representative Vietnamese H5N1 viruses of clade 2.3.2.1 and vaccine strains

Virus strains	Antigenic site					Receptor binding		Glycosylation sites									Cleavage site
	129	140	189	263	277	222	224	10	11	23	140	154	165	286	484	543	320–331
QB1207	L	N	R	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
OIE1287	L	N	K	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
OIE2202	L	N	R	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
LBM132	L	N	K	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
LBM315	L	N	R	T	K	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
LBM66	L	N	R	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQIERRRRKR/G
LBM57	L	N	R	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRR ~ KR/G
27265	L	N	R	T	R	Q	G	+	+	+	+	−	+	+	+	+	SPQRERRRRKR/G
GD/96	S	R	K	A	K	Q	G	+	+	+	−	−	+	+	+	+	TPQRERRRKKR/G
Anhui/06	S	K	K	A	K	Q	G	+	+	+	−	+	+	+	+	+	SPLRERRRK ~ R/G

QB1207 A/duck/Vietnam/QB1207/2012, *OIE1287* A/duck/Vietnam/OIE-1287/2012, *OIE2202* A/duck/Vietnam/OIE-2202/2012, *LBM132* A/duck/Vietnam/LBM132/2012, *LBM315* A/muscovy duck/Vietnam/LBM315/2012, *LBM66* A/Muscovy duck/Vietnam/LBM66/2011, *LBM57* A/muscovy duck/Vietnam/LBM57/2011, *27265* A/chicken/Vietnam/27265/2009, *Anhui/06* A/duck/Anhui/1/2006, *GD/96* A/goose/Guangdong/1/1996

vaccine twice a year, 6 months apart. In the duck from which Dk/VN/QB1207/12 was isolated, onset of disease was 6 days post vaccination at 59 days of age (average of the flock). This would imply that either the virus had been persistently circulating in the region or virus was newly introduced to the flock, despite the mass vaccination campaign occurring in Vietnam. In this case, it is possible that the decline in maternal antibody protection in juvenile ducks exposes them to infection with circulating virus. This case may also suggest that the 6 month interval between vaccinations could leave gaps in protection, allowing flock infection, requiring an improvement of vaccination protocol in Vietnam. However, investigating this possibility will require careful collection of data addressing the factors responsible for the persistent outbreaks in the country. Immunosurveillance studies also should be undertaken to monitor viral circulation in the field. Unfortunately, we could not obtain sera from the flock prior to or after the outbreak. This fact prevents us from determining if vaccination was performed in a time frame that provides protection against H5N1 infection. Additional studies including immunosurveillance using sentinel chickens/ducks would be useful to improve strategies for disease control.

The analysis of predicted amino acid sequence for the NA of Dk/VN/QB1207/12 and representative Vietnamese strains did not identify any of the amino acid substitutions known to relate to the oseltamivir resistance (Table 4) [35, 36]. In the M2 protein of Dk/VN/QB1207/12, the S31A mutation known to be involved in amantadine resistance was also not seen. However, the M2 of Dk/VN/QB1207/12 contained an amino acid substitution at position 27 (V27I), which may suggest a reduced susceptibility to amantadine (Table 4) [37, 38]. Recently, Govorkova et al. [36] systematically examined the prevalence of NA inhibitor and amantadine resistance among the HPAIVs circulating worldwide, and reported that most of the HPAIVs are likely to be susceptible to NA inhibitors while some proportion will also be susceptible to amantadine. The genetic features of Dk/VN/QB1207/12 seem to match that prediction. However, continued monitoring of the antiviral susceptibility of HPAIVs seems important to maintaining therapeutic approaches for control of the disease in humans.

Genetic analysis of other internal genes of Dk/VN/QB1207/12 did not reveal any mutation that increases virulence of the virus except in the NS gene. The analysis of predicted amino acid sequence for NS1 of Dk/VN/QB1207/12 showed a five amino acid deletion at positions 80–84 which has been reported to enhance cytokine expression by macrophages [39]. The predicted NS1 amino acid sequence of Dk/VN/QB1207/12 displayed an additional two mutations at S42A and E92D which might also increase virulence of this virus in chicken and mice as well as promoting inhibition of host immune responses as previously described [39, 40]. Furthermore, the PDZ binding sequence at the C-terminus of the NS1 protein was ESEV, which may correlate with H5N1 virulence in mammals [41]. Hence, the pathogenicity of Dk/VN/QB1207/12 in mammalian animal models should be explored for its possible public health significance.

This study provides a genetic characterization of the HPAI H5N1 virus recently isolated from a duck in a vaccinated flock in Vietnam. Genetic analysis revealed that

Table 4 Antiviral resistance markers in the NA and M2 of A/duck/Vietnam/QB1207/2012 and representative Vietnamese H5N1 viruses of clade 2.3.2.1

Virus strains	Amino acid position in the NA and M2											
	NA							M2				
	119	275	295	117	150	223	247	26	27	30	31	34
QB1207	E	H	N	I	K	I	S	L	I[a]	A	S	G
OIE1287	E	H	N	I	K	I	S	L	V	A	S	G
OIE2202	E	H	N	I	K	I	S	L	I	A	S	G
LBM132	E	H	N	I	K	I	S	L	V	A	S	G
LBM315	E	H	N	I	K	I	S	L	I	A	S	G
LBM66	E	H	N	I	K	I	S	L	V	A	S	G
LBM57	E	H	N	I	K	I	S	L	V	A	N[b]	G
27265	E	H	N	I	K	I	S	L	V	A	S	G

Amino acid in the NA and M2 proteins of the viruses at the position related to the antiviral resistance are shown

QB1207 A/duck/Vietnam/QB1207/2012, *OIE1287* A/duck/Vietnam/OIE-1287/2012, *OIE2202* A/duck/Vietnam/OIE-2202/2012, *LBM132* A/duck/Vietnam/LBM132/2012, *LBM315* A/muscovy duck/Vietnam/LBM315/2012, *LBM66* A/muscovy duck/Vietnam/LBM66/2011, *LBM57* A/muscovy duck/Vietnam/LBM57/2011, *27265* A/chicken/Vietnam/27265/2009

[a] Amino acid substitution for V27 in the M2 protein, which is reportedly related to a reduced Amantadine susceptibility

[b] Amino acid substitution for S31 in the M2 protein, which is known to be related to a reduced Amantadine susceptibility

mutation in the HPAI H5N1 isolate might have been driven by selection pressure due to herd immunity caused by natural infection or vaccination. In spite of control measures such as enforcement of biosecurity, culling of infected birds, and a vaccination program, HPAI H5N1 virus circulation has been persistent in Vietnam. Therefore, continuous monitoring of genetic changes in HPAI H5N1 viruses like the one presented here and evaluation of the efficacy of vaccines are extremely important for Vietnam and all the countries where H5N1 outbreaks have not been yet controlled. There is also an urgent need to develop a vaccine that matches the circulating strains and a standardized program to maximize the benefit of the vaccination campaign.

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References

1. X. Xu, K. Subbarao, N.J. Cox, Y. Guo, Virology **261**, 15–19 (1999)
2. I. Monne, A. Fusaro, M.H. Al-Blowi, M.M. Ismail, O.A. Khan, G. Dauphin, A. Tripodi, A. Salviato, S. Marangon, I. Capua, G. Cattoli, J. Gen. Virol. **89**, 2691–2697 (2008)
3. S.L. Salzberg, C. Kingsford, G. Cattoli, D.J. Spiro, D.A. Janies, M.M. Aly, I.H. Brown, E. Couacy-Hymann, G.M. De Mia, H. Dung do, A. Guercio, T. Joannis, A.S. Maken Ali, A. Osmani, I. Padalino, M.D. Saad, V. Savic, N.A. Sengamalay, S. Yingst, J. Zaborsky, O. Zorman-Rojs, E. Ghedin, I. Capua, Emerg. Infect. Dis. **13**, 713–718 (2007)
4. E. Starick, M. Beer, B. Hoffmann, C. Staubach, O. Werner, A. Globig, G. Strebelow, C. Grund, M. Durban, F.J. Conraths, T. Mettenleiter, T. Harder, Vet. Microbiol. **128**, 243–252 (2008)
5. Y. Guan, L.L. Poon, C.Y. Cheung, T.M. Ellis, W. Lim, A.S. Lipatov, K.H. Chan, K.M. Sturm-Ramirez, C.L. Cheung, Y.H. Leung, K.Y. Yuen, R.G. Webster, J.S. Peiris, Proc. Natl. Acad. Sci. USA **101**, 8156–8161 (2004)
6. J.S.M. Peiris, M.D. de Jong, Y. Guan, Clin. Microbiol. Rev. **20**, 243–267 (2007)
7. H.N.E.W.G. WHO/OIE/FAO, Influenza Other Respir. Viruses **6**, 1–5 (2012)
8. D.A. Boltz, B. Douangngeun, P. Phommachanh, S. Sinthasak, R. Mondry, C. Obert, P. Seiler, R. Keating, Y. Suzuki, H. Hiramatsu, E.A. Govorkova, R.G. Webster, J. Gen. Virol. **91**, 949–959 (2010)
9. K. Chaichoune, W. Wiriyarat, A. Thitithanyanont, R. Phonarknguen, L. Sariya, S. Suwanpakdee, T. Noimor, S. Chatsurachai, P. Suriyaphol, K. Ungchusak, P. Ratanakorn, R.G. Webster, M. Thompson, P. Auewarakul, P. Puthavathana, J. Gen. Virol. **90**, 216–222 (2009)
10. J. Chen, F. Fang, Z. Yang, X. Liu, H. Zhang, Z. Zhang, X. Zhang, Z. Chen, Virus Res. **146**, 19–28 (2009)
11. L. Duan, J. Bahl, G.J. Smith, J. Wang, D. Vijaykrishna, L.J. Zhang, J.X. Zhang, K.S. Li, X.H. Fan, C.L. Cheung, K. Huang, L.L. Poon, K.F. Shortridge, R.G. Webster, J.S. Peiris, H. Chen, Y. Guan, Virology **380**, 243–254 (2008)
12. D.C. Nguyen, T.M. Uyeki, S. Jadhao, T. Maines, M. Shaw, Y. Matsuoka, C. Smith, T. Rowe, X. Lu, H. Hall, X. Xu, A. Balish, A. Klimov, T.M. Tumpey, D.E. Swayne, L.P. Huynh, H.K. Nghiem, H.H. Nguyen, L.T. Hoang, N.J. Cox, J.M. Katz, J. Virol. **79**, 4201–4212 (2005)
13. X.F. Wan, T. Nguyen, C.T. Davis, C.B. Smith, Z.M. Zhao, M. Carrel, K. Inui, H.T. Do, D.T. Mai, S. Jadhao, A. Balish, B. Shu,

F. Luo, M. Emch, Y. Matsuoka, S.E. Lindstrom, N.J. Cox, C.V. Nguyen, A. Klimov, R.O. Donis, PLoS One **3**, e3462 (2008)
14. T. Nguyen, P. Rivailler, C.T. Davis, T. Hoa, A. Balish, N.H. Dang, J. Jones, D.T. Vui, N. Simpson, N.T. Huong, B. Shu, R. Loughlin, K. Ferdinand, S.E. Lindstrom, I.A. York, A. Klimov, R.O. Donis, Virology **432**, 405–416 (2012)
15. P.Q. Minh, B. Schauer, M. Stevenson, G. Jones, R.S. Morris, A. Noble, Transbound Emerg Dis. **56**, 311–320 (2009)
16. T. Nguyen, C.T. Davis, W. Stembridge, B. Shu, A. Balish, K. Inui, H.T. Do, H.T. Ngo, X.F. Wan, M. McCarron, S.E. Lindstrom, N.J. Cox, C.V. Nguyen, A.I. Klimov, R.O. Donis, Virology **387**, 250–256 (2009)
17. T. Dung Nguyen, T. Vinh Nguyen, D. Vijaykrishna, R.G. Webster, Y. Guan, J.S. Malik Peiris, G.J. Smith, Emerg. Infect. Dis. **14**, 632–636 (2008)
18. World Health Organization, Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO. http://www.who.int/influenza/human animal interface/H5N1 cumulative_table_archives/en. Accessed 10 Dec 2013
19. A. Creanga, D. Thi Nguyen, N. Gerloff, H. Thi Do, A. Balish, H. Dang Nguyen, Y. Jang, V. Thi Dam, S. Thor Jones, N. Simpson, B. Shu, S. Emery, L. Berman, H.T. Nguyen, J.E. Bryant, S. Lindstrom, A. Klimov, R.O. Donis, C.T. Davis, T. Nguyen, Virology **444**, 12–20 (2013)
20. World Health Orrganization, WHO Manual on Animal influenza diagnosis and surveillance. http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf. Accessed 10 Jul 2012
21. E. Hoffmann, J. Stech, Y. Guan, R.G. Webster, D.R. Perez, Arch. Virol. **146**, 2275–2289 (2001)
22. J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. **22**, 4673–4680 (1994)
23. K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, Mol. Biol. Evol. **30**, 2725–2729 (2013)
24. V.N. Bui, T.D. Dao, T.T. Nguyen, L.T. Nguyen, A.N. Bui, D.Q. Trinh, N.T. Pham, K. Inui, J. Runstadler, H. Ogawa, K.V. Nguyen, K. Imai, Virus Res. **179**, 125–132 (2014)
25. M. Hatta, P. Gao, P. Halfmann, Y. Kawaoka, Science **293**, 1840–1842 (2001)
26. M.N. Matrosovich, T.Y. Matrosovich, T. Gray, N.A. Roberts, H.D. Klenk, Proc. Natl. Acad. Sci. USA **101**, 4620–4624 (2004)
27. Y. Ha, D.J. Stevens, J.J. Skehel, D.C. Wiley, Proc. Natl. Acad. Sci. USA **98**, 11181–11186 (2001)
28. M. Prabakaran, F. He, T. Meng, S. Madhan, T. Yunrui, Q. Jia, J. Kwang, J. Virol. **84**, 11822–11830 (2010)
29. N.V. Kaverin, I.A. Rudneva, N.A. Ilyushina, N.L. Varich, A.S. Lipatov, Y.A. Smirnov, E.A. Govorkova, A.K. Gitelman, D.K. Lvov, R.G. Webster, J. Gen. Virol. **83**, 2497–2505 (2002)
30. D.C. Wiley, I.A. Wilson, J.J. Skehel, Nature **289**, 373–378 (1981)
31. W.L. Wu, Y. Chen, P. Wang, W. Song, S.Y. Lau, J.M. Rayner, G.J. Smith, R.G. Webster, J.S. Peiris, T. Lin, N. Xia, Y. Guan, H. Chen, J. Virol. **82**, 1798–1807 (2008)
32. Y. Abe, E. Takashita, K. Sugawara, Y. Matsuzaki, Y. Muraki, S. Hongo, J. Virol. **78**, 9605–9611 (2004)
33. D.J. Vigerust, V.L. Shepherd, Trends Microbiol. **15**, 211–218 (2007)
34. W. Wang, B. Lu, H. Zhou, A.L. Suguitan Jr, X. Cheng, K. Subbarao, G. Kemble, H. Jin, J. Virol. **84**, 6570–6577 (2010)
35. Q.M. Le, M. Kiso, K. Someya, Y.T. Sakai, T.H. Nguyen, K.H. Nguyen, N.D. Pham, H.H. Ngyen, S. Yamada, Y. Muramoto, T. Horimoto, A. Takada, H. Goto, T. Suzuki, Y. Suzuki, Y. Kawaoka, Nature **437**, 1108 (2005)
36. E.A. Govorkova, T. Baranovich, P. Seiler, J. Armstrong, A. Burnham, Y. Guan, M. Peiris, R.J. Webby, R.G. Webster, Antiviral Res. **98**, 297–304 (2013)
37. C.L. Cheung, J.M. Rayner, G.J. Smith, P. Wang, T.S. Naipospos, J. Zhang, K.Y. Yuen, R.G. Webster, J.S. Peiris, Y. Guan, H. Chen, J. Infect. Dis. **193**, 1626–1629 (2006)
38. H. Suzuki, R. Saito, H. Masuda, H. Oshitani, M. Sato, I. Sato, J Infect Chemother. **9**, 195–200 (2003)
39. S.H. Seo, E. Hoffmann, R.G. Webster, Nat. Med. **8**, 950–954 (2002)
40. P. Jiao, G. Tian, Y. Li, G. Deng, Y. Jiang, C. Liu, W. Liu, Z. Bu, Y. Kawaoka, H. Chen, J. Virol. **82**, 1146–1154 (2008)
41. J.C. Obenauer, J. Denson, P.K. Mehta, X. Su, S. Mukatira, D.B. Finkelstein, X. Xu, J. Wang, J. Ma, Y. Fan, K.M. Rakestraw, R.G. Webster, E. Hoffmann, S. Krauss, J. Zheng, Z. Zhang, C.W. Naeve, Science **311**, 1576–1580 (2006)