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Characterization of H5N1 influenza A viruses isolated from domestic green-winged teal

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Abstract Two avian influenza virus strains, A/domestic green-winged teal/Hunan/67/2005 (H5N1) (D-GWT/67) and A/domestic green-winged teal/Hunan/79/2005 (H5N1) (D-GWT/79), were isolated from healthy domestic green-winged teals (*Anas crecca*) in Hunan Province, South China. Genomic analysis showed that both isolates were reassortants. The hemagglutinin (HA) genes of the two isolates were closely related to that of an H5N1 strain isolated from tree sparrow (A/tree sparrow/Henan/1/04). The neuraminidase (NA) genes and the internal protein genes of both isolates were closely related to those from A/chicken/Shantou/4231/2003-like (H5N1) viruses, with exception of the matrix (M) gene of D-GWT/79, which was closely related to that of the H7N3 strain A/mallard/Netherlands/12/2000 isolated from wild mallard duck. The virulence of the two isolates was examined in chickens, ducks, and mice. Both strains were found to be highly pathogenic in chickens and ducks, but showed low pathogenicity in mice. These findings contribute to the realization that domestic green-winged teals carrying the H5N1 virus may play an important role in transmitting the virus among birds.

Keywords Domestic green-winged teal · H5N1 · Reassortment · Hemagglutinin

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI), caused by rapid transmission of avian influenza virus (AIV) subtype H5N1, among poultry in eight Asian countries (Cambodia, China, Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam) during late 2003 and early 2004, resulted in enormous damage to the poultry industry. Since then, the H5N1 virus has expanded its geographical range and caused high mortality in human cases [1–3]. As of September 10, 2007, about 330 people had been confirmed worldwide to be infected with the H5N1 virus and 202 had died [4]. In China, the first human case was reported in November 2005 [5] and a total of 25 cases were confirmed, of whom 16 died [4]. The persistent introduction of the H5N1 virus into the human population raises the possibility of the emergence of a human pandemic virus.

The H5N1 virus can become highly transmissible among birds via reassortment. Molecular characterization showed that the H5N1 virus prevalent in Hong Kong in 1997 was a reassortant virus. The hemagglutinin (HA) gene of the Hong Kong virus was genetically similar to that of A/goose/Guangdong/1/96 (H5N1) virus [6]. Its replicative complex was highly homologous with that of either the A/quail/Hong Kong/G1/97 (H9N2) virus [7] or the A/teal/

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Hong Kong/W312/97 (H6N1) virus [8]. A series of genetic reassortment events since 2001 gave rise to a dominant H5N1 genotype (Z) in chickens and ducks that was responsible for the regional outbreak in 2003–2004 [9]. Therefore, it is important to maintain surveillance of live poultry markets for the H5N1 virus.

During surveillance of poultry for AIV in a poultry market in Hunan Province, South China, in 2005, we noticed the trading of domestic green-winged teals (*Anas crecca*) besides chickens, ducks, and gooses. We collected cloacal and fecal specimens from apparently healthy domestic green-winged teals (*A. crecca*) and isolated two strains of H5N1 subtype influenza virus. Previously, no H5N1 viruses were reported to be isolated from domestic green-winged teals. We studied the virulence of the two isolates in chickens, ducks, and mice, and found that the two isolates were highly pathogenic H5N1 viruses. These findings indicate the potential impact of this species, i.e., domestic green-winged teals, on the epidemiology of AIV in China.

Materials and methods

Virus isolation

Cloacal swabs were collected from 14 marketed domestic green-winged teals (*A. crecca*), and were eluted with 2.0 ml phosphate-buffered saline (PBS) containing 0.1% bovine serum saline (BSA), 4×10^6 U/l penicillin G and 400 mg/l streptomycin sulfate. The samples were sterilized using a 0.22-μm filter 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 [10]. Aliquots of virus allantoic fluid stock were stored at −80°C before use.

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 (New England Biolabs). All segments were amplified with the Phusion™ High-Fidelity PCR Kit (New England Biolabs), using segment-specific primers shown in Table 1. The PCR products were purified with the Cycle-pure Kit and Gel Extraction Kit (Omega Bio-Tek, USA) and the fragments were cloned into pGEM-T easy vector. The fragments were sequenced by the dideoxy method with a 3730 DNA sequencer (Applied Biosystems). The sequencing reactions were performed according to the manufacturer's instructions. Data were edited and aligned by BioEdit software version 7.0.5.2.

Phylogenetic analysis

Phylogenetic analysis was based on nucleotides 35–1,659 (1,625 bp) of the HA gene, 1–873 (873 bases) of the neuraminidase (NA) gene, 21–981(961 bases) of the M gene, 1–974 (974 bases) of the nucleocapsid protein (NP) gene, 10–832 (823 bases) of the nonstructural protein (NS) gene, 1,424–2,140 (717 bases) of the polymerase

Table 1 Primers for amplification of eight genome segments from the two isolates

Gene name	Primer	D-GWT/67[a]	D-GWT/79[a]
PB2	Forward: 5′ TATATTCAGTATGGAGAGAATA 3′	EU430499	EU430505
	Reverse: 5′ AAACAATTCGACACTAATTGAT3′		
PB1	Forward: 5′GGCAAACCATTTGAATGGATGT 3′	EU430500	EU430509
	Reverse: 5′AAGCTAAATTCACTATTTTTGC 3′		
PA	Forward: 5′GGTACTGATCCAAAATGGAAGA 3′	EU430501	EU430508
	Reverse: 5′GTAGCATTGCCACAACTATTTC 3′		
HA	Forward: 5′AGCGAAAGCAGGGGTTCAATCTGTC 3′	EU430496	EU430511
	Reverse: 5′AGTAGAAACAAGGGTGTTTTAAC 3′		
NP	Forward: 5′AGCGAAAGCAGGGTAGATAATCACT 3′	EU430498	EU430504
	Reverse: 5′AGTAGAAACAAGGGTATTTTTCTTT 3′		
NA	Forward: 5′AGCGAAAGCAGGAGTTCAAAATGAA 3′	EU430497	EU430510
	Reverse: 5′AGTAGAAACAAGGAGTTTTTTGAAC 3′		
M	Forward: 5′GAAAGCAGGTAGATGTTGAAAG 3′	EU430502	EU430507
	Reverse: 5′AGAAACAAGGTAGTTTTTTACT 3′		
NS	Forward: 5′AGCGAAAGCAGGGTGACAAAAACAT 3′	EU430503	EU430506
	Reverse: 5′AGTAGAAACAAGGGTGTTTTTTATC 3′		

[a] D-GWT/67 represents isolate A/domestic green-winged teal/Hunan/67/2005(H5N1) and D-GWT/79 represents isolate A/domestic green-winged teal/Hunan/79/2005(H5N1)

acidic (PA) gene, 52–1,232 (1,181 bases) of the polymerase basic 1 (PB1) gene, and 1,009–2,226 (1,218 bases) of the polymerase basic 2 (PB2) gene. Multiple alignments were constructed using the clustalW multiple alignment function of the software BioEdit (version 7.0.5.2). Phylogenetic trees were generated with neighbor-joining bootstrap analysis (1,000 replicates) using the Tamura–Nei algorithm in MEGA version 3.1 [11].

Pathogenicity

For each kind of the tested animals, two groups were used, one for assessing D-GWT/67 and one for D-GWT/79. Six-week-old SPF chickens (Beijing MERIAL Ltd.), ten for each group, were tested according to the recommendation of the Office International des Épizooties (OIE). Each chicken was intravenously injected with 0.2 ml of a 1:10 dilution of stock virus (the titers of D-GWT/67 and D-GWT/79 stocks were $10^{9.2}$ EID_{50}/ml and $10^{8.7}$ EID_{50}/ml, respectively), and mortality was observed over a 10-day period. Six-week-old Shaoxing ducks (*Anas p. plathyrynchos domestica*), a Chinese local breed (obtained from the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences), eight for each group, were inoculated intranasally with 0.2 ml of a 1:10 dilution of stock virus, and mortality was also observed over a 10-day period. In the experiment with mice, 6–8-week-old female BALB/c mice, ten for each group, were anesthetized and intranasally inoculated with 20 μl of stock virus. Seven mice from each group were observed daily for signs of infection and survival until 14 days after infection. The remaining three mice were exsanguinated on day 3, and their lungs, brains, kidneys, recta, spleens, and heads were removed. The lungs were washed twice by injecting a total of 2 ml PBS containing 0.1% BSA. The head of the mouse was removed and the lower jaw was cut off. A syringe needle was inserted into the posterior opening of the nasopharynx and a total of 1 ml of PBS containing 0.1% BSA was injected thrice to collect the outflow as nasal washings. The lung and nasal washings were centrifuged to remove the cellular debris before being used for virus titration. Other organs were ground and homogenized in 1 ml of cold PBS, solid debris was collected by centrifugation at 2,000*g* for 10 min, and the supernatant were used to determine the EID_{50} in 10-day-old embryonated chicken eggs. Virus titers were given in units of $\log_{10}$ EID_{50} per ml ± SE. The limit of virus detection was 1.0 $\log_{10}$ EID_{50} per ml.

Nucleotide sequence accession numbers

All sequences have been deposited in GenBank. The accession numbers are EU430496–EU430511.

Results

Virus isolation and phylogenetic analysis

In January 2005, we isolated two H5N1 strains from domestic green-winged teals obtained from a poultry market in Hunan province, China. The two isolates were designated A/domestic green-winged teal/Hunan/67/2005(H5N1) (D-GWT/67) and A/domestic green-winged teal/Hunan/79/2005(H5N1) (D-GWT/79). All the eight gene segments of the two isolates were sequenced. Sequence analysis revealed that the two isolates shared a high sequence similarity with each other (96–99%), with the exception of their M genes (93%).

In order to determine whether these two H5N1 strains were related to representative GenBank entries, we performed phylogenetic analysis of all eight segments. Phylogenetic analysis of the HA genes revealed that D-GWT/67 and D-GWT/79 were closely related to A/tree sparrow/Henan/1/04 and fell into occupied a branch with the H5N1 virus A/chicken/Hong Kong/FY150/01 (Fig. 1). The NA gene tree showed that D-GWT/67 and D-GWT/79 formed a branch with the H5N1 virus A/chicken/Shantou/4231/2003 isolated in China in 2003 and Japanese and Korean isolates (A/chicken/Korea/ES/03, A/chicken/Yamaguchi/7/2004 and A/crow/Kyoto/53/2004) during 2003–2004 (Fig. 1). The other six segments of D-GWT/67 had the same relationship as the D-GWT/67 NA gene. The PB2, PB1, PA, NP, and NS genes of D-GWT/79 also showed the same relationship as the viral NA gene, while the M gene was closely related to that of A/mallard/Netherlands/12/00 (H7N3). Thus, when the M segment was considered, D-GWT/79 formed a distinct branch from D-GWT/67 (Fig. 1).

According to the above-described analysis of phylogenetic relationships, both isolates were reassortant viruses. It is apparent from the phylogenetic tree that A/chicken/Shantou/4231/2003-like virus acquired the HA gene from A/tree sparrow/Henan/1/2004-like virus to generate D-GWT/67, and that D-GWT/67 acquired the M gene from A/mallard/Netherlands/12/00-like virus to produce D-GWT/79.

Molecular characterization

Based on the deduced amino acid sequences of the HA genes, both D-GWT/67 and D-GWT/79 had the same multiple basic amino acids at the connecting peptide between HA1 and HA2 (REGRRKKR/G), which is considered to be a characteristic of influenza viruses that are highly pathogenic for chickens [12]. Unlike the majority of H5N1 isolates circulating during 1996–2004 (RERRRKKR/G), there is a mutation at −6 position of the HA1-connecting peptide

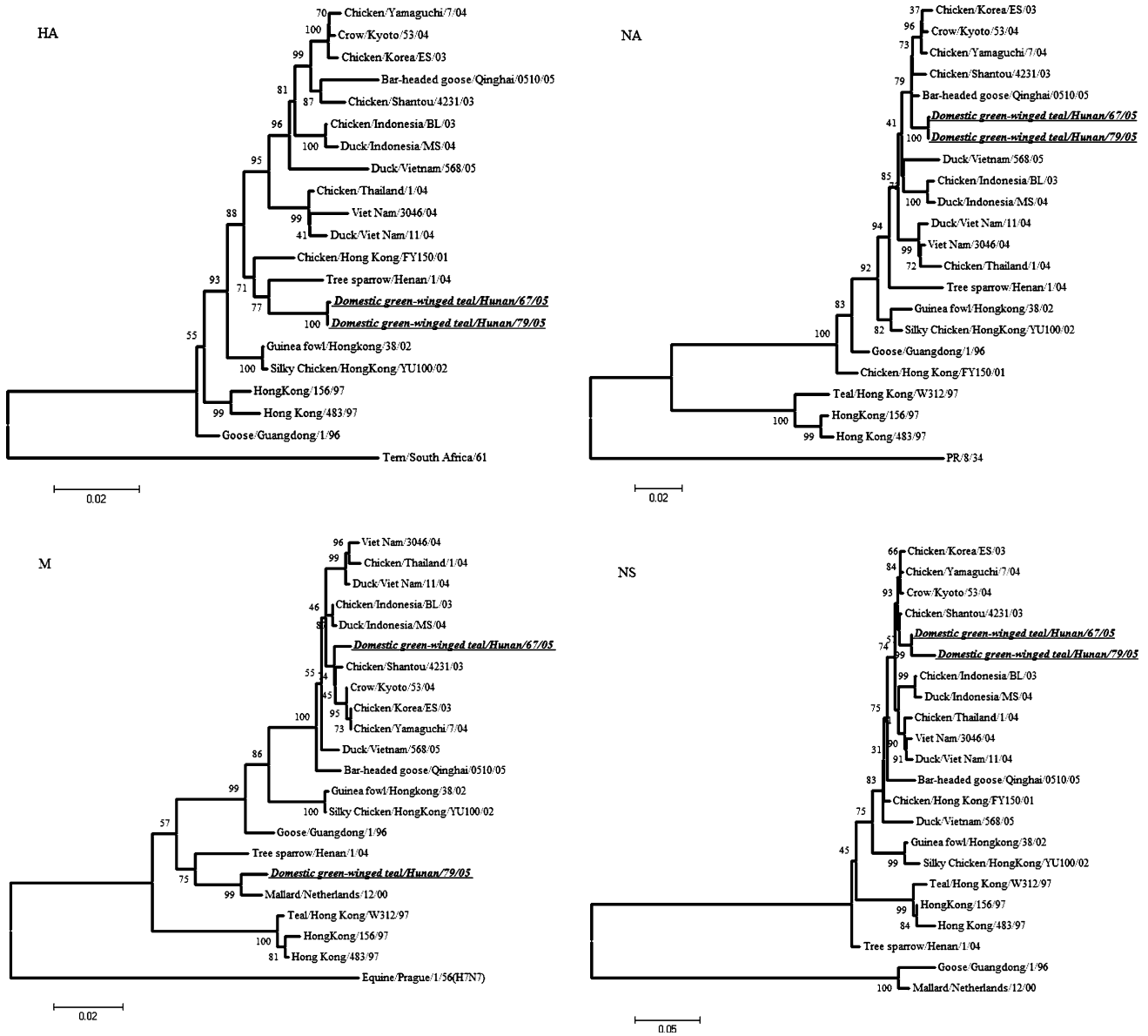

Fig. 1 Phylogenetic trees of viruses isolated from domestic green-winged teals. Trees were generated using neighbor-joining analysis with the Tamura–Nei model in the MEGA program (version 3.1). Numbers at the nodes indicate confidence levels of bootstrap analysis with 1,000 replications as a percentage value. Analysis was based on nucleotides 35–1,659 (1,625 bp) of the HA gene, 1–873 (873 bases) of the NA gene, 21–981 (961 bases) of the M gene, 1–974 (974 bases) of the NP gene, 10–832 (823 bases) of the NS gene, 1,424–2,140 (717 bases) of the PA gene, 52–1,232 (1,181 bases) of the PB1 gene, and 1,009–2,226 (1,218 bases) of the PB2 gene. The scale bar represents the distance unit between sequence pairs

(R → G). The receptor binding pockets of the HA1 proteins of both D-GWT/67 and D-GWT/79 retained amino acid residues Gln222 and Gly224 (H5 numbering used throughout) that preferentially bind to 2, 3-NeuAcGal linkages of avian cell-surface receptors [13].

By analyzing the deduced amino acid sequences of NA proteins, both D-GWT/67 and D-GWT/79 were found to have a 20-amino acid deletion (positions at 49–68) in the stalk region, which is also found in the dominant H5N1 virus in southern China (genotype Z) [9]. It has been reported that the presence of the mutation His274Tyr in the NA protein was associated with decreasing sensitivity to NA inhibitors [14]. No amino acid mutation was observed at this residue in the NA proteins of D-GWT/67 and D-GWT/79, which indicates that domestic green-winged teal isolates would be sensitive to NA inhibitors.

H5N1 viruses with mutation Lys627 in PB2 are highly virulent and systemically replicable in mice [15]. Sequence analysis revealed no Lys627 mutation in the PB2 proteins of D-GWT/67 and D-GWT/79. The two viruses also did not

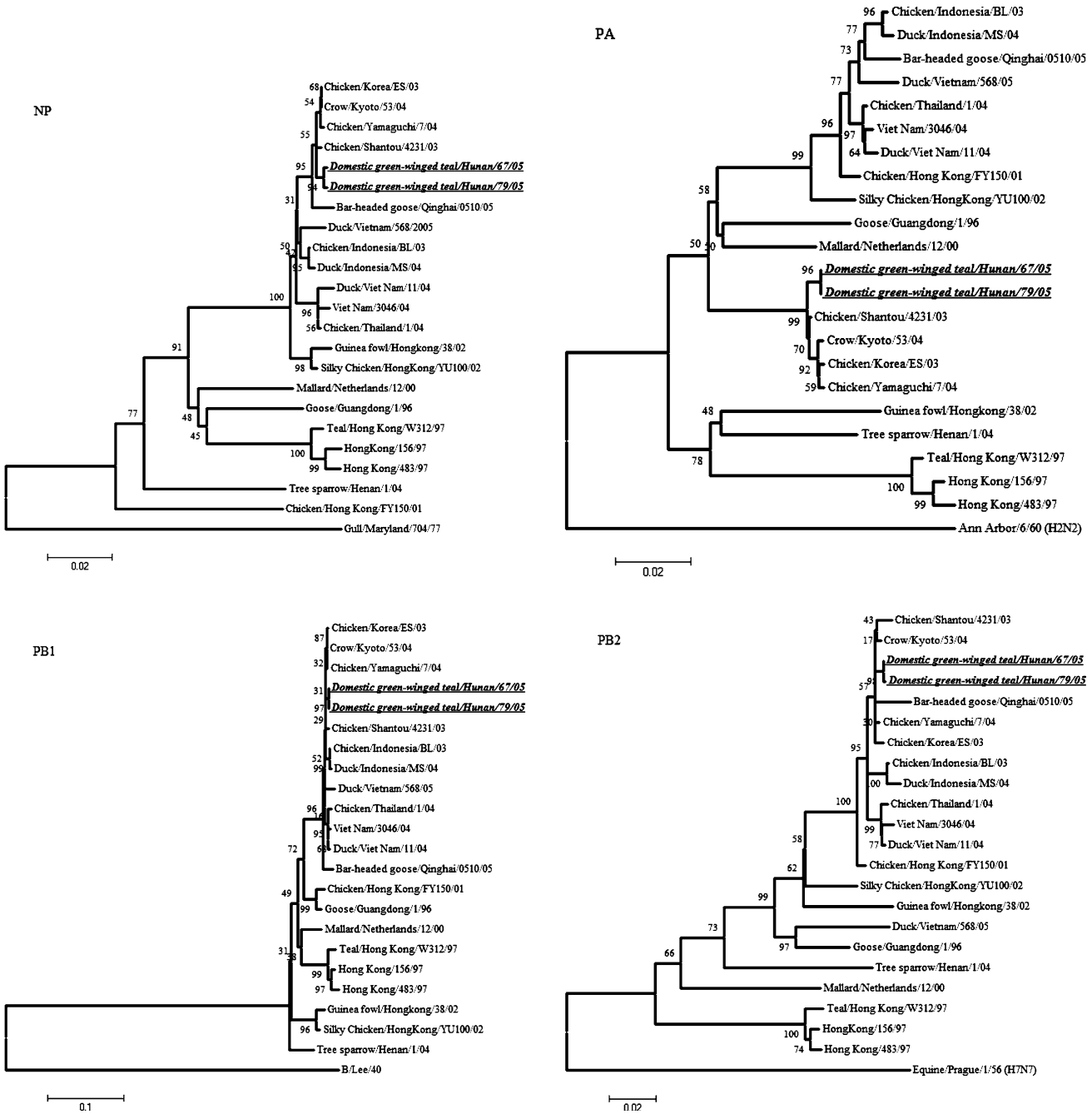

Fig. 1 continued

have a mutation of Glu92 in the NS1, which was associated with high virulence of H5N1 subtype in 1997 [16, 17]. Both D-GWT/67 and D-GWT/79 have a 5-aa deletion (aa 80–84) in the middle of the NS protein, which was found in the dominant H5N1 virus in southern China (genotype Z) [9]. Amantadine-resistant influenza A variants carry amino acid substitutions at residues 26, 27, 30, 31, or 34 of the M2 protein [18, 19]. Our sequence analysis did not show any substitutions at these residues. Therefore, the two isolates may be sensitive to this class of antiviral drugs.

Pathogenicity

In order to determine the pathogenicity of the two isolates in chickens, ducks, and mice, virus stocks were prepared as described in the "Materials and methods" section. The titers of D-GWT/67 and D-GWT/79 stocks were $10^{9.2}$ and $10^{8.7}$ EID_{50}/ml, respectively. Pathogenicities of D-GWT/67 and D-GWT/79 were tested in 6-week-old chickens according to the guidelines established by OIE. Each chicken was intravenously inoculated with 0.2 ml of a 1:10

Table 2 Pathogenicity of D-GWT/67 and D-GWT/79 in chickens and ducks

Virus	Titer of infection (log_{10} EID_{50})	Chicken[a]		Duck[b]	
		Number of survivors/ number of tested	IVPI	Number of survivors/ number of tested	MDT (days)
D-GWT/67	7.5	0/10	3.0	0/8	4.2
D-GWT/79	7.0	0/10	3.0	0/8	2.9

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

[b] Each duck was inoculated intranasally with 0.2 ml of a 1:10 dilution of virus allantoic fluid stock

Table 3 Pathogenicity of D-GWT/67 and D-GWT/79 in mice

Virus	Titer of infection (log_{10} EID_{50})	Mice		Virus titers in organs of mice (log_{10} EID_{50}/ml)[a]					
		Number of survivors/ number of tested (2 weeks)	Weight loss (%) (7 days)	Lung	Brain	Nasal	Kidney	Spleen	Rectums
D-GWT/67	7.5	7/7	23.2 ± 5.4	4.3 ± 0.5	–[b]	2.3 ± 0.4	–	–	–
D-GWT/79	7.0	6/7	24.9 ± 6.0	3.3 ± 0.4	–	2.6 ± 0.1	–	–	–

Groups of ten adult female BALB/c mice were intranasally inoculated with 20 μl of stock virus (the titers of D-GWT/67 and D-GWT/79 stocks were $10^{9.2}$ and $10^{8.7}$ EID_{50}/ml, respectively). Seven mice per group were observed for survival within 2 weeks. The remaining three mice from each experimental group were exsanguinated on day 3, and their lungs, brains, kidneys, spleens, recta, and heads were collected for virus titration in embryonated chicken eggs. The limit of detection was $10^{1.0}$ EID_{50}/ml

[a] Values represent mean ± SD

[b] Virus undetected

dilution of virus stock in PBS. The results showed that the two H5N1 isolates of domestic green-winged teals were highly pathogenic to chickens. All chickens died within 24 h and the intravenously pathogenicity index (IVPI) was 3.0 (Table 2). Six-week-old Shaoxing ducks (*Anas p. platyrynchos domestica*) were inoculated intranasally with the same doses of D-GWT/67 and D-GWT/79 and observed daily for clinical signs of disease. Similar to the chickens, ducks inoculated with either D-GWT/67 or D-GWT/79 showed 100% mortality. The mean death times (MDT) of D-GWT/67 and D-GWT/79 were 4.2 and 2.9 days, respectively (Table 2).

In order to determine the pathogenicity of D-GWT/67 and D-GWT/79 viruses in a mammalian host, BALB/c mice were inoculated intranasally with the viruses. Virus replication in various organs, weight loss, and mortality were tested. Infection of mice with each of the H5N1 viruses resulted in high titers of virus in the nasal and lung washes on day 3 post-infection (Table 3). However, mice infected with either D-GWT/67 or D-GWT/79 virus had undetectable viral titers in brain, kidney, spleen, and rectum. They also showed signs of illness, such as ruffled fur and hunched posture, and weight loss (maximal loss of weight 7 days after infection), but had survival rates of 100% (7/7) and 86% (6/7), respectively, during 2 weeks post-infection (Table 3). These results suggested that both D-GWT/67 and D-GWT/79 viruses could not kill mice without adaptation, but that they could replicate well in the lung of the mice, in contrast to their limited ability to spread in brains and other internal organs.

Discussion

In this study we characterized two H5N1 viruses isolated from domestic green-winged teals. Both isolates were reassortant viruses and highly pathogenic to chickens and ducks, but showed low pathogenicity in mice. The distribution of H5N1 HPAI viruses in domestic green-winged teals demonstrated the potential impact of this duck species on the epidemiology of the viruses in China. The domestic green-winged teals carrying H5N1 influenza A viruses may play an important role in transmitting H5N1 virus among birds.

It has been reported that genotype Z strains of H5N1 viruses were responsible for the regional outbreak in 2003–2004 [9]. On the other hand, the genotype V viruses could be isolated in southern China (such as A/chicken/Shantou/4231/2003) [9] and also in other eastern Asian countries. In South Korea, an outbreak of HPAI H5N1 disease among chickens and ducks occurred in December 2003 [2]. In Japan, between the end of December 2003 and March 2004, four outbreaks of HPAI H5N1 disease occurred in birds [1]. The H5N1 viruses isolated in Korea and Japan were genetically closely related to each other, i.e., >99% identity in the nucleotide sequences of all eight RNA

segments [20]. Phylogenetic analysis of all segments showed that the Korean and Japanese isolates belonged to the same cluster as the genotype V virus A/chicken/Shantou/4231/2003 [20]. In this study, it was apparent from the phylogenetic tree that D-GWT/67 was a reassortant of the genotype V virus, which acquired the HA gene from A/tree sparrow/Henan/1/04-like virus through gene reassortment. Phylogenetic analysis also suggested that D-GWT/79 was a descendant of D-GWT/67, with its M gene derived from the wild mallard virus strain A/mallard/Netherlands/12/2000 (H7N3). The M gene has been shown to act as a dominant factor that determined host restriction [21]. Recent studies showed that, besides the M gene, those genes encoding other internal proteins, i.e., RNA polymerase (PB2, PB1, PA), NP, and NS1, NS2/NEP, also contributed to the host range [22]. The contribution of individual proteins to host range restriction seems to vary, depending on virus strains and even the test system [22].

On the other hand, it has been shown that a series of genetic reassortment events had happened and given rise to a dominant H5N1 genotype Z in chickens and ducks [9]. It is reasonable to believe that the genetic reassortment events between influenza A viruses are ongoing among birds in China. The co-circulation of genotype V and Z strains of H5N1 viruses is a cause for more concern because they can reassort and recombine to create additional versions of influenza A viruses of H5N1 subtype.

In this study, we found that the D-GWT/67 and D-GWT/79 viruses isolated from the domestic green-winged teals were highly pathogenic to chickens, killing every exposed chicken within 1 day of intravenous injection. The results are consistent with Mase et al.'s [1] study, whose result showed that chickens inoculated with the Japanese isolates of 2003 (genotype V) died suddenly without clinical signs. Similarly, the two viruses were also highly pathogenic for ducks, resulting in 100% mortality. The mortality is higher than that of Korean isolates (genotype V), which resulted in a mortality rate of only 25% in Peking ducks (*Anas platyrhynchos domestica*) [2]. The mortality in ducks in this study is very similar to that of the H5N1 virus isolated in Qinghai birds, which caused 80% mortality when inoculated in Shaoxing ducks (*Anas p. plathyrynchos domestica*) [23]. Previously, a series of studies had reported that the pathogenicity for ducks varied in different strains. The H5N1 isolates from 1997 to 2001 did not cause significant symptoms [24], while the late-2002 H5N1 isolates (A/teal/Hong Kong/2978.1/2002, A/Rosybilled Pochard/Hong Kong/821/2002, and A/goose/Hong Kong/739.2/2002) caused significant symptoms and mortality [24]. The virulence of H5N1 isolates in mice also varied in different strains. Previous studies had established that viruses with LD_{50} values $>10^{6.5}$ EID_{50} were considered to be of low pathogenicity, while those with LD_{50} values $<10^{3.0}$ EID_{50} were considered highly pathogenic in mouse models [25]. Lee et al. [2] demonstrated that the chicken HPAI H5N1 virus (A/chicken/Korea/ES/03, genotype V) did not cause mortality in mice. Mase et al. [1] showed higher virulence of Japanese isolates (genotype V) in mice (with a dose lethal to 50% of mice $= 5 \times 10^5$ EID_{50}). Most of the H5N1 viruses isolated from Qinghai Lake birds were highly lethal. When administered to mice intranasally, the viruses had an LD_{50} of $<10^{0.5}$ EID_{50} [23, 26]. It has been shown that the molecular basis of the pathogenesis of H5N1 in mice is associated with Lys at position 627 of PB2 [25]. In this study, both isolates have Glu at position 627, indicating that they had low level of pathogenicity for mice. The result also showed that infection of mice with D-GWT/67 and D-GWT/79 at the dose of $>10^{7.0}$ EID_{50} resulted in survival rates of 7/7 and 6/7, respectively. Therefore the two viruses have a low-pathogenicity phenotype for mice.

Wild ducks represent the major natural low-pathogenic avian influenza (LPAI) virus reservoir and have a higher prevalence of influenza A virus than other species [27, 28]. The present study demonstrated that domestic green-winged teals, a kind duck bred for meat in southern China, were likely to harbor highly pathogenic influenza viruses even when they appeared healthy. To avoid the H5N1 viruses in domestic green-winged teals from crossing the species barrier and infecting humans, more understanding of the distribution and characteristics of H5N1 viruses in domestic green-winged teals are required.

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