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Characterization of highly pathogenic H5N1 avian influenza viruses isolated from poultry markets in central China

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ABSTRACT

H5N1 highly pathogenic avian influenza (HPAI) viruses have seriously affected the Asian poultry industry since their recurrence in 2003. While surveillance in southern China has revealed that H5N1 viruses underwent extensive genetic reassortment to generate many different viral genotype viruses, little is known concerning the genotypes of H5N1 virus that circulated in central China in recent years. In this study, 16 H5N1 influenza viruses were isolated from the poultry market in central China during late 2006 and early 2007, and the genotypes and pathogenicity of the viruses were identified and characterized. All eight segments of each virus were sequenced, and phylogenetic analysis showed that the two surface glycoprotein genes, hemagglutinin (HA) and neuraminidase (NA), of all the viruses were closely related to the H5N1 viruses isolated in poultry in southern China since 2006. Phylogenetic analysis of the internal protein genes indicated that four viral genotypes circulated in poultry markets in central China. The virulence of 7 of the 16 isolates was tested in chickens and mice. The results showed that the 7 isolates were highly pathogenic for SPF chickens, and had a varied virulence in mice. Our results indicate that the H5N1 viruses circulated in central China have diversified characteristics of genotype and virulence.

1. Introduction

In 1997, the H5N1 avian influenza virus crossed the species barrier in Hong Kong and resulted in 18 human infection cases, 6 of which were fatal. Molecular characterization showed that the avian influenza virus in Hong Kong was a reassortant virus, whose HA gene was provided by the Goose/Guangdong/1/96-like H5N1 virus (Xu et al., 1999), NA gene was from the Teal/Hong Kong/W312/97-like H6N1 virus (Hoffmann et al., 2000), and other internal protein genes were acquired from the Teal/Hong Kong/W312/97-like H6N1 virus or Quail/HK/G1/97-like H9N2 virus (Guan et al., 1999). The H5N1 virus has continued to evolve in poultry and the reassortants became the main form. In 2001, six viral genotypes were isolated from the poultry market in Hong Kong (genotype A, B, C, D, E and X0) (Guan et al., 2002). In 2002, the genotypes A, C, D, E and their common precursor, Goose/Guangdong/1/96, disappeared, but new genotypes, V, W, X1, X2, X3, Y, Z and Z$^+$, appeared in southern China. These later genotypes obtained a survival advantage through the adaptation (Guan et al., 2002). The genotype Z strains of H5N1 viruses, which appeared after 2002, became the prevalent virus in poultry (Li et al., 2004), and were responsible for outbreaks in poultry in China and seven other Southeast Asian countries during late 2003 and early 2004. Another pattern in the evolution of the H5N1 virus is a continuous mutation of HA, and the H5N1 virus circulated in southern China and Southeast Asian countries in 2004–2005 formed multiple sublineages and became endemic in poultry in different geographical regions (Chen et al., 2006). Since 2005, genetic analysis revealed that an H5N1 influenza variant, the Clade 2.3.4 (Fujian-like sublineage), had emerged and become predominant in each of the provinces, replacing those previously established multiple sublineages in different regions of southern China (Smith et al., 2006).

In China, the first human infected case was reported in November 2005, and 30 cases have been confirmed. Of these, 20 cases died (WHO, 2008). The human cases reported in China showed that some of the patients had close contact with poultry (Yu et al., 2006), and virus genetic information analysis showed that the human H5N1 virus had a close relationship with the H5N1 virus isolated from the poultry market (Abdel-Ghafar et al., 2008; Cheng et al., 2008; Yan et al., 2007; Zhou et al., 2007). As a result, analyses of the epidemic situation and the biological characteristics of the H5N1 virus in the poultry market may reveal the evolution of trends and successfully predict the appearance of new viruses that infect humans.

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Although we have considerable data concerning the epidemic situation of the H5N1 virus in southern China, data concerning the epidemic situation, genome and pathogenic characteristics of the H5N1 viruses in the poultry market in central China are sparse. For this purpose, we have isolated a total of 16 H5N1 viruses in poultry markets from Hubei Province, Hunan Province, Henan Province and Anhui Province at the end of 2006 and early 2007. We identified many genotypes of H5N1 virus strains in the poultry markets in central China, and these viral genotypes showed variation in virulence in mice.

2. Materials and methods

2.1. Virus isolation

Cloacal samples were collected once every month from apparently healthy poultry in live poultry markets in Hubei Province ($n = 1215$), Anhui Province ($n = 605$), Henan Province ($n = 1141$) and Hunan Province ($n = 1081$) (Fig. 1). The samples were eluted with 2.0 ml phosphate-buffered saline (PBS) containing penicillin G (2×10^6 U/l), polymyxin B (2×10^6 U/l), gentamicin (250 mg/l), nystatin (0.5×10^6 U/l), ofloxacin HCl (60 mg/l), and sulfamethoxazole (0.2 g/l). Specimens were first screened by cell culture for H5 subtype influenza virus using a hemagglutinin assay and RT-PCR. All PCR-positive swabs were transported to the BSL-3 Laboratory and inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated eggs (Beijing MERIAL Ltd.). After incubation at 37 °C for 48–72 h, the allantoic fluid of the inoculated eggs was collected. Fifty percent egg infectious dose (EID_{50}) titers were calculated by the method of Reed–Muench (Reed and Muench, 1938). Aliquots of virus allantoic fluid stock were stored at −80 °C before use.

2.2. RNA extraction and nucleotide sequencing

Viral RNA from the isolates propagated in 10-day-old embryonated eggs were extracted by lysing the viruses with Trizol LS reagent (Life Technologies, Inc.). The RNA was reverse-transcribed into single-stranded DNA with M-MuLV reverse transcriptase (Promega). All segments were amplified using Ex Taq™ DNA polymerase (Takara) with segment-specific primers (Hoffmann et al., 2001). The PCR products were purified using a Cycle-pure Kit and Gel Extraction Kit (Omega Bio-Tek, USA), and the fragments were cloned into pGEM-T easy vector and sequenced by the dideoxy method using a ABI 3730 DNA sequencer (Applied Biosystems). Data were edited and aligned by BioEdit Version 7.0.5.2.

2.3. Phylogenic analysis

Phylogenic analysis was based on nucleotides 57–1069 (1013 bp) of the HA, 67–1290 (1224 bases) of the NA, 22–956 (935 bases) of the M, 24–988 (965 bases) of the nucleoprotein (NP), 39–704 (666 bases) of the nonstructural protein (NS), 760–2139 (1380 bases) of the polymerase acidic (PA), 313–1484 (1172 bases) of the polymerase basic 1 (PB1), and 1015–2226 (1212 bases) of the polymerase basic 2 (PB2). Multiple alignments were constructed using ClustalW Multiple alignment of BioEdit (Version 7.0.5.2). Phylogenic trees were generated by neighbor-joining bootstrap

Fig. 1. Map of China shows the Hunan, Hubei, Henan and Anhui provinces, where influenza surveillance was conducted.

analysis (1000 replicates) using the Tamura–Nei algorithm in MEGA version 3.1 (Kumar et al., 2004).

2.4. Pathogenicity

Six-week-old SPF chickens (Beijing MERIAL Ltd.), 10 for each group, were tested according to the recommendation of the Office International des Épizooties (OIE). Each chicken was injected intravenously with 0.2 ml of a 1:10 dilution of stock virus (the titers of infection are shown in Table 3), and mortality was observed over a 10-day period. Ten 6-week-old SPF BALB/c mice were infected intranasally with each virus using 20 μl of $10^{6.75}$ EID_{50} under anesthesia. On day 5, 3–4 of the 10 inoculated mice were sacrificed for virus titration in the lung, kidney, spleen and brain. Tissue samples were homogenized in 1 ml cold PBS and centrifuged at 16,000 × *g* for 10 min before homogenates were titrated for virus infectivity in MDCK cells with initial dilutions of 1:10. The remaining 6 inoculated mice were monitored daily for weight loss and mortality. The mouse 50% minimal lethal dose (MLD_{50}) was determined for the virus that caused lethal infection in mice by i.n. inoculation. The MLD_{50} was calculated by the method of Reed and Muench (Reed and Muench, 1938).

2.5. Nucleotide sequence accession numbers

All sequences have been deposited in GenBank. The accession numbers are FJ784761–FJ784888.

3. Results

3.1. Virus isolation and homologous analysis

From November 2006 and January 2007, a total of 4042 cloacal samples were collected from the poultry markets from Wuhan City in Hubei Province, Yueyang City in Hunan Province, Hefei City in Anhui Province, and Zhengzhou City in Henan Province (Fig. 1) in central China. All samples were collected from apparently healthy birds. Sixteen H5N1 avian influenza viruses were isolated, including 6 viruses from chickens, 10 viruses from ducks and no strains from geese or pigeons. From the geographic distribution of the viruses, 8 strains were acquired from Hunan Province, 4 strains were acquired from Henan Province, 3 strains were acquired from Hubei Province and 1 strain was acquired from Anhui Province (Table 1).

The whole-genome of each of the 16 isolates was sequenced, and compared with each other for identity analysis. The results showed that six genes shared high nucleic sequence similarity with each other, including the HA gene (>97% identity), NA gene (>95% identity), NP gene (>98% identity), M gene (>97% identity), NS gene (>96% identity) and PB1 gene (>96% identity). The PA gene of four isolates (Chicken/Hunan/1793/2007, Duck/Hunan/3340/2006, Duck/Hunan/1994/2007, Duck/Hunan/1930/2007) shared high similarity with each other (>99% identity), while the identity between the remaining 12 isolates was >97%. The identity between the 4 isolates with high similarity and the remaining 12 isolates was 92–93%, which indicated that the two isolate groups originated from different ancestors. The similarity of the PB2 gene showed more diversity, from 87% to 99%, which suggests a multiple origin of the PB2 gene.

Table 1
H5N1 virus strains isolated from central China.

No.	Name	Date
1	A/Duck/Hunan/689/2006	2006.12.15
2	A/Chicken/Hunan/1793/2007	2007.1.23
3	A/Duck/Hunan/1930/2007	2007.1.23
4	A/Duck/Hunan/1964/2007	2007.1.23
5	A/Duck/Hunan/1994/2007	2007.1.23
6	A/Chicken/Hunan/3157/2006	2006.11.30
7	A/Duck/Hunan/3315/2006	2006.11.30
8	A/Duck/Hunan/3340/2006	2006.11.30
9	A/Chicken/Henan/1362/2006	2006.12.30
10	A/Duck/Henan/1647/2006	2006.12.30
11	A/Duck/Henan/1650/2006	2006.12.30
12	A/Duck/Henan/1652/2006	2006.12.30
13	A/Chicken/Hubei/2856/2007	2007.1.29
14	A/Duck/Hubei/2911/2007	2007.1.29
15	A/Chicken/Hubei/3002/2007	2007.1.29
16	A/Chicken/Anhui/1089/2007	2007.1.28

3.2. Phylogenic analysis

In order to better understand the relationship between the 16 strains of H5N1 viruses and the representative strains reported previously, we downloaded the sequence of the representative strains from GenBank and constructed phylogenetic trees. The representative strains include the H5N1 viruses isolated in the Hong Kong Special Administrative Region in 1997, the viruses isolated in Vietnam, Thailand, Malaysia, and Indonesia since 2004, the Qinghai-like viruses isolated from migratory birds since 2005, viruses isolated from the tree sparrow in 2004 in central China, viruses isolated in South Korea and Japan in 2004–2005, viruses isolated from poultry markets in southern China in 2005–2006, as well as human H5N1 strains.

In the HA gene tree, the 16 viral isolates clustered with H5N1 viruses isolated from poultry markets or humans in southern China since 2006, and formed an independent branch, Clade 2.3.4 (Fujian-like sublineage) (Fig. 2). This result suggests that the HA gene of H5N1 viruses in this study shared a common origin with the H5N1 viruses circulated in southern or central China since 2006. In the NA gene tree, the NA genes of 16 isolates were demonstrated to originate from a common mutant, while differentiate into two sister branches, which indicates that the NA and HA of the H5N1 viruses circulated in central China did not co-evolve. In the NP gene tree, the 16 strains are originated from the same ancestor, but divided into two branches. The NS gene tree is similar to the NP with 16 strains forming two branches. In the M gene tree, all of the M genes of the 16 strains fall into one branch. In the PA gene tree, the 16 strains originated from two different ancestors, of which 4 strains isolated in Hunan provinces locate at the top of the tree and 12 strains locate at the bottom of the evolutionary tree. In the PB1 gene tree, all of the 16 isolates cluster together, which suggests they originate from a common ancestor. In the PB2 gene tree, the 16 strains fall into three branches. Obviously, the PB2 genes of the 16 isolates originated from three different ancestors.

According to the above described phylogenetic analysis of the eight segments and the definition of genotype by Duan et al. (2008), we divided the 16 H5N1 strains into four different gene constellations and defined them as genotype V, G, V2 and V3 (Fig. 3). The HA, NA, NP, M, NS and PB1 genes of the four genotype viruses shared the same ancestor (China/GD01/06), although the NA, NP, NS and PB1 genes diversified into two sister branches. The PA and PB2 genes of the four genotype viruses have more than one origin. The PB2 gene of genotype V3 was provided by China/GD01/06-like strain, and the PA gene of genotype V3 was provided by Duck/Fujian/1734/2005-like strains (Table 2). The PB2 and PA genes of genotype G were provided by China/GD01/06-like and House crow/Hong Kong/2648/2006-like strain, respectively. The PB2 and PA genes of genotype V were provided by Duck/Fujian/1734/2005-like strain. The PB2 gene of genotype V2 was provided by the wild bird strains (Mallard/Netherlands/12/2000), the PA gene of genotype V2 was provided by Duck/Fujian/1734/2005-like strain.

3.3. Molecular characterization

Based on the deduced amino acid sequences of the HA genes, all 16 isolates had the same multiple basic amino acids at the connecting peptide between HA1 and HA2 (RRRKR↓G), which was considered a characteristic of influenza viruses that were highly pathogenic for chickens (Senne et al., 1996). Unlike the majority of H5N1 isolates circulating during 1996–2004 (RRRKKR/G), there was a Lys deletion mutation at −3 position of the HA1-connecting peptide. The receptor binding pocket of HA1 in these isolates retained the amino acid residues, Gln222 and Gly224 (H5 numbering used throughout), that preferentially bind to 2,3-NeuAcGal linkages of avian cell-surface receptors (Ha et al., 2001) (Table 3).

Analysis of the deduced amino acid sequences of NAs from these isolates showed a 20-amino acid deletion (positions at 49–68) in the stalk region, which was also observed in the dominant H5N1 virus in southern China (genotype Z) (Li et al., 2004). It has been reported that the presence of the His274Tyr mutation in the NA protein is associated with decreasing sensitivity to NA inhibitors (de Jong et al., 2005). No amino acid mutation was observed at this residue in the NA proteins of the present isolates, which indicates that these isolates may be sensitive to NA inhibitors.

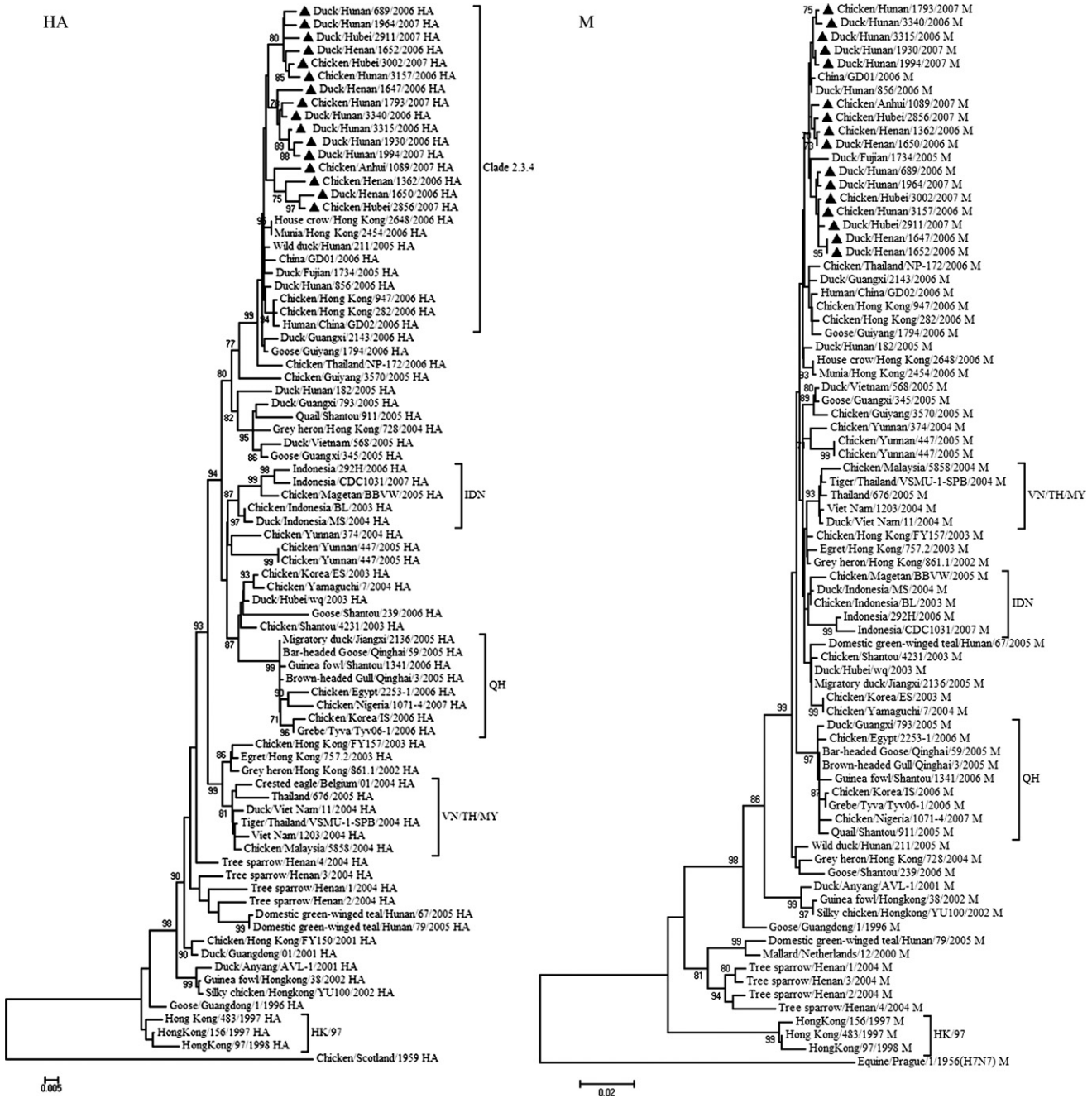

Fig. 2. Phylogenic trees of virus genes. Trees were generated by using neighbor-joining analysis with the Tamura–Nei model in the MEGA program (version 3.1). Numbers at the nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value. Analysis was based on nucleotides 57–1069 (1013 bp) of the HA, 67–1290 (1224 bases) of the NA, 22–956 (935 bases) of the M, 24–988 (965 bases) of the nucleoprotein (NP), 39–704 (666 bases) of the nonstructural protein (NS), 760–2139 (1380 bases) of the polymerase acidic (PA), 313–1484 (1172 bases) of the polymerase basic 1 (PB1), and 1015–2226 (1212 bases) of the polymerase basic 2 (PB2). The scale bar represents the distance unit between the sequence pair. Black triangles represent isolates in this study.

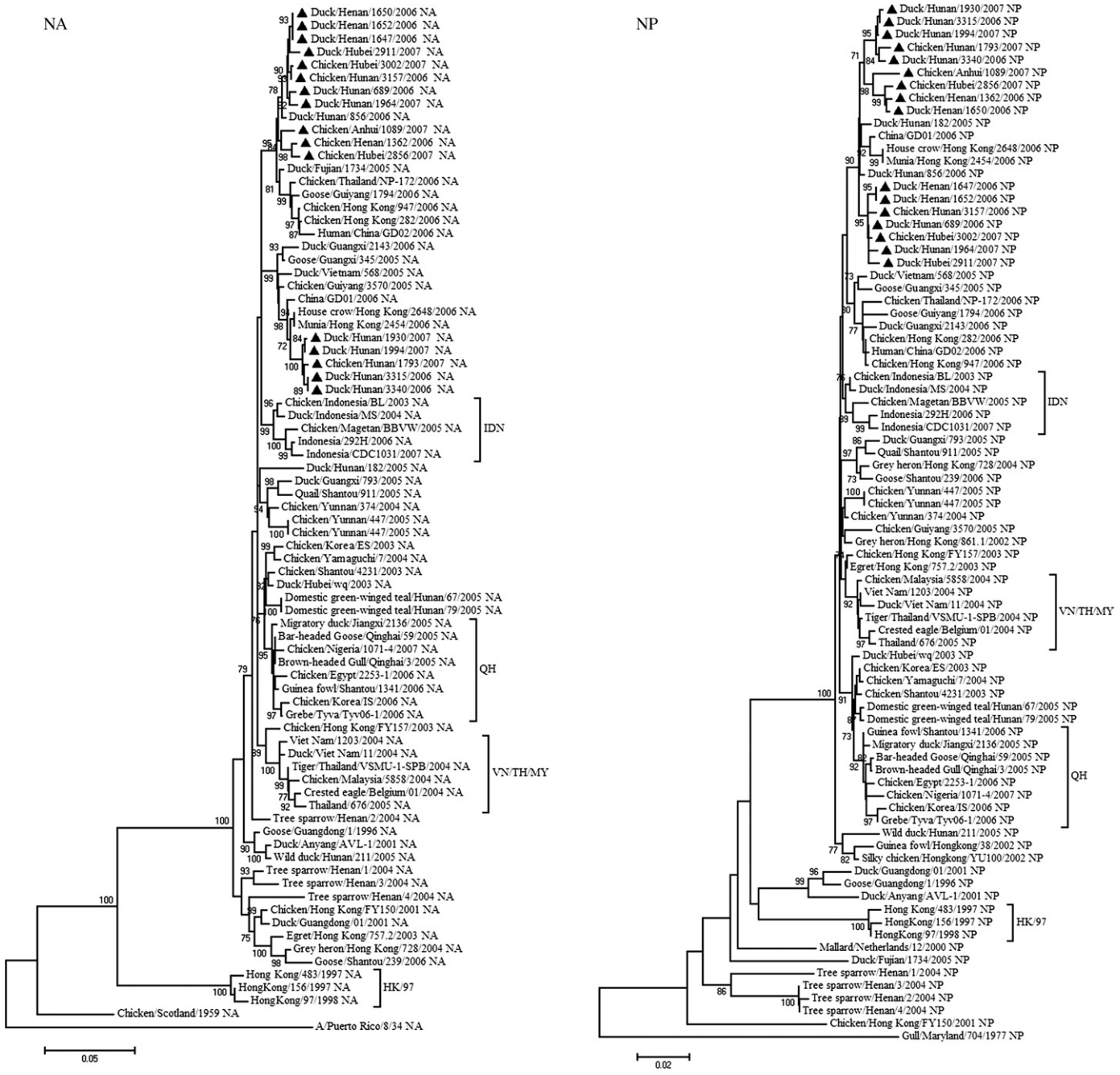

Fig. 2. (*Continued*)

It has been shown that H5N1 viruses with the Lys627 or Asn701 mutation in PB2 are highly virulent and systemically replicable in mice (Hatta et al., 2001; Li et al., 2005). Sequence analysis revealed no Lys627 or Asn701 mutation in the PB2 of these 16 isolates. In addition, these 16 isolated viruses did not have a mutation of Glu92 in the NS1, which has been associated with the high virulence of H5N1 subtype in 1997 (Lipatov et al., 2005; Seo et al., 2002). All 16 isolates had a 5-aa deletion (aa 80–84) in the middle of the NS protein, which was also found in the dominant H5N1 virus in southern China (genotype Z) (Li et al., 2004). Amantadine-resistant influenza A variants carried amino acid substitutions at residues 26, 27, 30, 31, or 34 of the M2 protein (Hay et al., 1985; Pinto et al., 1992). Our sequence analysis did not show any substitutions at these residues. Therefore, the two isolates may be sensitive to this class of antiviral drugs.

3.4. Pathogenicity

According to geographic distribution, 7 viruses were chosen for pathogenic analysis in animals, including 1 from Hunan Province, 3 from Henan Province, 2 from Hubei Province and 1 from Anhui Province. We evaluated the pathogenicity of 7 representative H5N1 viruses in 6-week-old SPF white leghorn chickens by intravenous (i.v.) inoculations. According to the Office International des Épizooties (OIE) criteria, each chicken was intravenously inoculated with 0.2 ml of a 1:10 dilution of virus stock in PBS, respectively. The titer of infection is indicated in Table 4. The results showed that the 7 H5N1 isolates were highly pathogenic to chickens. All chickens died within 48 h, and the intravenously pathogenicity index (IVPI) of these viruses was between 2.8 and 3.0 (Table 4).

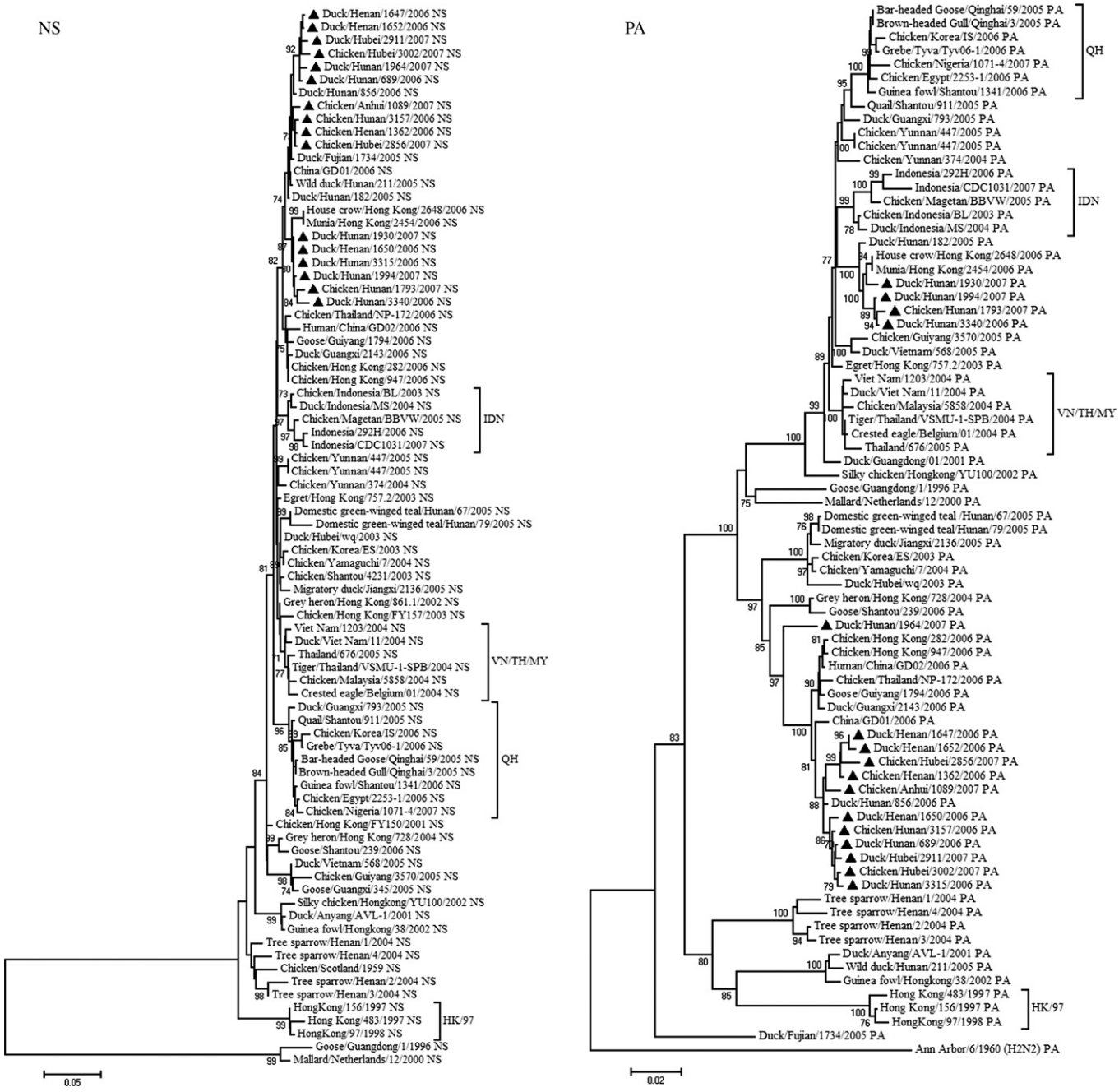

Fig. 2. (*Continued*)

In order to determine the pathogenicity of H5N1 viruses in a mammalian host, BALB/c mice were inoculated intranasally with a dose of $10^{6.75}$ EID_{50} viruses, and virus replication in organs, weight loss and mortality were examined. Two weeks after infection, the mortality of the mice infected with the strain of virus Duck/Henan/1650/2006, Chicken/Anhui/1089/2007, Chicken/Henan/1362/2006, Duck/Henan/1647/2006 and Chicken/Hubei/2856/2007 was higher (>67%) (Table 5). The mortality rate of the mice infected with Duck/Hunan/1994/2007 and Duck/Hubei/2911/2007 was 33.3% and 16.7%, respectively. The weight loss in the 7 groups of mice on the 5th day after infection corresponded with the mortality rate, with both an increased mortality rate and increased weight loss. In addition, the mice infected with Duck/Hubei/2911/2007 showed a loss of body weight that reached 23%, but these mice recovered the body weight, and the final mortality rate was 16.7% (Table 5).

Five days after infection, the titers of virus replication in lungs, spleen, kidney and brain were examined (Table 5). In general, high viral titers were detected in the lungs of mice in each experiment group. In addition, a higher lethality of the viruses correlated with a higher titer of viruses detected in brains or spleens. According to Katz's definition (Katz et al., 2000), $MLD_{50} > 10^{6.5}$ EID_{50} is a low-pathogenic virus, $<10^{3.0}$ EID_{50} is a high-pathogenic virus, and $10^{3.0}$ $EID_{50} < MLD_{50} < 10^{6.5}$ EID_{50} is a intermediate pathogenicity virus. The MLD_{50} determination showed that Duck/Henan/1647/2006, Chicken/Hubei/2856/2007, Duck/Henan/1650/2006, Chicken/Anhui/1089/2007, and Chicken/Henan/1362/2006 are high-pathogenic viruses, while

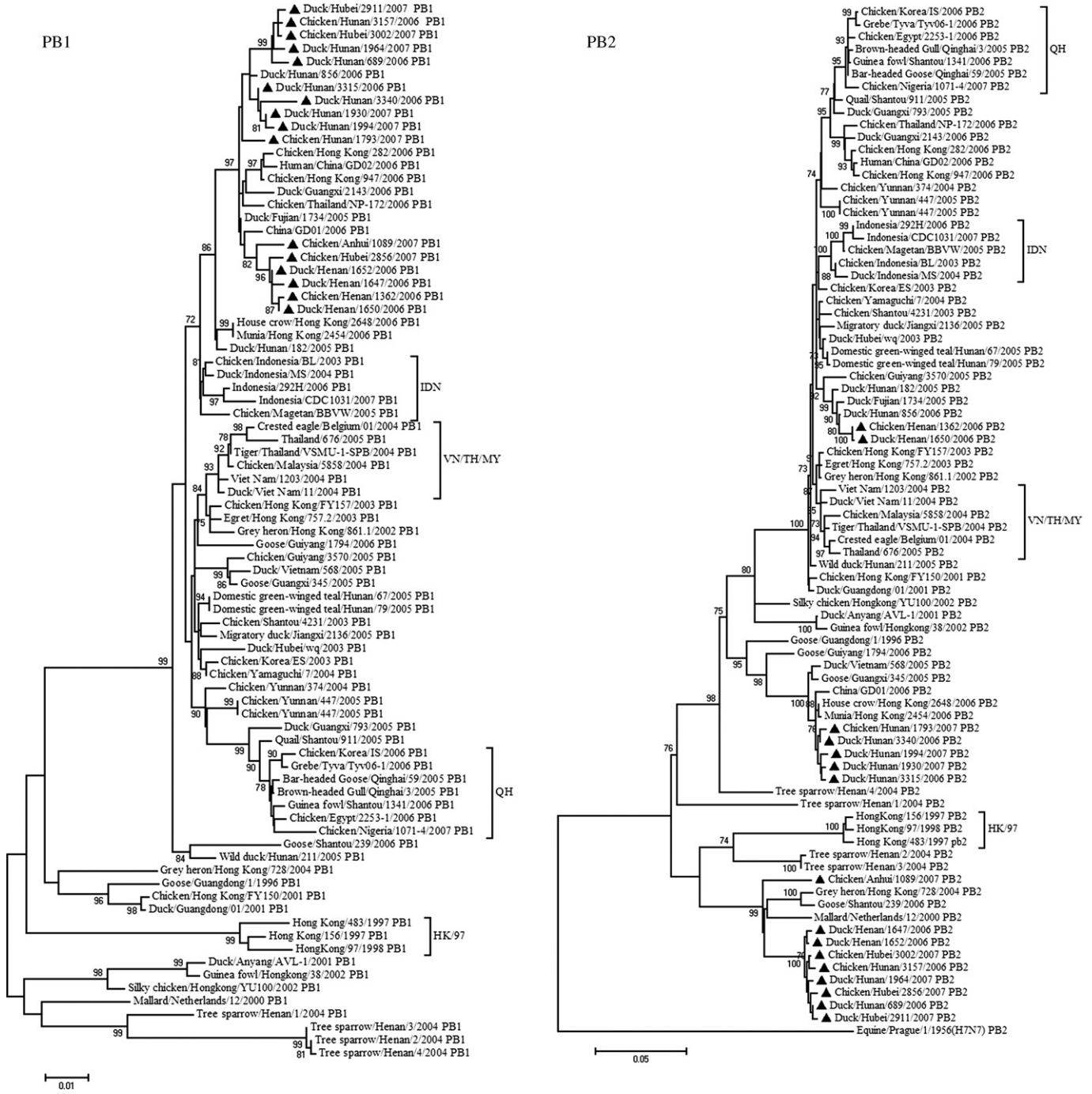

Fig. 2. (*Continued*).

Duck/Hunan/1994/2007 and Duck/Hubei/2911/2007 are low-pathogenic viruses (Table 5).

4. Discussion

The H5N1 viruses isolated in this study were all collected from apparently healthy poultry and no sick or dead poultry cases were included in this study. Poultry carrying the H5N1 viruses do not show the clinical symptoms, which reduces the caution people use when they breed, slaughter and trade the poultry. Effective protection measures may not be used, which poses a potential threat to be infected with the H5N1 virus. Apparently healthy poultry infected with the H5N1 viruses can discharge high titers of viruses from the cloaca and throat. Chen et al. showed that domestic ducks did not show clinical symptoms when experimentally infected with H5N1 virus isolated from southern China in 2000–2002, but the poultry discharged high titer H5N1 virus from the dejecta at 5 days post-infection (Chen et al., 2004). Webster et al. also found that domestic ducks infected with the H5N1 virus strain isolated in Hong Kong in 1999 showed no clinical symptoms, but high titers of virus can be detected from the throat and cloaca at 5 days post-infection (Webster et al., 2002). Therefore, seemingly healthy poultry carrying the H5N1 virus is a potential threat to humans.

The HA of H5N1 virus isolated in southern China and Southeast Asia in 2004–2005 showed an obvious geographical clustering (Chen et al., 2006). However, these H5N1 influenza viruses did not

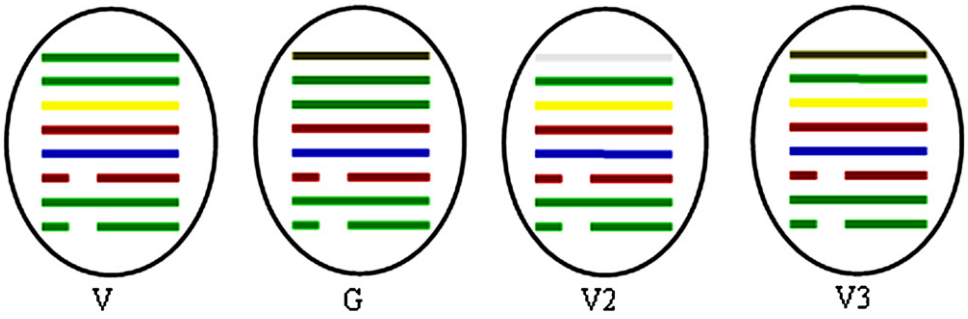

Fig. 3. Genotypes of H5N1 viruses during 2006 and 2007 in central China. The eight gene segments in each schematic virus particle are represented in the order (top to bottom) PB2, PB1, PA, HA, NP, NA, M, and NS genes. A different color is used to represent each distinct virus lineage. Genotype definitions are described in Section 3 and Duan et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Genotypes and their representative virus strains.

Genotypes	Representative strains	Location
V3	Duck/Hunan/3315/2006	Hunan
G	Chicken/Hunan/1793/2007, Duck/Hunan/1930/2007, Duck/Hunan/1994/2007, Duck/Hunan/3340/2006	Hunan
V	Chicken/Henan/1362/2006, Duck/Henan/1650/2006	Henan
V2	Duck/Hunan/689/2006, Chicken/Anhui/1089/2007, Duck/Hunan/1964/2007, Chicken/Hubei/2856/2007, Duck/Hubei/2911/2007, Chicken/Hubei/3002/2007, Chicken/Hunan/3157/2006, Duck/Henan/1647/2006, Duck/Henan/1652/2006	Hunan, Anhui, Hubei, Henan

circulate in 2005–2006, and were replaced by Clade 2.3.4 (Fujian-like sublineage) viruses (Smith et al., 2006). Although the 16 strains of H5N1 viruses isolated in 2006–2007 in this study came from different provinces, phylogenetic analysis indicated that the HA of the 16 strains clustered together and formed in a branch, and did not show an obvious geographical clustering. The strains of central China in 2006–2007 were closely related to the strains of southern China in 2005–2006. The spread of H5N1 viruses with similar characteristics may be due to the transport of poultry and the trade of live birds between the provinces of the central China and southern China. However, we cannot exclude other causes (Chen et al., 2006; Kou et al., 2005; Smith et al., 2006). In 2006–2007, cases of humans infected with the H5N1 viruses occurred in southern China and central China (Zhou et al., 2007), and the H5N1 viruses from these cases, such as China/GD01/2006 and Human/China/GD02/2006, were closely related to the strains isolated in this study. In addi-

Table 3
Molecular characterization of HA, NA, PB2, M2 and NS1 at representative sites.

Virus strain	HA		NA		PB2		M2					NS1		
	Connecting peptide	RBS	Deletion 49–68	274 His	627 Glu	701 Asp	26 Leu	27 Val	30 Ala	31 Ser	34 Gly	Deletion 80–84	42 Ser	92 Glu
Duck/Hunan/689/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Anhui/1089/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Henan/1362/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Henan/1647/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Henan/1650/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Henan/1652/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Hunan/1793/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hunan/1930/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hunan/1964/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hunan/1994/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Hubei/2856/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hubei/2911/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Hubei/3002/2007	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Chicken/Hunan/3157/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hunan/3315/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–
Duck/Hunan/3340/2006	RRRKR↓G	QSG	+	+	+	+	+	+	+	+	+	+	+	–

Table 4
Pathogenicity of H5N1 viruses in chickens[a].

Virus strain	Inoculation dose (log_{10} EID_{50})	i.v. inoculation		
		No. dead/inoculated	Death time (days)	IVPI
Chicken/Anhui/1089/2007	7.00	10/10	1	3.00
Chicken/Henan/1362/2006	6.75	10/10	1	3.00
Duck/Henan/1647/2006	7.00	10/10	1–2	2.98
Duck/Henan/1650/2006	7.00	10/10	1	3.00
Duck/Hunan/1994/2007	7.00	10/10	1	3.00
Chicken/Hubei/2856/2007	6.75	10/10	1	3.00
Duck/Hubei/2911/2007	6.25	10/10	1–2	2.82

[a] Each chicken was injected intravenously with 0.2 ml of a 1:10 dilution of virus allantoic fluid stock.

Table 5
Replication and virulence of the H5N1 viruses in BALB/c mice[a].

Virus strain	No. of survive/no. of tested	% weight loss (5 days)	Virus replication in organs, $\log_{10}$ $TCID_{50}$/0.1 ml				MLD_{50} ($\log_{10}$ EID_{50})[b]	Pathotype[c]
			Lung	Spleen	Kidney	Brain		
Chicken/Anhui/1089/2007	1/6	17.6 ± 5.1	1.75 ± 0.35	–[d]	–	–	2.00	High
Chicken/Henan/1362/2006	2/6	23.5 ± 3.9	1.84 ± 0.23	0.75 ± 0.35	–	–	0.75	High
Duck/Henan/1647/2006	0/6	30.3 ± 5.9	4.50 ± 0.24	2.42 ± 0.12	3.17 ± 0.23	1.84 ± 0.23	0.7	High
Duck/Henan/1650/2006	0/6	28.4 ± 5.9	2.83 ± 0.71	1.17 ± 0.23	1.17 ± 0.23	–	0.5	High
Duck/Hunan/1994/2007	4/6	2.6 ± 0.2	3.50 ± 0.24	1.42 ± 0.12	–	–	>6.75	Low
Chicken/Hubei/2856/2007	0/6	29.5 ± 3.4	5.00 ± 0.47	3.00 ± 0.00	5.1 ± 0.6	3.34 ± 0.47	0.5	High
Duck/Hubei/2911/2007	5/6	23.5 ± 4.4	2.17 ± 0.23	–	–	–	>6.75	Low

[a] Six-week-old BALB/c mice were infected intranasally with $10^{6.75}$ EID_{50} of each virus. Organs were collected on day 5 post-inoculation, and clarified homogenates were titrated for virus infectivity in MDCK cells at initial dilution of 1:10.
[b] Fifty percent of mouse lethal dose (MLD_{50}) was determined according the Reed and Muench method (Reed and Muench, 1938).
[c] Pathotypes were determined on the basis of replication and lethality in mice. Low, low pathogenicity; High, high pathogenicity.
[d] –, means virus was not detected (<1.00$TCID_{50}$/0.1 ml).

tion, Yu et al. reported that 24 of the total 26 cases of humans infected with the H5N1 viruses occurred in the 12 provinces of China between the October 2005 and April 2008 also belonged to Clade 2.3.4 (Yu et al., 2008). These results suggest that these H5N1 viruses have the potential to infect humans.

Based on the phylogenetic analysis, the HA, NA, NP, M, NS and PB1 gene of the 16 H5N1 viruses in central China may have originated from a common ancestor. Genes of the 16 H5N1 viruses in central China had been circulated and identified in 2005–2006 in southern China. The other two genes, PA and PB2, of the H5N1 viruses have more than one origin, of which PA has two different origins and may have circulated in the poultry in 2005–2006. PB2 originated from three different ancestor viruses, all of which may have circulated in the poultry (Fig. 2). A total of 9 H5N1 viruses carrying the wild ducks-like PB2 gene, and accounting for more than half of the strains isolated and distributed in the four provinces, may have advantages to proliferate in central China. But, it needs to be confirmed by further surveillance. In general, a novel genotype virus may obtain survival advantages more readily and cause large-scale epidemics, such as the pandemic strain in the poultry of Hong Kong in 1997, the genotype Z virus that led to the deaths of the poultry in China and other Southeast Asian countries in 2003–2004, and the strains that led to the deaths of the migratory birds in Qinghai lake in 2005. All of these case were gene reassortant strains (Guan et al., 1999; Hoffmann et al., 2000; Xu et al., 1999; Chen et al., 2005). Whether the new genotype strain carrying the PB2 gene of wild ducks-like virus can also replace the other genotypes to become the dominant H5N1 virus remains to be examined.

The challenge experiment showed that 7 strains had a high pathogenicity for the SPF chicken (Table 4). Based on the deduced amino acid sequence, the 7 isolates contained the multibasic amino acid motif RRRKR↓G at their HA cleavage sites, which is a characteristic of highly pathogenic avian influenza viruses (Senne et al., 1996). Concerning the pathogenicity in mice, 5 strains were high-pathogenic viruses (Table 5) and 2 strains were low-pathogenic viruses. We were able to detect the viruses in the lungs of the mice infected with the above 7 strains. Viruses were also detected in the kidneys and brain in mice infected with the more lethal strains. The mice with detectable H5N1 viruses in the brain showed the neurovirulence, which was observed in the H5N1 virus in poultry of Hong Kong in 2001 and 2003 (Guan et al., 2004; Lipatov et al., 2003). The replication of the H5N1 virus in the brain may enhance the virulence of the virus. The molecular mechanism of the pathogenicity of H5N1 viruses in mice is not clear, although many studies have demonstrated the relationship between virulence in mice and genetic mutation of the H5N1 viruses. For example, H5N1 viruses with the mutation of Glu → Lys in $PB2_{627}$ or Asp → Asn in $PB2_{701}$ enhanced the virulence of the virus of Hong Kong in 1997 in mice (Hatta et al., 2001; Li et al., 2005), the mutation of Asp → Glu in $NS1_{92}$ enhanced the virulence of the H5N1 virus in pigs (Seo et al., 2002), and the mutation of Pro → Ser in $NS1_{42}$ can enhance the pathogenicity of the viruses in mice (Jiao et al., 2008). The 5 highly pathogenic strains (Duck/Henan/1650/2006, Chicken/Anhui/1089/2007, Chicken/Henan/1362/2006, Duck/Henan/1647/2006, and Chicken/Hubei/2856/2007) described in this paper did not show mutations in $PB2_{627}$ and $NS1_{92}$ associated with increased pathogenicity. Although there were Pro → Ser mutations in the $NS1_{42}$ in all 7 strains, 5 were high-virulence strains and 2 were low-virulence strains. From these results, we conclude that there may be other unknown mutations relevant to the pathogenicity of H5N1.

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