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ORIGINAL ARTICLE

Characterization of H9N2 influenza viruses isolated from Dongting Lake wetland in 2007

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Abstract In 2007, a total of eight H9N2 influenza viruses were isolated from the water and fowl feces in Dongting Lake wetland, China. The genomes of the eight viruses were sequenced, and all eight gene segments were subjected to phylogenetic analysis. The results showed that all the isolates belonged to the same genotype, in which the HA, NA and NS gene segments were Chicken/Beijing/94-like; the PB2, PB1, PA and NP gene segments were Chicken/Shanghai/F/98-like; and the M gene was Quail/Hong Kong/G1/97-like. Animal experiments showed low pathogenicity of the selected viruses for chickens, although some chickens died after inoculation. The viruses showed no overt clinical signs in mice, but they could replicate in murine lungs prior to adaptation.

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Introduction

Highly pathogenic avian influenza (HPAI) has garnered attention worldwide. However, low-pathogenic avian influenza (LPAI) viruses that circulate widely in wild and commercial fowl, such as H9N2 virus, should not be ignored. H9N2 avian influenza (AI) viruses have been detected or isolated in every continent of the world since their first isolation [1–6]. Infection with H9N2 virus often decreases the laying rate of hens, and coinfection with other viruses or bacteria can cause severe morbidity and high mortality [6, 7].

Since H9N2 influenza viruses were isolated from humans in 1999 [8], they have been considered potential candidates for causing the next human influenza pandemic. Therefore, strengthening the epidemiological investigation of H9N2 AI viruses is of importance for public health.

An epidemiological study has shown a high incidence of H9N2 AI virus infections in chickens and other land birds in southern China [4]. Surveillance of AI in a live-poultry market in Hong Kong in 1997 has shown that H9N2 AI viruses were the second-most prevalent isolates, next to H5N1 viruses [9]. H9N2 AI viruses also have been isolated from domestic fowl in other Asian countries, such as the United Arab Emirates, Korea, and India [1, 6, 10]. Three distinct sublineages of H9N2 AI viruses are circulating in different types of poultry in several countries in the Eurasia region: Ck/Bei/94-like, represented by A/Chicken/Beijing/1/94; G1-like, represented by A/Quail/Hong Kong/G1/97; and Y439-like or Korean-like, represented by A/Chicken/Korea 38349-p96323/96 [10, 11]. The G1-like viruses provided an internal gene for human H5N1 influenza virus in 1997 [9].

The wild waterfowl that live close to wetlands or lakes are considered to be the natural host of AI viruses, from

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which all of the 16 HA and 9 NA subtype viruses have been isolated [12, 13]. Dongting Lake wetland is located in central and southern China and is an important habitat and overwintering area along the migratory route of East Asian migratory birds. In this area, many poultry are free-range, and mixing between chickens and domestic aquatic fowl (including ducks and geese) is common. Tens of thousands of migratory birds reach Dongting Lake wetland every autumn and share the same area of water with domestic ducks, which might result in the spread and transmission of AI viruses between migratory birds and domestic fowl, through water contaminated with influenza virus. Experimental studies have shown that AI viruses can remain detectable in water and wet feces for up to 4–6 days at 37°C and can remain infective in water for up to several months at 17°C [14–16]. Therefore, it is possible that AI viruses can be transmitted by a waterborne mechanism [17, 18].

Previous surveillance of AI viruses has focused mainly on live-poultry markets in southern China, but there are few data on the genetic evolution of the viruses outside the live-poultry markets, and the role of waterways in virus transmission remains poorly understood. Therefore, from March to December 2007, an influenza virus surveillance program was carried out in Dongting Lake wetland (including water and fecal samples). Five H9N2 viruses were isolated from the collected water samples, and three from fecal samples, and these eight viruses were characterized.

Materials and methods

Sample collection

Dongting Lake is an internationally important wetland. From the autumn to the next spring is the low-water period of Dongting Lake. In this period, Dongting Lake wetland is mainly comprised of smaller lakes, some shallow mudflats and meadow. During the low-water period of Dongting Lake, many wild birds (most of these birds are of the family Anatidae including mallard, spot-billed duck, whopper swan, bar-headed goose, and so on) live in the wetland. A mass of feces excreted by the wild birds is left on the shore of the lake and flushed by the lake water.

In 2007, water samples were collected from the areas that were heavily contaminated with feces or used by aquatic fowl. A 200-ml water sample was collected at each sampling site using a 250-ml sterile vial. Fecal samples were also collected from Dongting Lake wetland. Four hundred ninety-eight water samples and 316 fecal samples were collected during the investigation in 2007. All of the collected samples were stored in a portable refrigerator and sent to our laboratory, where they were frozen immediately at −80°C.

In the spring, the average temperature of water in Dongting Lake is between 10 and 12°C, while in the autumn and winter, the average temperature is between 6 and 10°C. Under these conditions, the viruses will have a considerably long persistence time in the water. Every summer, most areas of the Dongting lake wetland are covered by floodwater from the Yangtze River. It is difficult to collect water samples and fecal samples in summer, so the investigation was performed in the spring, autumn and winter.

Virus isolation

The viruses in the water samples were concentrated and isolated as described previously [17, 18]. To every 200-ml water sample, we added formaldehyde-fixed chicken erythrocytes to a final concentration of 0.1%. The samples were placed on ice for 1 h, during which the tube was inverted gently every 15 min to mix the contents, and the sample was then centrifuged at 3,000 rpm at 4°C for 5 min. The supernatant was discarded, and the precipitate was resuspended in 1 ml PBS (pH 7.2) containing 2×10^6 U/l penicillin G, 2×10^6 U/l polymyxin B, 250 mg/l gentamicin, 0.5×10^6 U/l nystatin, 60 mg/l ofloxacin HCl, and 0.2 g/l sulfamethoxazole, inoculated into 10-day-old SPF embryonated chicken eggs at a dose of 0.5 ml/egg, and incubated at 37°C for 72 h. The fecal samples were immersed and eluted with PBS (pH 7.2) and centrifuged at 3,000 rpm at 4°C for 5 min. The supernatant was collected, inoculated into 10-day-old SPF embryonated chicken eggs at a dose of 200 μl/egg, and incubated at 37°C for 72 h. All of the operations were performed under aseptic conditions. All of the viruses isolated were purified by three rounds of limiting dilution in 10-day-old SPF embryonated chicken eggs.

RNA extraction and nucleotide sequencing

Viral RNA from the isolates propagated in 10-day-old embryonated eggs was extracted by lysing the viruses with TRIzol LS reagent (Life Technologies). The RNA was reverse-transcribed into single-stranded DNA with M-MuLV reverse transcriptase (New England Biolabs). All segments were amplified using a Phusion™ High-Fidelity PCR Kit (New England Biolabs). The HA, NA, NP, M and NS gene segments of the viruses were amplified using segment-specific primers designed by Hoffmann et al. [19], and the PB2, PB1 and PA gene segments were amplified using primers designed by Li et al. [20]. The PCR products were purified using a Cycle-Pure Kit and a Gel Extraction Kit (Omega Bio-Tek). The fragments were cloned into

pGEM-T Easy Vector and sequenced by the dideoxy method with an ABI 3730 DNA sequencer (Applied Biosystems). Data were edited and aligned with BioEdit version 7.0.5.2.

Phylogenic analysis

Phylogenic analysis was based on nucleotides 94–1,556 (1,463 bp) of the HA gene, 38–1,371 (1,334 bp) of NA, 56–2,289 (2,234 bp) of PB2, 40–2,233 (2,194 bp) of PB1, 25–2,166 (2,142 bp) of PA, 39–1,388 (1,350 bp) of NP, 95–931 (837 bp) of M, and 38–806 (769 bp) of NS. Multiple alignments were constructed by using the ClustalW multiple alignment program of the software BioEdit (version 7.0.5.2). Phylogenic trees were generated by neighbor-joining bootstrap analysis (1,000 replicates) using the Tamura–Nei algorithm in MEGA version 3.1 [21].

Animal study

Six-week-old White Leghorn chickens (Beijing MERIAL), eight in each group, were tested according to the recommendations of the Office International des Épizooties (OIE). Each chicken was injected intravenously with 0.2 ml of a 1:10 dilution of virus (the titers are shown in Table 4). The inoculated chickens were observed for 10 days for death and clinical signs of disease. Pharyngeal and cloacal swabs were collected on days 3, 5 and 7 post-inoculation (p.i.) for virus isolation in 10-day-old SPF embryonated chicken eggs. The sera were harvested on day 21 p.i. for determination of seroconversion.

In order to evaluate the ability of H9N2 virus to replicate in BALB/c mice, groups of 6-week-old female BALB/c mice were infected intranasally with each virus in a volume 50 µl of $10^{6.5}EID_{50}$, under anesthesia. On day 5, three of ten inoculated mice were sacrificed, and the organs were collected for virus titration. The remaining inoculated mice were monitored daily for death and clinical signs of disease. Meanwhile, in order to determine the 50% mouse lethal doses (MLD_{50}) of the viruses, groups of mice ($n = 5$ mice each) were inoculated intranasally with virus-containing allantoic fluid without any dilution and tenfold serial dilutions of the virus in a volume of 50 µl. The MLD_{50} were calculated by the method of Reed and Muench [22].

Antigenic analysis

Sera were harvested from the four groups of inoculated chickens on day 21 p.i. for confirmation of seroconversion and antigenic analysis by hemagglutination inhibition (HI) assays with 0.5% chicken erythrocytes according to the recommendation of OIE.

Results

Virus isolation and genetic analysis

In 2007, a total of eight H9N2 AI viruses were isolated from the Dongting Lake wetland, of which, five were isolated from water samples and three from fecal samples in the surrounding environment (Table 1). The whole genomes of the eight viruses isolated in this study were sequenced, and all sequences are available from GenBank under accession numbers GU474547–GU474610.

The nucleotide sequences for each gene segment of the eight H9N2 viruses were aligned with ClustalW, which showed that the corresponding gene segments of the eight viruses had high nucleotide similarity (Table 2). Homology analysis was also performed to compare the nucleotide sequences of all eight gene segments with other available sequences in GenBank. The results showed that several gene segments of the viruses isolated in the present study were highly homologous to those of H9N2 AI viruses isolated from chickens in other regions of China in recent years, such as Shandong Province and Hunan Province. In addition, the PB2, PB1 and PA genes of the current isolates

Table 1 H9N2 viruses isolated in the present study

Virus	Isolation date[a]	Virus source	Abbreviation	Accession numbers
A/environment/Hunan/1-18/2007(H9N2)	03/2007	Water sample	Environment/HN/1-18/07	GU474547–GU474554
A/feces/Hunan/1-23/2007(H9N2)	03/2007	Fecal sample	Feces/HN/1-23/07	GU474555–GU474562
A/environment/Hunan/1-70/2007(H9N2)	03/2007	Water sample	Environment/HN/1-70/07	GU474563–GU474570
A/environment/Hunan/1-81/2007(H9N2)	03/2007	Water sample	Environment/HN/1-81/07	GU474571–GU474578
A/feces/Hunan/2-28/2007(H9N2)	04/2007	Fecal sample	Feces/HN/2-28/07	GU474579–GU474586
A/environment/Hunan/2-84/2007(H9N2)	04/2007	Water sample	Environment/HN/2-84/07	GU474587–GU474594
A/feces/Hunan/3-91/2007(H9N2)	10/2007	Fecal sample	Feces/HN/3-91/07	GU474595-GU474602
A/environment/Hunan/5-38/2007(H9N2)	12/2007	Water sample	Environment/HN/5-38/07	GU474603–GU474610

[a] Isolation date is shown as month/year

Table 2 Homology (%) of nucleotide sequences of eight genes of virus strains isolated in this study to themselves and relevant sequences available in GenBank

Gene	Homology (%)[a]	Homologous virus[b]	Homology (%)[b]
PB2	96.6–99.9	Ck/SD/B3/07(H9N2)	95.6–96.3
		Sw/SD/w4/03(H9N2)	95.5–96.2
PB1	98.8–99.9	Ck/SD/B3/07(H9N2)	97.4–98.4
		PF/HK/2142/08(H5N1)	97.5–98.0
		Sw/SD/w4/03(H9N2)	95.9–96.3
PA	98.6–99.9	Ck/SD/B2/07(H9N2)	97.4–98.1
		Sw/SD/w4/03(H9N2)	96.8–97.5
		Ck/SH/F/98(H9N2)	96.8–97.5
HA	96.3–99.9	Ck/GX/55/05(H9N2)	97.7–98.6
		Ck/HN/HG29/07(H9N2)	96.8–97.9
NP	98.4–99.9	Ck/SD/B2/07(H9N2)	98.3–98.7
NA	98.4–99.8	Ck/SD/B2/07(H9N2)	98.5–99.3
M	99.0–99.9	Ck/GX/1032/05(H9N2)	99.0–99.4
		Ck/HN/4444/05(H9N2)	98.7–99.1
		Chukkar/ST/89/05(H6N1)	98.7–99.0
NS	98.7–99.9	Ck/SD/B4/07(H9N2)	98.9–99.7

Ck chicken, *Sw* swine, *PF* peregrine falcon, *SD* Shandong, *GX* Guangxi, *HN* Hunan, *HK* Hong Kong

[a] The identity among the eight H9N2 virus strains isolated in this study for each of the gene sequences

[b] The best match for each gene of the H9N2 isolates and the identity between them. Influenza A viruses with highest nucleotide sequence identity to the H9N2 viruses isolated in the present study were determined by BLAST search in the Influenza Sequence Database

showed homology of 95.5–97.5% with H9N2 viruses isolated from pigs in Shandong in 2003 (Table 2). In addition, the PB1 genes of the current isolates showed high homology (97.5–98.0%) with A/peregrine falcon/Hong Kong/2142/2008(H5N1) isolated from wild fowl in Hong Kong in 2008, which indicates that the isolated H9N2 viruses might provide internal genes for AI viruses of other subtypes.

Phylogenic analysis

Phylogenic analysis proved that the HA and NA gene segments of all eight viruses isolated in this study were Ck/Bei/94-like (Fig. 1a, b). The phylogenic tree of the HA gene also showed that Ck/Bei/94-like viruses were widely epidemic in domestic fowl in the mainland of China during 2007–2008, because these viruses were isolated from chickens in other provinces such as Liaoning, Yunnan and Hubei (Fig. 1a).

Phylogenic analysis of internal genes of the eight viruses isolated in this study showed that the viruses might have been generated by multiple reassortment (Figs. 1, 2). All of the PB2, PB1, PA and NP gene segments of the isolated viruses belonged to the Chicken/Shanghai/F/98-like (Ck/SH/F/98-like) cluster in phylogenic trees (Fig. 1c–f), while the M gene belonged to the G1-like cluster (Fig. 1g). The NS gene segments, like HA and NA, were Ck/Bei/94-like (Fig. 1h). The phylogenic analysis of the eight gene segments of the viruses isolated in this study also showed that all of the strains belonged to the same genotype.

Molecular characterization

Amino acid analysis showed that the sequences of all of the connecting peptides between HA1 and HA2 of the viruses isolated in this study were RSSR (Table 3). Almost all of the connecting peptide sequences of the HA genes of the epidemic H9N2 viruses in Mainland China are RSSR, which is characterized as a low-pathogenic virus, but it might be recognized and cleaved by some bacterial proteases [23], and the substitution of basic amino acids could enhance the pathogenicity of the virus [1, 24]. All of the viruses isolated in this study possessed leucine (L) at position 226 of the HA gene (Table 3), which indicates the potential of the isolated viruses to cause human infection [25]. All of the viruses had a 3-amino-acid deletion at sites 63–65 in the NA gene (Table 3), resulting in the deletion of a potential glycosylation site in the NA protein, but its biological significance needs to be investigated further. The M2 protein is the target of amantadine and rimantadine, and the mutation of amino acid V to A or T at site 27 and S to N or R at site 31 of the M2 protein might result in viral resistance to these drugs [26]. However, no such mutations were observed in the viruses isolated in this study, suggesting sensitivity of the isolates to these antiviral agents.

Animal study

Of the eight viruses isolated in this study, four were selected for pathogenicity tests in chickens and mice. The SPF chickens, aged 6 weeks, were divided into four groups of eight. Each chicken was inoculated with the virus in a volume of 0.2 ml by intravenous injection (Table 4). Pharyngeal and cloacal swabs on days 3, 5 and 7 after inoculation were collected for virus isolation. On day 3 p.i., viruses were isolated from the pharynges of all of the surviving chickens, but only from the cloaca in some chickens. On day 7 p.i., viruses were not detected in the cloaca of any chickens, but they were still isolated from the pharynges in some birds. This showed that the H9N2 viruses isolated in this study mainly replicated in the respiratory tract of fowl, which suggests that these viruses are mainly spread via the respiratory route. The maximum and minimum mortality rates of chickens after intravenous injection with the selected viruses were 62.5% (5/8) and

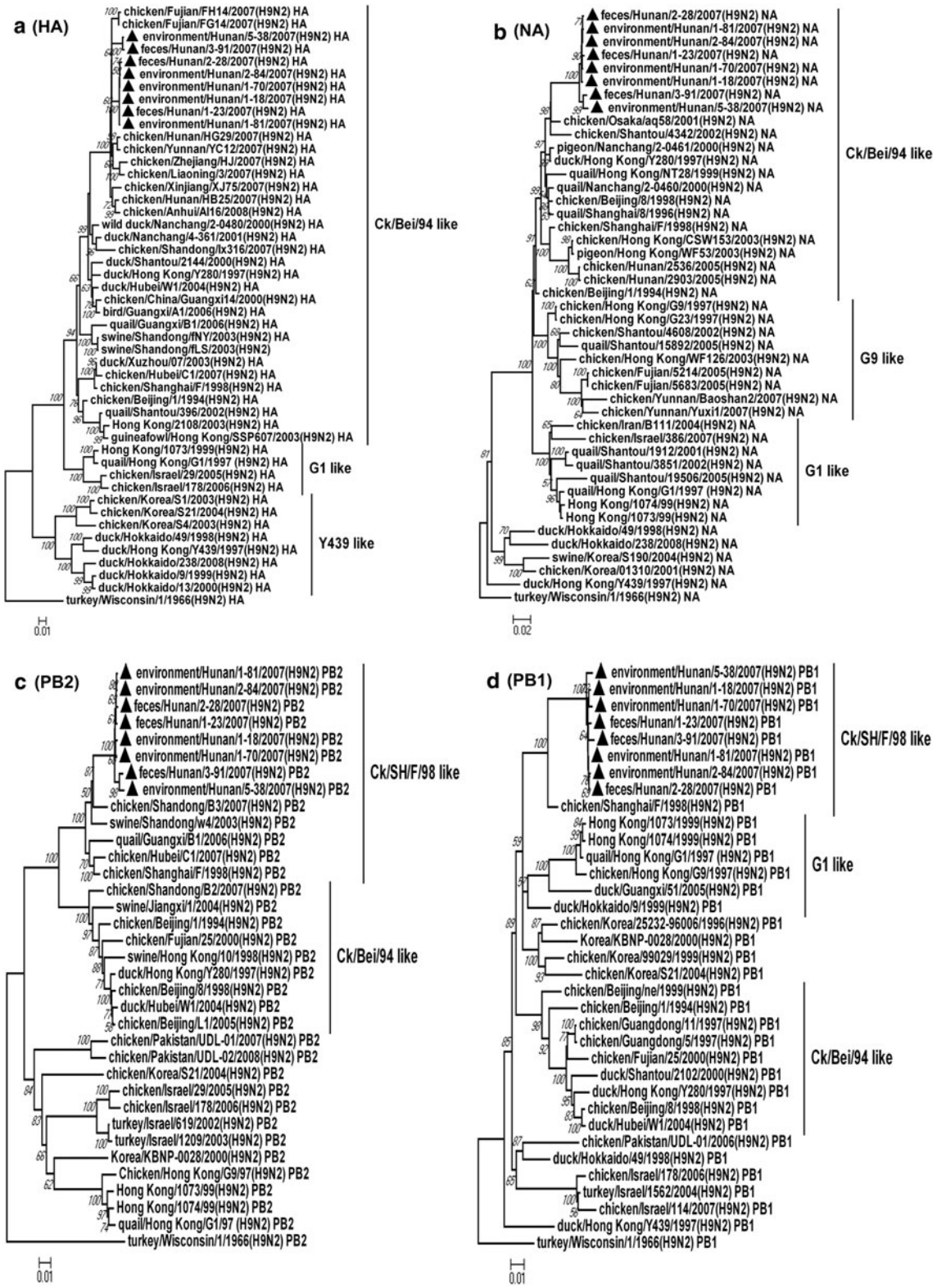

Fig. 1 Phylogenic trees of the HA (**a**), NA (**b**), PB2 (**c**), PB1 (**d**), PA (**e**), NP (**f**), M (**g**) and NS (**h**) genes of H9N2 influenza viruses. Trees were generated by using neighbor-joining analysis with the Tamura–Nei model in the MEGA program (version 3.1) and were rooted to A/turkey/wisconsin/1966 (H9N2). *Numbers* at the nodes indicate confidence levels of bootstrap analysis with 1,000 replications as a percentage value

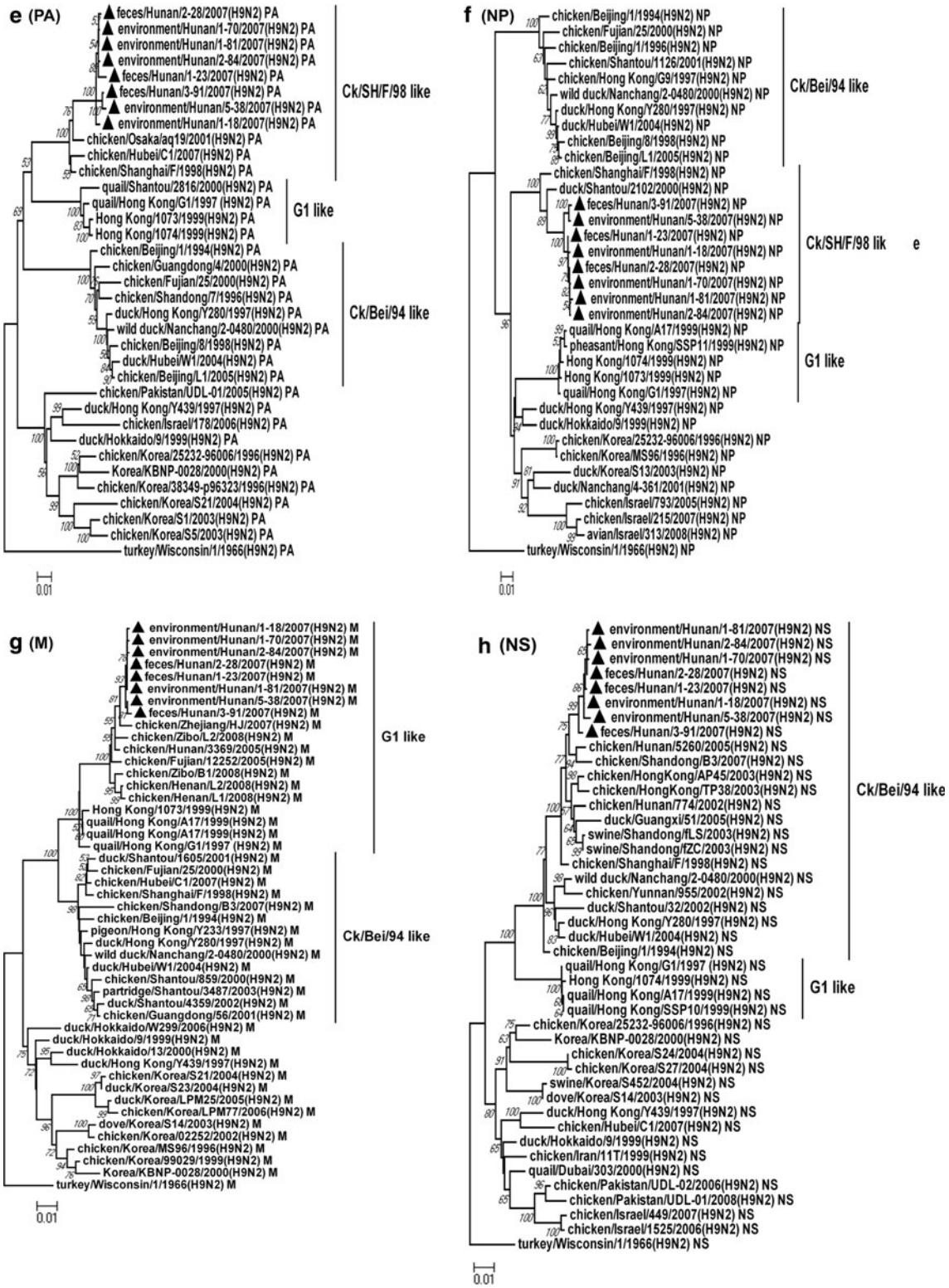

Fig. 1 continued

37.5% (3/8), respectively (Table 4), which indicates that none of the viruses met the criteria for highly pathogenic viruses.

When the mice were inoculated with the selected viruses at a dose of $10^{6.5}$EID$_{50}$, none of the mice showed overt clinical signs after inoculation. The inoculated mice

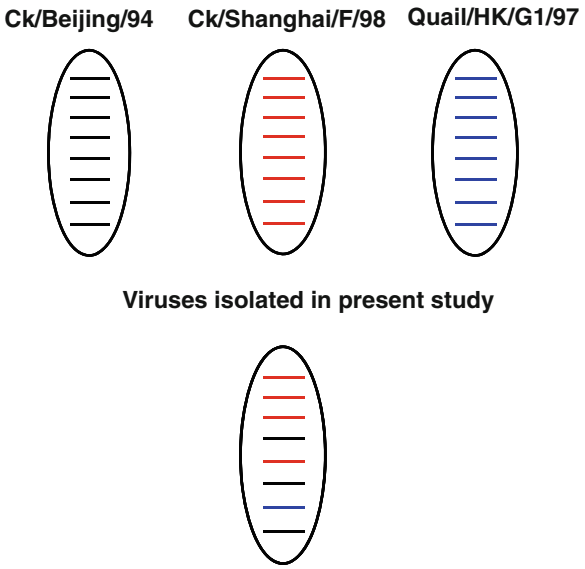

Fig. 2 Genotype of the viruses isolated in this study. The eight gene segments in each schematic virus particle (*from top to bottom*) are the PB2, PB1, PA, HA, NP, NA, M, and NS genes. Genes of the same sublineage are shown in the same color. *Capital letters* indicate genotypes. *Ck* chicken; *HK* Hong Kong

showed slight loss of body weight after infection, which was then restored to normal. The mice were killed on day 5 p.i., and their lungs, brain, spleen and kidneys were removed for virus titration. The viruses only replicated in the respiratory system (lungs) because no virus was detected in other organs (Table 5).

The mouse 50% minimal lethal dose (MLD_{50}) of each selected viruses was calculated by the method of Reed and Muench. The viruses isolated in the present study have a $MLD_{50}>10^{6.5}EID_{50}$ (Table 5), so they were all classified as having low pathogenicity to mice [27]. During the investigation of MLD_{50}, some mice were found dead that had been inoculated with the viruses environment/Hunan/1-18/2007(H9N2) and environment/Hunan/2-84/2007(H9N2) at a dose of approximately $10^{9.0}EID_{50}$.

Antigenic analysis

Antisera were collected on day 21 p.i. for antigenic analysis. We investigated the cross-reactivity of all of the isolated viruses by HI assay, in accordance with the instructions of OIE, using antisera to the selected four

Table 3 Comparison of amino acid sequences of HA, NA, and M2 genes of virus strains isolated in the present study

Virus	Residue at RBS[a]		Linker peptide	NA deletion (aa)	Residue at sites 27 and 31 of M2	
	226	228			27	31
Environment/HN/1-18/07	L	G	RSSR/G	63–65	V	S
Feces/HN/1-23/07	L	G	RSSR/G	63–65	V	S
Environment/HN/1-70/07	L	G	RSSR/G	63–65	V	S
Environment/HN/1-81/07	L	G	RSSR/G	63–65	V	S
Feces/HN/2-28/07	L	G	RSSR/G	63–65	V	S
Environment/HN/2-84/07	L	G	RSSR/G	63–65	V	S
Feces/HN/3-91/07	L	G	RSSR/G	63–65	V	S
Environment/HN/5-38/07	L	G	RSSR/G	63–65	V	S

[a] Numbering according to H3 HA

Table 4 Pathogenicity and replication of H9N2 influenza viruses in chickens

Virus	Virus titer ($log_{10}EID_{50}$)	Virus isolation from swabs	No. of birds shedding virus/total survivors on days 3,5 and 7 p.i.			No. of survivors on day 10/total	No. of seroconverted birds/total survivors[a]
			3	5	7		
Environment/HN/1-18/07	8.7	Trachea	7/7	5/5	0/3	3/8	3/3
		Cloaca	7/7	0/5	0/3		
Environment/HN/1-70/07	7.7	Trachea	5/5	5/5	3/5	5/8	5/5
		Cloaca	1/5	1/5	0/5		
Environment/HN/2-84/07	9.0	Trachea	4/4	3/3	0/3	3/8	3/3
		Cloaca	1/4	0/3	0/3		
Feces/HN/3-91/07	7.5	Trachea	5/5	5/5	2/5	5/8	5/5
		Cloaca	3/5	1/5	0/5		

One group of eight six-week-old specific-pathogen-free white leghorn chickens were inoculated intravenously with 0.2 ml of 1:10-diluted virus stock and observed for 10 days after infection

[a] Sera were harvested 3 weeks after infection, and seroconversion was confirmed by agar gel precipitin and HI test

Table 5 Replication of H9N2 influenza viruses in mice

Virus	Weight loss (%) 3 days p.i.	Virus titers in organs of mice ($log_{10}EID_{50}$/ml ± SD)				MLD_{50} ($log_{10}EID_{50}$)
		Lung	Spleen	Brain	Kidney	
Environment/HN/1-18/07	5.7 ± 0.8	4.2 ± 0.6	–[a]	–[a]	–[a]	>6.5
Environment/HN/1-70/07	5.9 ± 0.8	3.9 ± 0.2	–[a]	–[a]	–[a]	>6.5
Environment/HN/2-84/07	6.0 ± 1.6	4.9 ± 0.4	–[a]	–[a]	–[a]	>6.5
Feces/HN/3-91/07	7.0 ± 2.0	4.1 ± 0.5	–[a]	–[a]	–[a]	>6.5

Six-week-old BALB/c mice were infected intranasally with $10^{6.5}EID_{50}$ of the viruses. Organs were collected on day 5 after infection, and clarified homogenates were titrated for virus infectivity in 10-day-old SPF embryonated chicken eggs

[a] Virus was not detected in these samples

Table 6 Antigenic analysis of H9N2 influenza viruses

Virus	HI titers with post-infection antisera[a]			
	Environment/HN/1-18/07	Environment/HN/1-70/07	Environment/HN/2-84/07	Feces/HN/3-91/07
Environment/HN/1-18/07	2560[b]	1280	2560	1280
Feces/HN/1-23/07	1280	1280	2560	1280
Environment/HN/1-70/07	1280	2560[b]	2560	1280
Environment/HN/1-81/07	2560	2560	2560	2560
Feces/HN/2-28/07	1280	1280	2560	1280
Environment/HN/2-84/07	2560	2560	2560[b]	2560
Feces/HN/3-91/07	1280	1280	2560	1280[b]
Environment/HN/5-38/07	1280	1280	2560	1280

[a] Antisera were first diluted tenfold. HI titers represent the reciprocal of the dilution that resulted in complete inhibition of agglutination of 0.5% chicken RBCs

[b] HI titers for matching antisera

viruses isolated in the present study. The antisera to the selected four viruses could cross-react well with all of the viruses isolated in this study (Table 6), which indicated that there was no antigenic variation of the epidemic H9N2 viruses in the Dongting Lake region during the study period.

Discussion

H9N2 AI viruses have been isolated from waterfowl in North America and migratory ducks in Japan [5, 28], which indicates that migratory birds carry H9N2 viruses during migration. The Dongting Lake wetland is an important overwintering area along the migratory route of East Asian migratory birds, where mixed and scattered raising of chickens and ducks is popular, and migratory birds and domestic ducks often share the same water. The migratory birds that carry AI viruses might release viruses into the environment along their migratory route. After the birds leave, non-biotic environmental factors play important ecological roles in the transmission of AI viruses, for which the body of water is considered to be a virus stock [29]. Recently, genomes of AI viruses have been detected in precipitates in the water of the habitats of migratory birds, which are believed to cause infection in birds [29, 30]. Other studies have also shown that AI viruses are stable below 17°C, pH 7.4–8.2, and low salt concentration [14]. Webster et al. also have suggested that waterways are an important transmission route of AI viruses between different species of birds [17, 18].

In the present study, viruses isolated from water samples in Dongting Lake wetland and fecal samples in the surrounding environment belonged to the same genotype, and the nucleotide sequences of the genomes had high homology. This indicates that the body of water might play an important role in the perpetuation and spread of H9N2 AI viruses. Ck/Bei/94-like viruses have been introduced into various species of fowl [11] and have become established in terrestrial poultry in Mainland China, and Ck/Bei/94-like viruses might have been transmitted back to domestic ducks [31, 32]. Two-way interspecies transmission exists between different types of poultry and has been observed in aquatic and terrestrial birds in southern China, i.e., transmission from aquatic to terrestrial birds and vice versa [31]. Two-way interspecies transmission of H9N2

viruses might also occur in the Dongting Lake region. Influenza virus might be transmitted from chickens to domestic ducks because of mixed raising. The viruses could be released into the water by infected ducks, resulting in infection with chicken-derived virus of wild waterfowl that use the same area of water. Alternatively, viruses could be carried there by wild waterfowl during migration and then released into the water, resulting in infection with migratory-bird-derived virus of domestic ducks that share the same water with the wild aquatic birds. The infected domestic ducks could then transmit the viruses to chickens. However, there is a lack of evidence of H9N2 virus infection in migratory birds and domestic aquatic fowl in this region. It is difficult to confirm the origin or natural host of the H9N2 viruses isolated from water samples in the present study, but it is important that the viruses were successfully isolated from the water area contaminated by domestic or wild aquatic fowl. Therefore, these results at least show that the waterway might play an important role in the transmission of AI viruses between domestic fowl and migratory birds.

Since the 1990s, G1-like and Ck/Bei/94-like viruses have been prevalent mainly in quails and chickens in southern China [9, 11, 24]. Ck/SH/F/98-like viruses are a special type of virus in which the HA, NA, M and NS genes are Ck/Bei/94-like, while the PB2, PB1, PA and NP genes are neither Ck/Bei/94-like nor G1-like, and form an independent Ck/SH/F/98-like clade in the phylogenetic tree [28]. All of the PB2, PB1, PA and NP genes of viruses isolated in the present study were Ck/SH/F/98-like, and the HA, NA and NS genes belonged to the Ck/Bei/94-like genotype. The M gene is involved in host range restriction of AI viruses [33, 34]. Both human H5N1 and H9N2 influenza viruses isolated in Hong Kong contain internal genes provided by a G1-like virus [9]. The H9N2 viruses isolated in the present study obtained their M gene segment from a G1-like virus by reassortment, but the significance of this genetic reassortment with respect to the determinants of influenza virus pathogenicity and the ability of the virus to cross species barriers to mammalian hosts needs to be studied further.

All the H9N2 viruses isolated in the present study were generated by gene reassortment of Ck/Bei/94-like, Ck/SH/F/98-like and G1-like viruses, but all of the isolates belonged to the same genotype. During the study, which lasted for nearly a year, viruses of this genotype were continuously isolated in this area, indicating that the viruses of this genotype might have become predominant in this area and that they were genetically stable to a certain degree. It also suggests that the domestic poultry and migratory birds of Dongting Lake wetland might be infected with viruses belonging to the same genotype. The AI viruses might be transmitted between domestic fowl and migratory birds in Dongting Lake wetland, and the ecology of this area might play an important role in the transmission process. However, whether the H9N2 viruses isolated in this study are predominant in other areas outside the Dongting Lake wetland needs the support of further data from molecular epidemiological investigations. In an influenza virus surveillance program of a live-fowl market in Hong Kong in 2003, H9N2 viruses of genotypes A, B, C, D, E and F were isolated within only 4 months [35], which might have been caused by reassortment of various virus strains carried there by live fowl transported from different areas. Compared to the multiple genotypes that were detected in a comparatively short-term influenza virus surveillance program in Hong Kong [35], there was only one genotype isolated in the Dongting Lake region in our study, suggesting that these viruses are genetically stable and not a transient gene constellation.

According to the recommendations of OIE, eight SPF chickens were inoculated intravenously with 0.2 ml of a 1:10 dilution of bacteria-free allantoic fluid that contained virus, and if more than six chickens died within 10 days after infection, the virus was classified as highly pathogenic. The viruses isolated in the present study caused 62.5% (5/8) mortality in chickens at most, and therefore failed to meet the criteria for a highly pathogenic influenza virus. The H9N2 viruses isolated in recent years have not induced signs of disease or caused death of inoculated chickens [6, 36], but Guo et al. have reported that the Chicken/Beijing/1/94 virus caused a mortality rate of 40% in 6-week-old and 80% in 12-week-old SPF chickens [24]. It should be noted that all of the viruses had an additional basic amino acid (–R–S–S–R) at the connecting peptide of their HA, like most of the H9N2 viruses isolated from chickens in the mainland of China. A single additional basic amino acid substitution in the connecting peptide could probably convert the virus to a highly pathogenic strain [24]. Notably, the viruses isolated in the present study also caused death in 6-week-old SPF chickens, which suggests that these isolates could cause great losses to the poultry industry.

Guo et al. also have found that some H9N2 viruses cause death in mice [24]. In the present study, although the mice inoculated with $10^{6.5}EID_{50}$ viruses showed slight loss of body weight, neither virus caused death during the 14-day experiment. However, viruses were detected in the lungs of the inoculated mice, indicating that the isolated virus was able to replicate in murine lungs without prior adaptation. In addition, when the mice were inoculated with the viruses environment/HN/1-18/2007 (H9N2) and environment/HN/2-84/2007(H9N2) at a dose of approximately $10^{9.0}EID_{50}$, some mice were found dead, and a 20% body weight loss was caused within the first 5 days p.i. It should be noted that all of the viruses isolated in the present

study possessed 226-L (numbered according to H3) at the receptor-binding site, and the human H9N2 isolate also possessed 226-L, suggesting its potential to infect humans.

In our study, H9N2 viruses that belonged to the same genotype were isolated from water and fecal samples in Dongting Lake wetland, which implied that fowl were probably infected with AI viruses via a route that involved feces-contaminated water. To prevent the transmission of AI viruses in domestic poultry and to interrupt the two-way transmission between different poultry species, poultry should be reared in bird-proof confinement, or at least be fenced, and chickens and ducks should be raised separately. In addition to establishing good biosecurity measures on poultry farms, regular environmental disinfection, including decontamination of birds' feces, is also important for preventing AI virus transmission. On the other hand, strengthening influenza epidemiological monitoring of the water and surrounding environment of Dongting Lake wetland is of major significance for research on transmission of AI viruses between migratory birds and domestic fowl and is helpful for taking more effective measures to prevent and control AI in this area and its neighboring region.

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