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Characterization of H5N1 Influenza Viruses Isolated from Migratory Birds in Qinghai Province of China in 2006

Fumin Lei,[AF] Shuang Tang,[BF] Delong Zhao,[A] Xiaowei Zhang,[C] Zheng Kou,[B] Yongdong Li,[B] Zhong Zhang,[B] Zuohua Yin,[A] Shengliang Chen,[B] Sandan Li,[D] Dehai Zhang,[D] Baoping Yan,[E] and Tianxian Li[BG]

[A]Institute of Zoology, Chinese Academy of Sciences, Beijing, 100080, People's Republic of China
[B]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430071, People's Republic of China
[C]Beijing Genomics Institute, Chinese Academy of Sciences, Beijing, 101300, People's Republic of China
[D]Forestry Administration of Qinghai Province, Xining, 810007, People's Republic of China
[E]Information Center of Computer Network, Chinese Academy of Sciences, Beijing, 100080, People's Republic of China

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SUMMARY. Avian influenza H5N1 viruses pose a significant threat to human health because of their ability to infect humans directly. In the paper, three highly pathogenic H5N1 influenza viruses were isolated from three species of migratory birds in Qinghai Province of China in 2006. The analysis of the genome sequences indicated that the three isolates shared high homology with each other (94% to 99%). Three isolates shared a common ancestor and were closest to strains isolated from Qinghai and Siberia in 2005, but distinct from poultry viruses found in Southeast Asia. In experimental infection, all three viruses were highly pathogenic to chickens and mice. The results suggest that highly pathogenic avian influenza H5N1 viruses still exist in the migratory birds and could spread to other regions with wild bird migration.

RESUMEN. Caracterización de virus H5N1 de influenza aviar aislados de aves migratorias en la provincia de Qinghai en China en el año 2006.

Los virus de influenza aviar tipo H5N1 son una amenaza significante para la salud humana debido a su capacidad de infectar directamente a los humanos. En este trabajo, se aislaron tres virus de influenza aviar H5N1 altamente patógenos a partir de tres especies de aves migratorias en la provincia de Qinghai en China en el año 2006. El análisis de las secuencias del genoma indicó que los tres aislados compartían altos porcentajes de homología entre ellos (94% a 99%). Los tres aislados compartían un ancestro común y estaban muy cercanos a las cepas aisladas en Qinghai y en Siberia en el año 2005, pero fueron diferentes a los virus aislados de aves domésticas en el Sureste de Asia, En la infección experimental, todos los tres virus fueron altamente patógenos para pollos y ratones. Los resultados sugieren que los virus de influenza aviar H5N1 de alta patogenicidad aún existen en aves migratorias y pueden diseminarse a otras regiones mediante la migración de aves silvestres.

Key words: influenza virus, H5N1 subtype, migrating birds

Abbreviations: EID_{50} = 50% egg infectious dose; HA = hemagglutinin glycoprotein; HPAI = highly pathogenic avian influenza; M = matrix protein; NA = neuraminidase glycoprotein; NP = nucleocapsid protein; NS = nonstructure protein; ORF = open reading frame; PA = polymeras acid protein; PB1 = polymeras basic protein 1; PB2 = polymeras basic protein 2; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; SPF = specific-pathogen-free

Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype continue to be epidemic in Asia since their appearance in 1996 and have spread to Europe and Africa by migratory birds recently, which have posed a threat to poultry and public health (3,5,6,7,8,14,15,21,23). Wild birds have been considered as the natural pool for avian influenza viruses (1,2,13,18,20,24). In May 2005, more than 6000 migratory birds infected by H5N1 virus died at Qinghai Lake, the nature reserve in central China. It was the first observation of sustained transmission within migratory waterfowls (4,16).

In the paper, we described the isolation of three HPAI viruses of the H5N1 subtype from three species of migratory birds in the Qinghai Province of China during 2006. All the viruses were sequenced and their pathogenicity to chickens and mice were tested. The results indicated that they are the closest to strains isolated from Qinghai and Siberia in 2005. HPAI H5N1 viruses still exist in migratory birds and could spread likely to other regions with wild bird migration.

MATERIALS AND METHODS

Virus sampling. We picked out 93 fresh fecal samples from apparently healthy migratory waterfowls of different species (34 from bar-headed geese [*Anser indicus*], 12 from great brown-headed gulls [*Larus brunnicephalus*], 19 from whooper swans [*Cygnus Cygnus*], and 28 from great black-headed gulls [*Larus ichthyaetus*]) and tissue samples gathered from one dead whooper swan and one dead great brown-headed gull, in habitable wetlands in the Gangca and Maduo districts in Qinghai Province from April 3, 2006, to April 20, 2006.

Virus isolation and characterization. The 93 fecal samples were diluted with 0.5 to 1 ml of 10% w/v phosphate-buffered saline (PBS). After centrifugation at 6000 × *g*/min for 5 min at 4 C, the supernatant was mixed with an equal volume of PBS containing antibiotics (penicillin G, 40,000 IU/ml; streptomycin sulfate, 8000 IU/ml) for 4 hr at 4 C, and inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated eggs (Merial Ltd. Co., Beijing, China). After incubation at 37 C for 24 to 72 hr, the allantoic fluid of the inoculated eggs was collected. Additionally, samples of multiple organs (lung, spleen, liver, and intestine) of two dead birds (whooper swan, great brown-headed gull) were collected and mixed with an equal volume of PBS containing antibiotics. After tissue homogenizations were done by tapered tissue grinders, the samples were centrifuged to collect supernatants, which were then inoculated into the

[F]These authors contributed equally to this work.
[G]Corresponding author. Email: Litx@wh.iov.cn

Table 1. Percent sequence similarity between the migrant waterfowl isolates and other influenza viruses.[A]

Segment[B]	Viruses with the highest similarity	% Similarity
PB2	A/Great black-headed gull/Qinghai/1/05 (H5N1)	99
PB1	A/*Cygnus olor*/Astrakhan/Ast05-2-3/05(H5N1)	99
PA	A/*Cygnus olor*/Astrakhan/Ast05-2-7/05(H5N1)	99
HA	A/*Cygnus olor*/Astrakhan/Ast05-2-10/05(H5N1)	99
NP	A/goose/Krasnoozerka/627/05(H5N1)	99
NA	A/bar-headed goose/Qinghai/0510/05 (H5N1)	99
M	A/bar-headed goose/Qinghai/0510/05 (H5N1)	99
NS	A/black-headed gull/Qinghai/1/05 (H5N1)	99

[A]Percent sequence similarity was calculated based on the nucleotide sequences of the complete open reading frame (ORF) of eight genes with GBHGull/QH/3/06 as the representative strain. The nucleotide sequences of GBHGull/QH/3/06 were compared to those in GenBank.

[B]PB2=polymeras basic protein 2; PB1=polymeras basic protein 1; PA=polymeras acid protein; HA=hemagglutinin glycoprotein; NP=nucleocapsid protein; NA=neuraminidase glycoprotein; M=matrix protein; NS=nonstructure protein.

allantoic cavities of 10-day-old SPF embryonated eggs. After incubation at 37 C for 24 to 72 hr, the allantoic fluid was harvested 3 days after injection, and influenza A virus was detected by using hemagglutination assays with rooster erythrocytes. When no influenza A virus was detected on the initial virus isolation attempt, the allantoic fluid was passaged once more in embryonated eggs. Virus isolates were characterized with a hemagglutination inhibition assay with rooster erythrocytes and the influenza A virus subtype-specific antiserum were from Animal Influenza Laboratory of the Ministry of Agriculture China. The hemagglutination assays and hemagglutination inhibition tests as described previously. Fifty percent egg infectious dose (EID_{50}) titers were calculated using the method of Reed–Muench for the allantoic fluid. All allantoic fluids containing virus stocks were stored at −70 C before being used.

RNA extraction and nucleotide sequencing. Viral RNA was extracted from virus-infected allantoic fluids by Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using a one-step RNA PCR kit (Takara Bio, Dalian, China) to amplify the viral genes. PCR primers used in this study were described by Hoffmann *et al.* (11). The amplified DNA products were run on a 1.0% agarose gel and pieces of gel containing DNA bands of the expected sizes were cut out and purified using a gel extraction kit (Takara Bio). After purification, the PCR products were sequenced using the Amersham ET Dye Terminator kit (Amersham Pharmacia Biotech, Piscataway, NJ) and a ABI PRISM 370 DNA sequencer (PE Applied Biosystems, Foster City, CA). All sequence data were edited with BioEdit (version 5.0.9) and aligned with Clustal X (version 1.8). Phylogenetic trees were generated by MEGA (version 3.1).

Pathogenicity tests. To determine the pathogenicity of the isolated virus, it was inoculated into chickens and mice.

Chickens. Groups of eight 6-wk-old SPF chickens (Merial) were tested according to the recommendations of the World Organization for Animal Health. Each chicken was intravenously injected with 0.2 ml of a 1:10 dilution of allantoic fluid and mortality was observed over a 10-day period. The inoculated viral doses of the allantoises fluid were $10^{6.5}$ to $10^{7.0}$ EID_{50}.

Mice. Groups of 10 6-to-8-wk-old female BALB/c mice (Experimental Animal Center of Hubei Control Disease Center, Wuhan, China) were inoculated intranasally with 10^6 EID_{50} of the virus in a volume of 50 μl and were observed daily for 14 days for signs of disease. On the third day, two mice of each group were sacrificed, and the EID_{50} of the viruses in the lungs and brains were determined. The remaining mice were monitored daily for mortality. Control groups of chickens and mice were inoculated with PBS. The inoculated viral doses of the allantoises fluid were $10^{6.5}$ to $10^{7.0}$ EID_{50}. All the animal experiments were performed in a biosafety level 3 laboratory.

Histopathological studies. Tissue samples from chicken and mice infected by one isolate (Swan/QH/01/06) were collected. The samples were fixed in 4% (v/v) formaldehyde in PBS and dehydrated by embedding in paraffin. Serial sections were prepared and stained with hematoxylin and eosin (H&E) solution for detection of viral pathological changes by light microscopy.

Nucleotide sequence accession numbers. All sequences have been deposited in GenBank. The accession numbers are DQ822542–DQ822565.

RESULTS

Virus isolation and molecular characterization. Three avian influenza H5N1 viruses were isolated from three species of migratory birds: one from the feces of the bar-headed goose (*A. indicus*) and two from tissue samples of one dead whooper swan (*C. cygnus*) and one dead great brown-headed gull (*L. ichthyaetus*). All three isolates were identified to be highly pathogenic H5N1 influenza viruses. The three H5N1 influenza isolates were named A/Bar-headed Goose/Qinghai/F/2006(H5N1) (BHGs/QH/F/06), A/blackheaded gull/Qingha/3/2006(H5N1) (GBHGull/QH/3/06), and whooperswan/Qinghai /01/2006(H5N1) (Swan/QH/01/06). Nucleotide sequence analysis revealed that three isolates shared a high homology with each other (99%) except for the PB1 genes of BHGs/QH/F/06 and Swan/QH/01/06, which shared 94% identity. The results of genome sequencing analysis of the three virus isolates suggested that they were closed to strains isolated from Qinghai and Siberia in 2005 (Table 1).

The hemagglutinin (HA) genes of the three isolates had the same multiple amino acid sequence (-RRRKKR-) at the connecting peptide between HA1 and HA2, which is considered to be a distinguishing characteristic of highly pathogenic influenza viruses in chickens (12). All amino acids relevant to receptor binding (amino acids 99, 130–134, 149, 151, 186, 190–191, and 220–225) were identical to that of Gs/Gd/1/96 (9).The three virus isolates also had a 20-amino-acid deletion in the neuraminidase (NA) stalk (residues 49 to 68), but did not have mutations at the amino acids (Leu26, Val27, Ala30, Ser31) in the transmembrane region of the M2 protein, which has been suggested to be associated with amantadine resistance (22). All viruses had a 5-amino-acid deletion in the middle of the NS1 molecule. The three isolates did not have a mutation of Glu92 in the NS1 protein, and had acquired a Lys residue at position 627 of PB2 protein, which has been suggested to be associated with increased virulence of H5N1 viruses (10,19). Additionally, the three virus isolates had a PDZ domain ligand at the C terminus of NS1 (ESEV–COOH), which plays an important role in many key signaling pathways of viral replication (17).

As shown in Fig. 1a, the phylogenetic trees of HA gene revealed that three distinct branches were formed: one included viruses isolated from Siberia and Qinghai in 2005; the second included viruses isolated from Vietnam, Thailand, and Malaysia; and the third included viruses isolated from Indonesia, Yunnan, and Guangxi (24). The phylogenetic analysis of the other seven genes

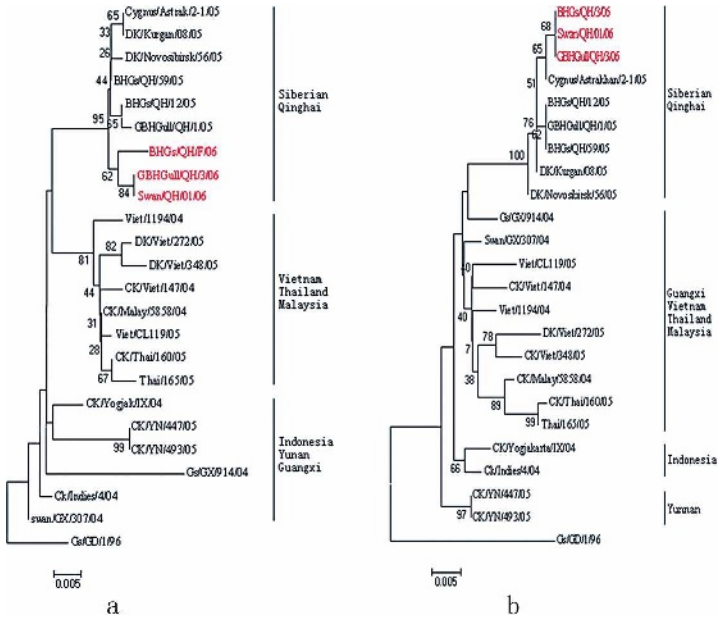

Fig. 1. Phylogenetic trees of the HA and PB1 genes of strains isolated from migratory birds in Qinghai Province, China. Trees were generated by using neighbor-joining analysis with the Tamura–Nei gamma model in the MEGA program (version 3.1). Numbers below branches indicate bootstrap value percent from 1000 replicates. Analysis was based on nucleotides 51–1330 (1279 bp) of the HA gene (a), 53–1510 (1457 bp) of the PB1 gene (b). Scale bar represents the distance unit between sequence pairs. Abbreviations: Thai = Thailand; Viet = Vietnam; Indies = Indonesia; Malay = Malaysia; GD = Guangdong; YN = Yunnan; QH = Qinghai; GX = Guangxi; Ck = chickens; Dk = ducks; GBHGull = black-headed gulls; BHGs bar-headed geese; Gs = geese. The sequences are named in concordance with their GenBank nomenclature. The genes studied in this paper are marked in red.

were consistent with those for the HA gene and revealed that all eight gene of the three Qinghai virus isolates formed a branch in the phylogenetic tree with the Qinghai and Siberia isolates from 2005 (Fig. 1b, only PB1 was shown).

Pathogenicity tests. The three virus isolates were inoculated into chicken and mice. As shown in Table 2, all the isolates killed 7/8 to 8/8 of infected chickens within 6 days. According to the criteria of World Organization for Animal Health, these isolates were highly pathogenic to chicken. In mouse experiments, no deaths of the mice were observed for 3 days after viruses were inoculated with $10^{6.5}$–$10^{7.0}$ EID_{50} (Table 2). Although the viruses did not kill all the mice, 5/8 to 7/8 of infected mice were killed within 9 days and virus could be detected from lungs and brains of the dead mice (Table 2). These isolates were pathogenic to mice.

Histopathologic slices were taken from the lungs and liver of one chicken and the lung and brain of one mouse. The chicken and mouse were euthanatized 3 days after inoculated with Swan/QH/01/06. Lung tissue from the chicken (Fig. 2a) and the mouse (Fig. 2b) showed pneumonia with hyperplasia of fibroblasts, congestion and expansion of blood vessels, and cytoclasis. The brain tissue from the mouse (Fig. 2c) showed apparent tumefaction in the hippocampus sections of brain cells, and congestion of blood vessels. The liver from the chicken (Fig. 2d) showed a balloon-like swelling of the cells.

DISCUSSION

Qinghai Lake, located in Northwest China and on the Central Asia flyway is an important breeding site for migratory birds (3,16). During May to June 2005, a serious epidemic of highly pathogenic H5N1 avian influenza broke out in the immediate Qinghai Lake region, and led to the death of more than 6000 migratory birds (4,16). In late July 2005, the highly pathogenic H5N1 avian influenza virus spread geographically beyond its original location in Asia to affect poultry and wild birds in Russia and adjacent parts of Kazakhstan (22). We conducted a study of avian influenza virus on migratory birds in Qinghai in April 2006. Three influenza viruses were isolated from three species of migratory birds and were identified as highly pathogenic H5N1 influenza viruses. In experimental infection, all three viruses were pathogenic to chicken and mice.

Nucleotide sequence and phylogenetic analysis revealed that the three Qinghai viruses were very similar to each other and were closest to isolates obtained from Qinghai and Siberia in 2005, but distinct

Table 2. The results of animal infection experiments.

		Chicken infection	Mouse infection[A]		
Virus	Titer, $\log_{10}$ EID_{50}[B]	Number dead/number inoculated (days to death)	Number dead/number inoculated (days to death)	Virus in lung, $\log_{10}$ $EID5_0$	Virus in brain, $\log_{10}$ EID_{50}
GBHGull/QH/3/6	6.5	8/8(2–5)	7/8(4–8)	4.7	4.2
BHGs/QH/F/06	6.7	7/8(2–4)	5/8(5–7)	5.0	4.0
Swan/QH/01/06	7.0	8/8(2–6)	6/8(4–9)	5.3	3.1

[A]On the third day, two mice of each group were sacrificed, and the EID_{50}'s of the viruses in the lungs and brains were determined.
[B]The inoculated viral doses of the allantoic fluids were 106.5 to 107.0 EID_{50} for chickens and mive, respectively.

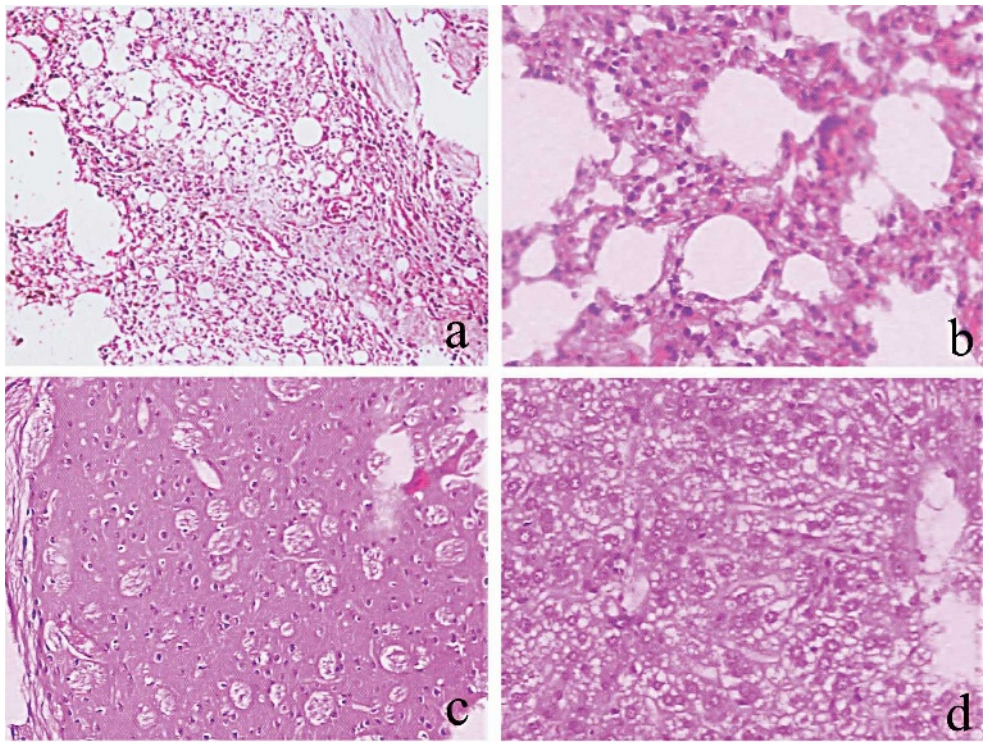

Fig. 2. Histopathology slices from the tissue of chickens and mice. The lung tissue infected for 3 days showed fibroblasts, expansion and congestion of blood vessels. ([a] chicken; [b] mouse). The brain tissue showed apparent tumefaction in the hippocampus sections of brain cells, and congestion of blood vessels ([c] mouse). The liver showed a reduction in the number of cells ([d] chicken). H&E. (a) 200×; (b, c, d) 400×.

from other poultry viruses found in Southeast Asia. Additionally, the data demonstrate that viruses isolated from this region can spread to other regions via wild bird migration.

The Qinghai territory is one of the most important breeding locations for migratory birds wintering in Southeast Asia, Tibet, and India (4,16,22). During the field investigation, the whooper swan, the bar-headed goose, and the great brown-headed gull were found sharing the same microhabitat in a freshwater area, which may facilitate the gene exchange of viruses among these species. The whooper swan overwinters at Qinghai Lake but breeds in Siberia, whereas the bar-headed goose and great brown-headed gull breed in Qinghai but overwinter in Thailand, Indonesia, Malaysia, and the Guangdong and Yunnan provinces of China. Breeding birds in Qinghai Lake may spread the viruses northward to the new breeding site through migration by overwintering birds (eg., whooper swan). Conversely, the whooper swan may also carry viruses from its breeding site to its wintering site (e.g., Qinghai Lake).

It was also suggested that viruses isolated from Qinghai Province could spread to other regions via wild bird migration. Viruses could be undergoing reassortment in Qinghai, at the intersection of two flyways (from Siberia to Southeast Asia and from Siberia to India). Countries that lie along the flight pathways of birds migrating from central Asia may face a persistent risk of virus transmission. This finding emphasizes the importance of the surveillance and control of the virus along migrating bird flyways.

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