Isolation and characterization of H6N1 and H9N2 avian influenza viruses from Ducks in Hanoi,

#### Vietnam

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#### Abstract

We report the genetic characterization of low pathogenic avian influenza (LPAI) viruses isolated from domestic ducks in northern Vietnam in 2009. In total, 22 influenza A viruses consisting 21 H6N1 subtypes and one H9N2 subtype were isolated from 1488 ducks collected in February, March, and April 2009, accounting the overall virus isolation rate for 1.5%. No H5N1 strain was isolated in this study. Phylogenetic analysis indicated that all the eight genes of the H6N1 and H9N2 subtypes analyzed in this study were similar to those isolated in Korea, southeast China and northern Japan, and wild birds which migrate along the coastal East Asian Flyway are estimated to transmit these viruses. There was no evidence that the H6N1 and H9N2 subtypes share the gene segments with H5N1 subtypes. However, it is important to monitor the prevalence and genetical backgrounds of LPAI viruses among poultry in an area where several different influenza A subtypes are in circulation.

# Highlights

This is a survey for avian influenza viruses among poultry in northern Vietnam in the year 2009. We isolated 21 H6N1 viruses and one H9N2 virus from domestic ducks. Phylogenetic analysis identified that they were related to those of the Eurasian origins. Monitoring on avian influenza in an area where different subtypes are in circulation is important.

**Keywords**: avian influenza viruses, H6N1, H9N2, LPAI, Vietnam, molecular epidemiology

# 1. Introduction

Wild waterfowls are the natural hosts for all of the 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes of the influenza A virus (Alexander, 2000; Fouchier et al., 2005). The virus increases its genetic diversity by undergoing extensive reassortments with other influenza A viruses of multiple origins.

Since the end of 2003, a series of outbreaks caused by highly pathogenic avian influenza (HPAI) H5N1 viruses had occurred in poultry in Vietnam, and sporadic human infections have followed (Hien et al., 2009). In order to cope with the unprecedented public and animal health threats, nationwide disease-control activities including the restriction of animal movements, pre-emptive culling, a ban on waterfowl hatching, and a mass vaccination campaign had started in Vietnam in 2005, and in a consequence, H5N1 virus outbreaks had been absent in the period between December 2005 and October 2006 in the country (Dung Nguyen et al., 2008). However, H5N1 viruses has re-emerged and cased outbreaks among poultry in 2007 again in Vietnam (Minh et al., 2009).

H6 and H9 AI viruses are widely distributed subtypes among poultry and wild birds in Asia (Huang et al., 2010; Moon et al., 2009; Nagarajan et al., 2009). The first H6 influenza

virus was isolated from a turkey in 1965, and since then, H6 viruses have been isolated with increasing frequency from wild birds and poultry in southern China and Southeast Asia (Webster et al., 1992). Similarly, H9N2 viruses have circulated in domestic poultry in mainland China since 1994 and have been the most prevalent subtype in chicken populations (Liu et al., 2003a; Liu et al., 2003b). Gene segments analyses on AI viruses revealed that A/teal/Hong Kong/W312/97 (H6N1) and A/quail/Hong Kong/G1/97 (H9N2) were closely related to the Hong Kong H5N1 viruses (Chin et al., 2002; Guan et al., 1999; Hoffmann et al., 2000; MMWR, 1997). Although the event was observed merely in the Hong Kong "bird flu" incident in 1997, and was not the case with the large diversity of subsequent H5N1 viruses, it is worthy to monitor the distribution of H6 and H9 AI viruses among poultry raised in farms in Hanoi and to study their genetical backgrounds, considering the geographical vicinity of northern Vietnam to southern China, and the close interaction of influenza viruses between these areas (Nguyen et al., EID, 2008). In addition, there are several reports implicating potential for AI viruses to cause avian-to-human interspecies transmission. For instance, H9N2 subtypes of avian origin and closely related to viruses circulating in poultry in the market have been reported to cause human infection with mild clinical symptoms in Hong Kong (Butt et al., 2005), and farmers

participating in backyard or free-ranging turkey production methods are likely to be associated with the increased risks of H6 subtype infection (Kayali et al., 2009).

In the present study, we report the genetic characteristics of AI viruses isolated from ducks in Hanoi in northern Vietnam in the period between February and April 2009. HA, NA, and NS genes of all AI isolates, and the remaining 5 genes of strains examined in the study were sequenced and compared to those of publically available AI strains deposited in GenBank to explore their genetic background.

#### 2. Materials and Methods

### 2.1. Surveillance site and specimen collection

Surveillance was conducted from February to April 2009 in Lien Chau village in Hanoi. The village is located approximately 30 km south of Hanoi, the capital of Vietnam, and supplies livestock to Hanoi and neighboring areas. Typical poultry production is practiced in the village wherein domestic ducks are released into a neighboring common waterway where wild migratory birds are freely accessible. Each month, 10 poultry farms which rear 200-1000 ducks were chosen, and approximately 50 healthy ducks per farm were collected. Throat and cloacae secretion specimens from each duck were taken by cotton swabs and suspended in 2 ml of PBS supplemented with 0.5% bovine serum albumin, 10,000 units/ml penicillin, 10 mg/ml streptomycin sulfate, and 0.3 mg/ml gentamicin sulfate. All specimens were aliquoted into two; one for RT-PCR and the other for subsequent virus isolation, respectively, and kept at 4°C for approximately 2 h during transportation to the laboratory and then kept at -80°C until use.

#### 2.2. RNA isolation and M gene detection of influenza A viruses by RT-PCR

Viral RNA was extracted from a 50-µl portion of all specimens using an RNA isolation kit (MagMAX-96 viral RNA Isolation Kit, Ambion) with an automated Thermo Scientific King Fisher magnetic particle processor, following the manufacturer's instructions. A 13.5-µl portion of the extracted RNA was converted to cDNA using 100 units of M-MLV reverse transcriptase (Promega) in a 25-µl reaction mixture containing 10 mM of the Uni12 primer (5'-AGCTAAAGCAGG-3') and 0.5 mM each of dNTPs, at 42°C for 60 min (Hoffmann et al., 2001). To estimate the prevalence of influenza A virus among ducks collected, the influenza A virus-specific M gene was detected by PCR (WHO 2009) for all the specimens. In brief, 20 µl of a reaction mixture containing 5 µl of the synthesized cDNA, a pair of M-specific primers

# (A/M30F; 5'-TTCTAACCGAGGTCGAAACG-3' and A/M264R2; 5'-ACAAAGCGTCTACGCTGCAG-3'), and 10 μl of GoTaq (Takara, Japan) was prepared for each specimen, and a 40-cycle PCR at 94°C for 30 s, 50°C for 40 s, and 72°C for 60 s was performed. The PCR products were separated on a 2% agarose gel (Sigma), and amplified fragments were visualized by ethidium bromide staining with ultraviolet illumination.

#### 2.3. Virus isolation and identification

The specimens positive for the influenza A-specific M gene were inoculated into two 10-day-old embryonated chicken eggs that were free from specific pathogens and incubated at 35°C for 72 h, unless the embryo died before this time. The allantoic fluid was harvested from each egg and tested for HA activity using 1% chicken erythrocytes. All of the allantoic fluids containing HA agents were further tested to determine subtypes for HA and NA by HA-inhibition and NA-inhibition tests (Aymard, 1973; Salk, 1944) using antisera specific for A/PR/8/34 A/swine/Iowa/15/30 (H1N1), A/Singapore/1/57 (H1N1), (H2N2), A/duck/Ukraine/1/63 (H3N8), A/duck/Czech/56 (H4N6), A/whistling swan/Shimane/499/83 Massachusetts/65 (H5N3), A/turkey/ (H6N2), A/seal/Massachusetts/1/80 (H7N7),

A/turkey/Ontario/6118/68 (H8N4), A/turkey/Wisconsin/66 (H9N2), A/chicken/Germany/"N"/49 (H10N7), A/duck/England/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), and A/duck/Memphis/564/74 (H11N9), as described in previous studies (Kida and Yanagawa, 1979). Viral RNA was extracted from fluids containing HA using the QIAamp viral RNeasy Mini Kit (Qiagen) and reverse transcribed. The synthesized cDNAs were used as templates for PCR.

# 2.4. DNA sequencing and phylogenetic analyses

The HA, NA, and NS genes of all isolated influenza A viruses were amplified by PCR using specific primers (Hoffmann et al., 2011) and purified using the QIAquick gel extraction kit (Qiagen). The DNA was then sequenced on an automated DNA sequencer, the ABI PRISM 3130 genetic analyzer with the ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit, version 3.1 (Applied Biosystems) (Hoffmann et al., 2001). The PB2, PB1, PA, NP, and M genes extracted from representative isolates with distinct subtypes were amplified and sequenced as described elsewhere (Hoffmann et al., 2001). The sequence data of each amplicon was aligned and compared with those of corresponding data deposited in GenBank. The sequence

corresponding to 13-1370, 64-1014, 67-1380, 32-1395, 43-2228, 187-2209, 21-2129, 31-999, 270-760, and 56-709 nt portion was used for the alignment and comparison of H6, H9, N1, N2, PB2, PB1, PA, NP, M, and NS1 gene, respectively. The GENETYX software was used for the analysis, and the phylogenetic trees were generated by neighbor-joining (NJ) methods using the ClustalW software with 1,000 NJ bootstrap replicates.

# 3. Results

# 3.1. Specimen collection, detection of the influenza A-specific M gene, and virus isolation

On one day in February, March, and April 2009, 489, 499, and 500 ducks were collected, and of those, the influenza A-specific M gene was detected in XX (AA%). In total, 22 strains of avian influenza viruses, consisting 21 H6N1 and one H9N2, were isolated (1.5%; 22/1488) (Table 1). No H5N1 strain was isolated in the study. One H6N1 strain was isolated from a specimen taken from throat, whereas, the remaining 21 strains were from cloacae. The H9N2 strain was isolated from a specimen collected in February, and all H6N1 strains were isolated from specimens collected in April (Table 1). Ducks from which influenza viruses were isolated in the present study had been raised at two farms that were approximately 200 m apart

from each other.

#### 3.2. DNA sequencing and phylogenetic analyses

The HA, NA, and NS genes of all the 21 H6N1 strains isolated in this study were almost identical, with more than 99% homology. One strain was chosen as a representative strain and designated as A/Duck/Vietnam/3T7-8/09 (H6N1). Phylogenetic analysis indicated that the H6 gene sequence of A/Duck/Vietnam/3T7-8/09 (H6N1) was categorized into a gene cluster originating from wild birds in Korea and southeast China, but not from poultry of Korean origin (Fig. 1A). The sequence of the N1 gene of A/Duck/Vietnam/3T7-8/09 (H6N1) was 99% homologous to that of A/Md/Hokkaido/24/09 (H5N1), and was close to a cluster of AI viruses that had been isolated in Europe in 2003-2008. Contrary to the H6, the N1 gene of A/Duck/Vietnam/3T7-8/09 (H6N1) did not cluster with those of AI viruses isolated from birds in the Korean peninsula or southeast China (Fig. 1B). The H9N2 strain isolated in the present study, designated as A/Duck/Vietnam/C9-423/09 (H9N2), carried the H9 gene which was more than 98% homologous in sequence to that of A/Duck/Hokkaido/238/08 (H9N2) isolated in northern Japan. The cluster to which the H9 genes of A/Duck/Vietnam/C9-423/09 (H9N2) and

A/Duck/Hokkaido/238/08 (H9N2) belonged was phylogenetically closer to the one where AI viruses isolated in Europe in 2006–2008 belonged, rather than the one where AI isolates of Korean poultry origin belonged (Fig. 2A). Of note, the H9 gene of A/Duck/Vietnam/C9-423/09 (H9N2) did not cluster with those of AI strains isolated in Vietnam even in the year 2009 (Fig. 2A). The N2 gene of A/Duck/Vietnam/C9-423/09 (H9N2) was placed in a cluster that included AI viruses isolated from coastal areas of Far East Asia, including northern Japan, Korea, and southeast China (Fig. 2B).

The PB2, PB1, PA, NP, M genes of nine randomly chosen H6N1 isolates, including A/Duck/Vietnam/3T7-8/09 (H6N1), H9N2 and those of the sole isolate; A/Duck/Vietnam/C9-423/09 (H9N2), were sequenced and compared. Alignment of each of the five genes was found to be almost identical among the nine H6N1 strains examined, with more than 99% homology. The PB2, PB1, PA, NP, and M genes of A/Duck/Vietnam/3T7-8/09 (H6N1) and A/Duck/Vietnam/C9-423/09 (H9N2) clustered together with the corresponding genes of recent AI isolates from the coastal regions of Far East Asia (data not shown). The NS gene of A/Duck/Vietnam/3T7-8/09 (H6N1) was categorized into the allele B lineage in which influenza A viruses of avian origin principally cluster, while that of A/Duck/Vietnam/C9-423/09 (H9N2) was categorized into the allele A lineage in which viruses originating from various animal species cluster (Fig. 3F).

#### 3.3. Molecular analyses of H6N1 and H9N2 analyzed in the study

Deduced amino acid analysis identified the sequences PQIETR/GLF and PAASDR/GLF at the cleavage site on HA for A/Duck/Vietnam/3T7-8/09 (H6N1), representing all 21 H6N1 strains, and the sole H9 strain A/Duck/Vietnam/C9-423/09 (H9N2), respectively (Table 2). At the amino acid position 42 of NS1, S was used by all of the H6N1 strains isolated, but D was used by the H9N2 strain, instead. Amino acid A was universally used at the position 149 of NS1 of all of the H6N1 and the H9N2 strains. The PDZ domain-ligand ESEV sequence was found at C-terminal of the gene in all of the H6N1 strains and H9N2 strain isolated (Table 2).

# 4. Discussion

A wide variety of HA and NA subtype combinations found in the influenza A virus enables the virus to infect a broad range of animals and raises public health concerns for the genesis of a new type of virus (Webster et al., 1992). Preparedness for an influenza virus pandemic principally focuses on the H5 and H7 subtypes, because these subtypes include strains that are highly pathogenic in poultry and cause serious illness in humans even if infections are sporadic (Malik Peiris, 2009). It is meaningful to carry out a study on monitoring the distribution of LPAI viruses in an area proximity to southern China where LPAI viruses are believed to be involved in the genesis of the Hong Kong H5N1 virus (A/Hong Kong/156/97) (Chin et al., 2002; Guan et al., 1999; Hoffmann et al., 2000).

Since late 2003, there have been many reports describing the epidemiological features of human H5N1 infections as well as outbreaks in poultry (3-5 references). However, studies on LPAI viruses including H6 and H9 subtypes circulating among poultry in northern Vietnam are few (one or two references). In the present study, samples from domestic ducks had been collected from February to April 2009 in Hanoi, and analyzed for the prevalence of AI viruses among poultry and for determining genetic backgrounds of these viruses. The H6N1 subtype was isolated from 21 specimens and the H9N2 subtype was isolated from one specimen. No H5N1 strain was isolated in the present study.

Phylogenetic analyses indicated that all of the eight gene segments of

A/Duck/Vietnam/3T7-8/09 (H6N1) and A/Duck/Vietnam/C9-423/09 (H9N2) isolated in the present study are closely related to those isolated from the coastal regions of Far East Asia, indicating the presence of transmission routes for H6N1 and H9N2 subtypes that extend from Far East Asia to northern Vietnam. A recent report has demonstrated one migratory corridor for waterfowl along the Asian coast that encompasses several lakes used as congregation or breeding sites; the general movement for more than 100 water bird species was depicted (Takekawa et al., 2010). Many studies have indicated that diversity of wild bird species plays an important role in the interspecies transmission of LPAI viruses (Chen et al., 2001; Hinshaw et al., 1985) and contributes to the spread of the viruses. Therefore, it is highly plausible that the migration of wild birds along the coastal East Asian Flyway is directly involved in the transmission of H6N1 and H9N2 subtypes, and is responsible for the spreading of the viruses among poultry in northern Vietnam. In addition, poultry grazing in a common waterway, a farming practice frequently observed in the area maximizes the potential for contact between wild birds and domestic poultry and consequently increases the chances of AI virus transmission. With regard to phylogenetic analyses, no evidence exists that viral reassortments occurred between H6N1, H9N2 and H5N1 strains in northern Vietnam.

The deduced amino acid sequence at the cleave site of HA of all isolates are the ones typically found in LPAI strains (Table 2), as expected. In addition, the N-terminal and C-terminal end of the RBS of HA carried by A/Duck/Vietnam/3T7-8/09 (H6N1) was flanked with GQRGR, and GVTKA, respectively, and those of A/Duck/Vietnam/C9-423/09 (H9N2) was flanked with GQQGR and GTSKA, respectively, a feature found typically in AI strains of avian origin. However, molecular analyses in the present study revealed that all of the H6N1 strains use S and A at amino acid position 42 and 149 of NS1, respectively (Table 2). The presence of serine (S) at the amino acid position 42 of the NS1 protein in H5N1 strains is reportedly contributes to an increased pathogenicity in mice by interfering innate immunity induction in host cells (Jiao et al., 2008). The V149A mutation in the NS1 region is estimated to increase the ability of subtype H5N1 virus to antagonize interferon induction in chicken embryonic fibroblast cells (Li et al., 2006). These cases may not apply directly to the LPAI viruses; however, it is still of significance for any AI viruses to monitor protein regions which are vulnerable to mutations associated with increased pathogenicities in a host, which observed precedently in HPAI subtypes. Because of their low pathogenic characteristics, AI viruses including H6N1 and H9N2 subtypes are prone to infect asymptomatically and transmit among

birds beyond species, which can increase the chance of mutations as well as gene reassortments during co-infection with other AI viruses. Phylogenetic and genotype analysis of these isolates provide valuable insight into the ecology and evolution of AI viruses in northern Vietnam and also underscores the significance of long-term monitoring on the evolution of AI viruses in ducks in northern Vietnam.

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#### References

- Alexander, D.J. (2000) A review of avian influenza in different bird species. Vet Microbiol 74(1-2), 3-13.
- Aymard, M., Coleman, M.T., Dowle, W.R., Laver, W.G., Schild, G.C., Webster, R.G. (1973) Influenza virus neuraminidase and neuraminidase-inhibition test procedures. Bulletin of WHO 48, 199-202.
- Bean, W.J., Threlkeld, S.C. and Webster, R.G. (1989) Biologic potential of amantadine-resistant influenza A virus in an avian model. J Infect Dis 159(6), 1050-6.
- Butt, K.M., Smith, G.J., Chen, H., Zhang, L.J., Leung, Y.H., Xu, K.M., Lim,
  W., Webster, R.G., Yuen, K.Y., Peiris, J.S. and Guan, Y. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003.
  J Clin Microbiol 43(11), 5760-7.
- 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. and Yewdell, J.W. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7(12), 1306-12.
- Chin, P.S., Hoffmann, E., Webby, R., Webster, R.G., Guan, Y., Peiris, M. and Shortridge, K.F. (2002) Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. J Virol 76(2), 507-16.
- Dung Nguyen, T., Vinh Nguyen, T., Vijaykrishna, D., Webster, R.G., Guan, Y., Malik Peiris, J.S. and Smith, G.J. (2008) Multiple sublineages of influenza A virus (H5N1), Vietnam, 2005-2007. Emerg Infect Dis 14(4), 632-6.
- Fang, R., Min Jou, W., Huylebroeck, D., Devos, R. and Fiers, W. (1981) Complete structure of A/duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human H3 Hong Kong 1968

influenza hemagglutinin. Cell 25(2), 315-23.

- Fornek, J.L., Gillim-Ross, L., Santos, C., Carter, V., Ward, J.M., Cheng, L.I., Proll, S., Katze, M.G. and Subbarao, K. (2009) A single-amino-acid substitution in a polymerase protein of an H5N1 influenza virus is associated with systemic infection and impaired T-cell activation in mice. J Virol 83(21), 11102-15.
- Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B. and Osterhaus, A.D. (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79(5), 2814-22.
- Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H.D. and Stech, J. (2005) The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci U S A 102(51), 18590-5.
- Gething, M.J., Bye, J., Skehel, J. and Waterfield, M. (1980) Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature 287(5780), 301-6.
- Gillim-Ross, L., Santos, C., Chen, Z., Aspelund, A., Yang, C.F., Ye, D., Jin, H., Kemble, G. and Subbarao, K. (2008) Avian influenza h6 viruses productively infect and cause illness in mice and ferrets. J Virol 82(21), 10854-63.
- Guan, Y., Shortridge, K.F., Krauss, S. and Webster, R.G. (1999) Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A 96(16), 9363-7.
- Hatta, M., Gao, P., Halfmann, P. and Kawaoka, Y. (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293(5536), 1840-2.
- Hay, A.J., Wolstenholme, A.J., Skehel, J.J. and Smith, M.H. (1985) The molecular basis of the specific anti-influenza action of amantadine. EMBO J 4(11), 3021-4.
- Hien, N.D., Ha, N.H., Van, N.T., Ha, N.T., Lien, T.T., Thai, N.Q., Trang, V.D., Shimbo, T., Takahashi, Y., Kato, Y., Kawana, A., Akita, S. and Kudo, K.

(2009) Human infection with highly pathogenic avian influenza virus (H5N1) in northern Vietnam, 2004-2005. Emerg Infect Dis 15(1), 19-23.

- Hinshaw, V.S., Wood, J.M., Webster, R.G., Deibel, R. and Turner, B. (1985) Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bull World Health Organ 63(4), 711-9.
- Hoffmann, E., Stech, J., Guan, Y., Webster, R.G. and Perez, D.R. (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146(12), 2275-89.
- Hoffmann, E., Stech, J., Leneva, I., Krauss, S., Scholtissek, C., Chin, P.S., Peiris, M., Shortridge, K.F. and 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(14), 6309-15.
- Huang, Y., Hu, B., Wen, X., Cao, S., Gavrilov, B.K., Du, Q., Khan, M.I. and Zhang, X. (2010) Diversified reassortant H9N2 avian influenza viruses in chicken flocks in northern and eastern China. Virus Res 151(1), 26-32.
- Ito, T., Suzuki, Y., Takada, A., Kawamoto, A., Otsuki, K., Masuda, H., Yamada, M., Suzuki, T., Kida, H. and Kawaoka, Y. (1997) Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. J Virol 71(4), 3357-62.
- Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R. and Lamb, R.A. (2008) A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc Natl Acad Sci U S A 105(11), 4381-6.
- Jiao, P., Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., Liu, W., Bu, Z., Kawaoka, Y. and Chen, H. (2008) A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol 82(3), 1146-54.

Kawaoka, Y., Bean, W.J. and Webster, R.G. (1989) Evolution of the

hemagglutinin of equine H3 influenza viruses. Virology 169(2), 283-92.

- Kawaoka, Y. and Webster, R.G. (1988) Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A 85(2), 324-8.
- Kayali, G., Ortiz, E.J., Chorazy, M.L. and Gray, G.C. (2009) Evidence of previous avian influenza infection among US turkey workers. Zoonoses Public Health 57(4), 265-72.
- Kida, H. and Yanagawa, R. (1979) Isolation and characterization of influenza a viruses from wild free-flying ducks in Hokkaido, Japan. Zentralbl Bakteriol Orig A 244(2-3), 135-43.
- Li, Z., Jiang, Y., Jiao, P., Wang, A., Zhao, F., Tian, G., Wang, X., Yu, K., Bu, Z. and Chen, H. (2006) The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 80(22), 11115-23.
- Liu, H., Liu, X., Cheng, J., Peng, D., Jia, L. and Huang, Y. (2003a) Phylogenetic analysis of the hemagglutinin genes of twenty-six avian influenza viruses of subtype H9N2 isolated from chickens in China during 1996-2001. Avian Dis 47(1), 116-27.
- Liu, J.H., Okazaki, K., Shi, W.M., Wu, Q.M., Mweene, A.S. and Kida, H. (2003b) Phylogenetic analysis of neuraminidase gene of H9N2 influenza viruses prevalent in chickens in China during 1995-2002. Virus Genes 27(2), 197-202.
- Malik Peiris, J.S. (2009) Avian influenza viruses in humans. Rev Sci Tech 28(1), 161-73.
- Marjuki, H., Scholtissek, C., Franks, J., Negovetich, N.J., Aldridge, J.R., Salomon, R., Finkelstein, D. and Webster, R.G. (2010) Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks. Arch Virol 155(6), 925-34.
- Minh, P.Q., Morris, R.S., Schauer, B., Stevenson, M., Benschop, J., Nam, H.V. and Jackson, R. (2009) Spatio-temporal epidemiology of highly pathogenic avian influenza outbreaks in the two deltas of Vietnam during 2003-2007. Prev Vet Med 89(1-2), 16-24.
- MMWR. (1997) Isolation of avian influenza A(H5N1) viruses from

humans--Hong Kong, May-December 1997. MMWR Morb Mortal Wkly Rep 46(50), 1204-7.

- Moon, H.J., Song, M.S., Cruz, D.J., Park, K.J., Pascua, P.N., Lee, J.H., Baek, Y.H., Choi, D.H., Choi, Y.K. and Kim, C.J. (2009) Active reassortment of H9 influenza viruses between wild birds and live-poultry markets in Korea. Arch Virol 155(2), 229-41.
- Nagarajan, S., Rajukumar, K., Tosh, C., Ramaswamy, V., Purohit, K., Saxena, G., Behera, P., Pattnaik, B., Pradhan, H.K. and Dubey, S.C. (2009) Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Vet Microbiol 133(1-2), 154-63.
- 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. and Naeve, C.W. (2006)
  Large-scale sequence analysis of avian influenza isolates. Science 311(5767), 1576-80.
- Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L., Lai, R.W., Orr, W.K. and Shortridge, K.F. (1999) Human infection with influenza H9N2. Lancet 354(9182), 916-7.
- Perdue, M.L., Garcia, M., Senne, D. and Fraire, M. (1997) Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. Virus Res 49(2), 173-86.
- Salk, J.E. (1944) A simplified procedure for titrating hemagglutinating capacity of influenza-virus and the corresponding antibody. Journal of Immunology 49, 87-98.
- Seo, S.H., Hoffmann, E. and Webster, R.G. (2002) Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8(9), 950-4.
- Shinya, K., Hamm, S., Hatta, M., Ito, H., Ito, T. and Kawaoka, Y. (2004) PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320(2), 258-66.
- Suarez, D.L. (2000) Evolution of avian influenza viruses. Vet Microbiol 74(1-2), 15-27.
- Suzuki, Y. (2005) Sialobiology of influenza: molecular mechanism of host

range variation of influenza viruses. Biol Pharm Bull 28(3), 399-408.

- Takekawa, J.Y., Newman, S.H., Xiao, X., Prosser, D.J., Spragens, K.A., Palm, E.C., Yan, B., Li, T., Lei, F., Zhao, D., Douglas, D.C., Muzaffar, S.B. and Ji, W. (2010) Migration of waterfowl in the East Asian flyway and spatial relationship to HPAI H5N1 outbreaks. Avian Dis 54(1 Suppl), 466-76.
- Vines, A., Wells, K., Matrosovich, M., Castrucci, M.R., Ito, T. and Kawaoka, Y. (1998) The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol 72(9), 7626-31.
- Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56(1), 152-79.
- WHO. (2009) WHO information for laboratory diagnosis of pandemic (H1N1)
   2009 virus in humans update. World Health Organization.
- Yamada, H., Chounan, R., Higashi, Y., Kurihara, N. and Kido, H. (2004) Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria. FEBS Lett 578(3), 331-6.
- Zamarin, D., Ortigoza, M.B. and Palese, P. (2006) Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J Virol 80(16), 7976-83.

Month		<u> </u>			
	Collected N	A gene detected $(\%)^*$ \	/irus isolated $(\%)^{*}$	Subtype	
February, 2009	489	X (A)	1 (0.2)	H9N2	
March, 2009	499	Y (B)	0 (0)		
April, 2009	500	Z (C)	21 (4.2)	H6N1	
Total	1488	XX (AA)	22 (1.5)		

Table 1Detection and isolation of influenza A viruses among ducks collected in 2009

\*; denominator is the number of ducks collected

# Table 2

Amino acid usage at sites indicated in HA and NA genes of AI strains isolated in the study

Icolata (Subturna)	HA		NS1		
	Cleavage site	42	149	C-terminal end	
Dk/VN/3T7-8/09 (H6N1)*	PQIETR/GLF	S	А	ESEV	
Dk/VN/C9-423/09 (H9N2)	PAASDR/GLF	D	А	ESEV	

\*; Dk/VN3T-8/09 (H6N1) is the representative strain of all 21 H6N1 starins isolted in the present study.

A (H6)

B (N1)



**Figure 1**: Phylogenetic trees of the A/Duck/Vietnam/3T7-8/09 (H6N1) isolated in the study were generated by the distance-based neighbor-joining method using ClustalW. The reliability of the trees was assessed by bootstrap analysis with 1,000 replications. The H6 (Fig. 1A) and N1 (Fig 1B) gene of the strain was aligned and compared with the corresponding region of influenza A viruses deposited in GenBank. Nucleotide length used for analysis is 13-1370 nt for the H6 and 67-1389 nt for the N1. The length of horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. Bootstrap value is appeared for each key node in the tree. Viruses isolated in this study are highlighted in red. Abbreviations: AB, aquatic bird; AW, American wigeon; BG, bean goose; BJ, Beijin; BS, bewick's swan; CA, California; CGE, common goldeneye; Chuk, chukkar; Ck, chicken; CO, Colorado; Dk, duck;

DW, Delaware; EC, Eastern China; envDtLake, environment Dongting Lake; EW, Eurasian wigeon; FJ, Fujian; Gb, Grebe; GCG, great crested grebe; GD, Guangdong; Gs, goose; Gw, gadwall; GX, Guangxi; GY, Guiyang; HB, Hebei; HK, Hong Kong; Hen, Henan; Hun, Hunan; JX, Jiangxi, Kor Korea; Md, Mallard; MDk, Muscovy duck; Mg, magpie; NC, Nanchang; Pa, partridge; Ph, pheasant; Qa, quail; QH, Qinghai; RCP, red crested pochard; RSDK, ruby sheduck; SB, shorebird; SBD, spot-billed duck; SCk, silky chicken; SD, Shandong; SH, Shanghai; ST, Shantou; Thai, Thailand; Tw, Taiwan; Ty, turkey; VN, Vietnam; WB, wild bird; WI Wisconsin; WS, whooper swan; YN, Yunnan; ZJ, Zhejiang.



**Figure 2**: Phylogenetic trees of the A/Duck/Vietnam/C9-423/09 (H9N2) isolated in the study were generated by the distance-based neighbor-joining method using ClustalW. The reliability of the trees was assessed by bootstrap analysis with 1,000 replications. The H9 (Fig. 2A) and N2 (Fig 2B) gene of the strain was aligned and compared with the corresponding region of influenza A viruses deposited in GenBank. Nucleotide length used for analysis is 64-1014 nt for the H9 and 32-1395 nt for the N2. The length of horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. Bootstrap value is appeared for each key node in the tree. Viruses isolated in this study are highlighted in red. Virus abbreviations are the same as those shown in the Figure 1.



**Figure 3**: Phylogenetic trees for the NS of the A/Duck/Vietnam/3T7-8/09 (H6N1) and the A/Duck/Vietnam/C9-423/09 (H9N2) isolated in the study were generated by the distance-based neighbor-joining method using ClustalW. NS gene of two strains was aligned and compared with the corresponding region of influenza A viruses deposited in GenBank. Nucleotide length used for the analysis was 56-709 nt. The length of horizontal lines is proportional to the minimum number of nucleotide differences required to join nodes. Bootstrap value is appeared for each key node in the tree. Viruses isolated in this study are highlighted in red. Virus abbreviations are the same as those shown in the Figure 1.