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Highly Pathogenic Avian Influenza A(H5N1) Virus Struck Migratory Birds in China in 2015

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Approximately 100 migratory birds, including whooper swans and pochards, were found dead in the Sanmenxia Reservoir Area of China during January 2015. The causative agent behind this outbreak was identified as H5N1 highly pathogenic avian influenza virus (HPAIV). Genetic and phylogenetic analyses revealed that this Sanmenxia H5N1 virus was a novel reassortant, possessing a Clade 2.3.2.1c HA gene and a H9N2-derived PB2 gene. Sanmenxia Clade 2.3.2.1c-like H5N1 viruses possess the closest genetic identity to A/Alberta/01/2014 (H5N1), which recently caused a fatal respiratory infection in Canada with signs of meningoencephalitis, a highly unusual symptom with influenza infections in humans. Furthermore, this virus was shown to be highly pathogenic to both birds and mammals, and demonstrate tropism for the nervous system. Due to the geographical location of Sanmenxia, these novel H5N1 viruses also have the potential to be imported to other regions through the migration of wild birds, similar to the H5N1 outbreak amongst migratory birds in Qinghai Lake during 2005. Therefore, further investigation and monitoring is required to prevent this novel reassortant virus from becoming a new threat to public health.

The H5N1 subtype of highly pathogenic avian virus (HPAIV), initially identified during 1996 in China, infected 18 humans with 6 deaths during 1997 in Hong Kong¹. This virus was highly pathogenic in chickens and humans, and posed a significant threat to public health. As of 31 March 2015, H5N1 virus caused at least 826 laboratory-confirmed human infections, including 440 deaths across 16 countries². Direct or indirect contact with diseased poultry is the primary route of HPAIV infections in humans³. Despite measures to prevent HPAIV spread by vaccination or the culling of infected birds, several H5 influenza subtypes are already prevalent in Asia, Europe, and Africa⁴. Wild waterfowl are currently regarded as a

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natural reservoir for avian influenza viruses (AIV)^{5–8}, and contribute to the geographical spread of AIV via long-distance migration, before transmission to domestic poultry^{7,9–11}.

Apart from sporadic cases, widespread infections and deaths of wild birds from HPAIV infection have never been reported before 2005. However, a HPAIV of the H5N1 subtype killed thousands of bar-headed geese (*Anser indicus*), great black-headed gulls (*Larus ichthyaetus*), and brown-headed gulls (*Larus brunnicephalus*) in Qinghai Lake, China during May 2005^{9,12}. In 2006, a Qinghai-like Clade 2.2 virus re-emerged in Qinghai Lake and caused more infections in wild birds, including bar-headed geese and great black-headed gulls. The Qinghai-like Clade 2.2 virus was found to possess a high genetic relationship with viruses isolated from other countries on the migratory flyway of wild birds⁴, suggesting that the migration of wild birds played an important role in circulating H5N1 HPAIV viruses between the different avian populations. The virus eventually spread into other parts of Asia, as well as Europe and Africa, resulting in the deaths of millions of birds, and hundreds of cases in humans^{9,13–15,2}.

In 2009, another novel H5N1 virus (Clade 2.3.2) emerged and caused an outbreak resulting in the deaths of more than 200 wild birds, including great cormorants (*Phalacrocorax carbo*), brown-headed gulls, great black-headed gulls, great-crested grebes (*Podiceps cristatus*), bar-headed geese, ruddy shelducks (*Tadorna ferruginea*), and common coots (*family Rallidae*) in Qinghai Lake^{16,17}. Further analysis showed that the viruses belonged to Clade 2.3.2.1, which had first emerged in whooper swans (*Cygnus cygnus*) during 2008 in Japan^{16,17}. This virus subsequently re-emerged in Mongolia during 2009–2010 in the bar-headed geese, whooper swans, common goldeneyes (*Bucephala clangula*), and ruddy shelducks¹⁸; in Russia during 2009–2010 with the great-crested grebes and great black-headed gulls¹⁸; as well as in Korea in mallard ducks, and Japan including whooper swans, greater scaups (*Aythya marila*), pintails (*Anas acuta*), peregrine falcons (*Falco peregrinus*), tufted ducks (*Aythya fuligula*), common pochards (*Aythya ferina*), great crested grebes (*Podiceps cristatus*), tundra swans (*Cygnus columbianus*), ural owls (*Strix uralensis*), mandarin ducks (*Aix galericulata*), and goshawks (*Accipiter gentilis*) during 2010–2011^{13,19}. Interestingly, the novel Clade 2.3.2.1 virus circulated mainly in wild waterfowl and was only occasionally isolated in domestic poultry.

In January of 2015, the deaths of approximately 100 migratory wild birds (mainly whooper swans and pochards) in the Sanmenxia Reservoir Area of China were investigated. Located approximately 1150 kilometers (km) east of Qinghai Lake, this location is an important wintering habitat for whooper swans and various species of wild ducks including pochards (*Aythya ferina*) migrating to China from Mongolia and Siberia. The deaths sparked concerns of another HPAIV outbreak being spread by migratory wild birds.

To investigate this outbreak, samples were taken from sick and dead wild birds, and H5N1 virus was identified as the causative agent. In this study the gene evolution, phylogenetics, virulence to chickens and mammals, as well as receptor binding properties of these H5N1 viruses are presented, in addition to its potential spread along the migratory flyway of wild birds.

Results

Virus isolation. Samples were collected from sick or dead birds, in addition to the surrounding environment, in the Sanmenxia Reservoir Area of China on January 4th–5th, 2015. All samples were inoculated in 10-day-old, embryonated chicken eggs, which were found to have died within 24–41 hours after inoculation. Allantoic fluids tested positive by a hemagglutination (HA) assay with titers between 128–256 reciprocal dilutions. These suggested that this virus is highly pathogenic to poultry. H5N1 influenza virus was confirmed and Newcastle disease virus (NDV) was ruled out by HA inhibition (HI) assay and RT-PCR (specific primer sequences for both viruses are available from the corresponding author upon request). In addition, no bacteria were detected in the organs and tissues of the sick or dead birds when the samples were plated onto LB agar.

A total of 12 influenza A (H5N1) viruses were isolated, with ten isolates from whooper swans, one from a dead pochard, and one isolate from 12 environmental samples containing the feces of apparently healthy birds.

Genetic identity analysis. The H5 and N1 sequences of the 12 viruses were found to possess high genetic identities with each other. To avoid redundancy, six of 12 viruses (Tables 1 and 2) were selected to further analyze the gene evolution of the Sanmenxia H5N1 viruses isolated from wild birds. Genetic analysis showed that all eight gene segments of the novel H5N1 viruses shared between 99.3–100% nucleotide identity with each other.

Sequence alignments and BLAST search in the GISAID and NCBI Flu database showed that the genomes of the Sanmenxia H5N1 viruses showed the highest genetic homology to a previously described H5N1 virus (A/Alberta/01/2014, Alberta2014). Alberta2014 was responsible for causing the first human H5N1 infection in North America, and was likely an imported case from Asia²⁰. The nucleotide identities of the novel virus isolates with Alberta2014 ranged between 98.7 to 99.6%, and protein identities ranged between 98.2–100%. These new isolates also possessed high nucleotide (91.6–98.7%) and amino acid (97.6–100%) identities to another mammalian H5N1 strain (A/tiger/Jiangsu/01/2013, Tiger2013), which was isolated from a tiger that had died from respiratory failure in 2013²¹ (Table 1).

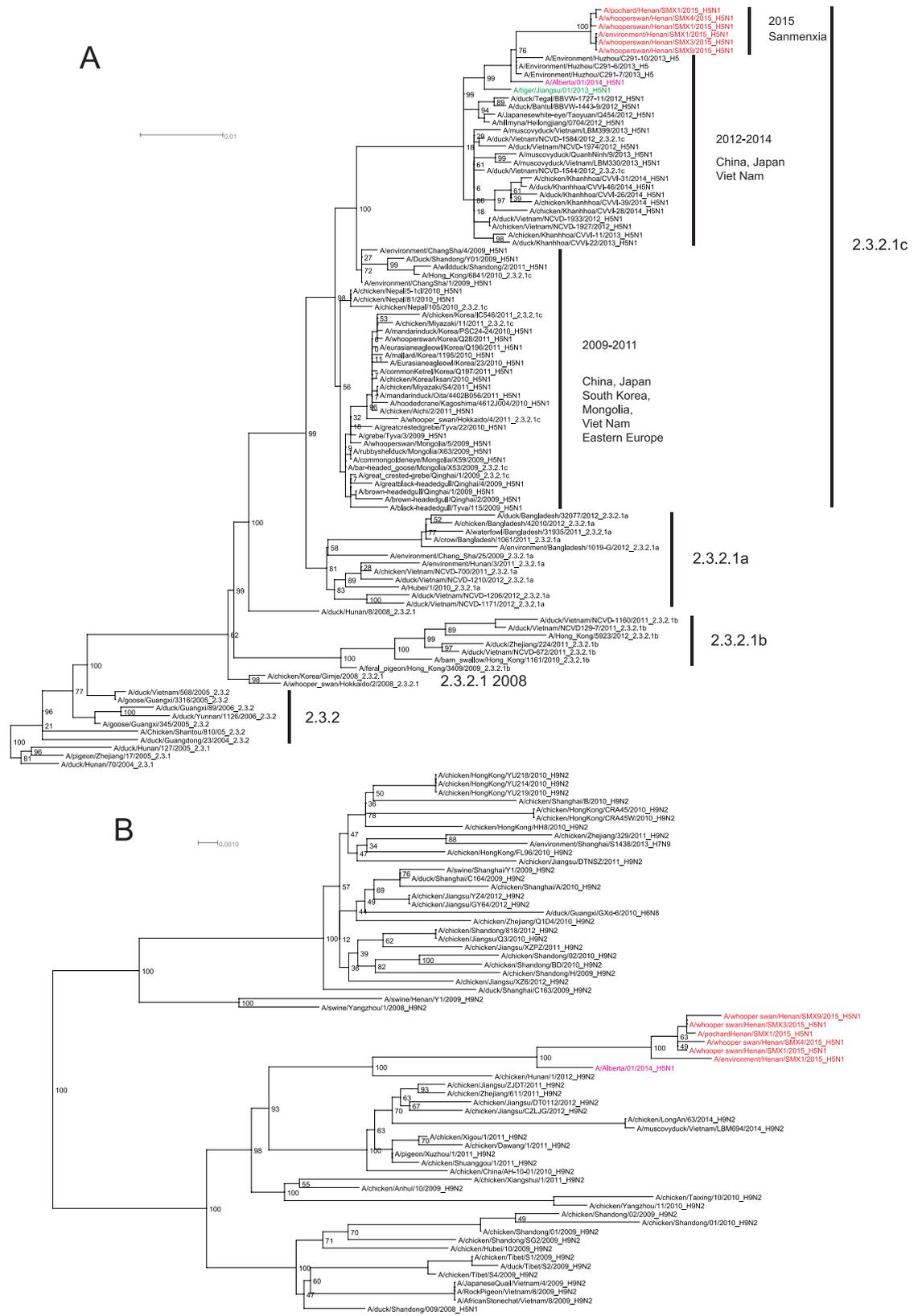


Figure 1. Phylogenetic analyses of the HA and PB2 gene sequences from Sanmenxia Clade 2.3.2.1c H5N1 isolates. The reference sequences were downloaded from the GISAID and GenBank database. All of the phylogenetic analyses were performed using Raxml, with 1000 bootstrap replicates. **(A)** phylogenies of Clade 2.3.2 HA gene for H5N1 HPAIVs. **(B)** phylogenetic tree constructed using the PB2 gene sequences. Segments of the whooper swan-H5N1, A/Alberta/01/2014(H5N1), and A/tiger/Jiangsu/01/2013(H5N1) viruses are colored in red, pink and green, respectively.

Virus ^a	Gene	Nucleotide (amino acid) identity (%)					
		Ws/HN/SMX1/15	Ws/HN/SMX3/15	Ws/HN/SMX4/15	Ws/HN/SMX9/15	Po/HN/SMX1/15	En/HN/SMX1/15
A/Alberta/01/2014	HA	98.8 (99.1)	98.8 (98.9)	98.8 (99.1)	98.8 (98.9)	98.7 (98.8)	98.8 (98.9)
	NA	98.8 (99.1)	98.8 (99.1)	98.8 (99.1)	98.8 (99.1)	98.8 (99.1)	98.8 (99.1)
	PB2	98.9 (98.9)	99.0 (90.0)	98.9 (98.9)	98.8 (98.5)	98.9 (99.0)	98.8 (99.0)
	PB1	99.0 (99.7)	99.0 (99.7)	99.0 (99.7)	99.0 (99.6)	99.0 (99.7)	99.0 (99.7)
	PA	99.3 (99.4)	99.4 (99.5)	99.3 (99.5)	99.4 (99.5)	99.3 (99.5)	99.4 (99.5)
	NP	99.3 (99.5)	99.1 (99.5)	99.3 (99.5)	99.2 (99.5)	99.2 (99.5)	99.2 (99.5)
	M	99.6 (M1:100, M2:100)	99.6 (M1:100, M2:100)	99.6 (M1:100, M2:100)	99.6 (M1:100, M2:100)	99.6 (M1:100, M2:100)	99.6 (M1:100, M2:100)
A/tiger/Jiangsu/01/2013	NS	99.0 (NS1:98.6, NS2:99.1)	98.9 (NS1:98.2, NS2:99.1)	NS2:99.1)	98.9 (NS1:98.2, NS2:99.1)	98.9 (NS1:98.2, NS2:99.1)	98.9 (NS1:98.2, NS2:99.1)
	HA	98.5 (98.9)	98.5 (98.7)	98.5 (98.9)	98.5 (98.7)	98.5 (98.7)	98.5 (98.7)
	NA	97.8 (98.4)	97.8 (98.4)	97.8 (98.4)	97.8 (98.4)	97.8 (98.4)	97.8 (98.4)
	PB2	91.8 (98.2)	91.8 (98.1)	91.6 (98.0)	91.6 (97.6)	91.8 (98.1)	92.0 (98.1)
	PB1	97.9 (98.9)	97.9 (98.9)	97.9 (98.9)	97.9 (98.8)	97.9 (98.9)	98.0 (98.9)
	PA	98.6 (98.7)	98.7 (98.8)	98.6 (98.8)	98.7 (98.8)	98.6 (98.8)	98.7 (98.8)
	NP	98.6 (99.5)	98.5 (99.5)	98.6 (99.5)	98.5 (99.5)	98.5 (99.5)	98.5 (99.5)
M	M	95.9 (M1:100; M2:98.9)	95.9 (M1:100; M2:98.9)	95.9 (M1:100; M2:98.9)	95.9 (M1:100; M2:98.9)	95.9 (M1:100; M2:98.9)	95.9 (M1:100; M2:98.9)
	NS	97.3 (NS1:98.2; NS2:98.3)	97.2 (NS1:97.7; NS2:98.3)	97.2 (NS1:97.7; NS2:98.3)	97.2 (NS1:97.7; NS2:98.3)	97.2 (NS1:97.7; NS2:98.3)	97.2 (NS1:97.7; NS2:98.3)

Table 1. Genetic identity (%) of the H5N1 viruses isolated from wild birds, humans, and tigers. ^aWs: whooper swan; Po: pochard; En: environment; HN: Henan.

Viruses ^a	Collection date	HA (H3 numbering)						PB2			NA		M2		PB1-F2
		Connecting peptide	110	158	160	224–228	318	591	627	701	274 ^b	Stalk deletion	27	31	58–90 truncated
A/Alberta/01/2014 ^c	2014.1.3	RERRRKR/G	H	D	A	NRQSG	T	Q	E	D	H	49–68	I	S	Yes
A/tiger/Jiangsu/01/2013 ^c	2013.1.20	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
Ws/HN/SMX1/2015	2015.1.4	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
Ws/HN/SMX3/2015	2015.1.4	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
Ws/HN/SMX4/2015	2015.1.5	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
Ws/HN/SMX9/2015	2015.1.5	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
Po/HN/SMX1/2015	2015.1.5	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes
En/HN/SMX1/2015	2015.1.5	RERRRKR/G	H	D	A	NGQSG	T	Q	E	D	H	49–68	I	S	Yes

Table 2. Molecular characterizations of the H5N1 viruses on HA, PB2, NA, M2 and PB1-F2. ^aWs: whooper swan; Po: pochard; En: environment; HN: Henan. ^bN2 numbering. ^cThe sequences of the reference strains are downloaded from GISAID and GenBank databases.

Phylogenetic analysis. To further analyze the genetic evolution of these novel H5N1 viruses, phylogenetic analysis of the whole genomes was performed with reference H5N1 strains. The HA gene of the new isolates was found to belong to Clade 2.3.2.1c (Figs 1A and S1). Notably, the topology of Clade 2.3.2.1c formed a “ladder-like” phylogenetic structure, and H5N1 strains causing the Sanmenxia outbreak formed a novel independent “ladder” (Fig. 1A). The other distinct “ladder-like” branches include the 2009–2011 sub-clade circulating in Asia and Eastern Europe, and the 2012–2014 sub-clade circulating in Eastern Asia. Therefore, Clade 2.3.2.1c has been circulating in Eastern Asia since 2009, undergoing a lineage replacement every 2–3 years. However, the gap between the sub-clade 2.3.2.1 in 2008 and the recent 2015 sub-clade is quite large, as indicated by branch length (Fig. 1A), but it is currently unknown whether these variations in gene sequences influence viral antigenicity/pathogenicity.

Consistent with results obtained from the comparison of sequence identities, isolates from the 2015 outbreak were most closely related to a H5 lineage circulating in China from 2013 in the HA phylogenetic

Virus ^a	Amino acid residue sites (H3 numbering)								
	77	193	225	246	260	397	475	509+	531
A/Alberta/01/2014	D	K	R	E	V	R	N	I	M
A/tiger/Jiangsu/01/2013	D	R	G	E	V	R	N	I	I
Ws/HN/SMX1/2015	S	K	G	E	I	K	N	V	M
Ws/HN/SMX3/2015	S	K	G	E	I	K	D	V	M
Ws/HN/SMX4/2015	S	K	G	E	I	K	N	V	M
Ws/HN/SMX9/2015	S	K	G	E	I	K	D	V	M
Po/HN/SMX1/2015	S	K	G	K	I	K	N	V	M
En/HN/SMX1/2015	S	K	G	E	I	K	D	V	M

Table 3. Different amino acid residues in the HA of the H5N1 viruses isolated from wild birds, humans and tigers. 509+: there is a gap after the 509th residue in H3 protein compared with these H5 proteins. ^aWs: whooper swan; Po: pochard; En: environment; HN: Henan.

tree, including Alberta2014 and Tiger2013 (Fig. 1A). Similar topologies were also observed in the phylogenetic trees constructed using the NA, NP, MP, NS, PA, and PB1 gene sequences (Figs S2–S7).

The PB2 genes of the novel isolates and Alberta2014 were found to cluster together with previously circulating H9N2 viruses in China (Fig. 1B). Interestingly, the PB2 gene of Tiger2013 was also not derived from the H5 lineage (Fig. S8), but rather it belonged to an AIV gene pool that mainly existed in wild birds (Fig. S8), confirming the results of a previous study²¹. Therefore, the novel isolates from Sanmenxia and Alberta2014 belong to the same genotype, with the HA gene from Clade 2.3.2.1c, and the PB2 gene from H9N2 origin. The Tiger2013 isolate belongs to a different genotype, with the HA gene from Clade 2.3.2.1c, and the PB2 gene from an AIV gene pool.

Genetic signature analysis. The genetic signatures of the Sanmenxia Clade 2.3.2.1c-like H5N1 isolates were analyzed for their potential biological characteristics in order to lay the groundwork for future experiments regarding virus pathogenicity, host adaptation and drug sensitivity. The results showed that the novel isolates identified in this outbreak possessed multiple basic amino acids at the cleavage sites of HA, RERRRKR/G (Table 2), which is the typical molecular signature for HPAIVs²². In addition, the HA proteins of these isolates had the amino acids Q226 and G228 (H3 numbering) in the receptor-binding site. This suggested that these viruses favor binding to avian-like receptors, but the N158D and T160A substitutions in these viruses may enhance their capacity to also bind human-type receptors^{23–25}. Amino acids Q591, E627 and D701 were observed in the PB2 protein, suggesting that these novel H5N1 isolates are not yet fully adapted to infect mammals.

In light of the close genetic relationship between the H5N1 viruses isolated from wild birds and mammals, the genetic signatures of these viruses were also compared with Alberta2014 and Tiger2013. Interestingly, there are only five amino acid mutations in the HA proteins between H5N1 virus isolates from wild birds and human/tiger (Tables 2 and 3). The D225R substitution was found only in Alberta2014, which may influence viral receptor binding properties, according to the results of a previous study²⁶.

Furthermore, the NA stalk of these novel H5N1 viruses possessed a 20 amino acid deletion (positions 49 to 68). Past studies had shown that AIVs with this deletion demonstrated better adaptation to terrestrial birds after they were introduced from wild birds, and showed increased virulence in mammals^{27,28}. Moreover, these H5N1 isolates had a C-terminal (aa 58–90) truncated PB1-F2 protein with only 57 amino acids, which has also been shown to contribute to their virulence in mammals^{29,30}. Hence, the novel H5N1 isolates may be highly pathogenic to mammals based on the molecular characteristics on NA stalk and PB1-F2, even though they possess the avian adaptation residues with Q591, E627 and D701 on the PB2 protein.

The NA-inhibitor resistance mutation H274Y was not found in the NA antigen of these novel H5N1 reassortants. However, the V27I substitution was observed in the M2 protein, indicating that these H5N1 isolates are sensitive to NA inhibitors, but may be resistant to M2 inhibitors².

Pathogenicity in birds and mice. To further characterize the virulence of these new H5N1 viruses, one representative strain (whooper swan/HN/SMX4/2015) was selected for studies in chickens and mice. All ten specific-pathogen free (SPF) chickens inoculated with whooper swan/HN/SMX4/2015 died 1–3 days post-infection (d.p.i.) and the intravenous pathogenicity index (IVPI) was 2.79 (Table 4), indicating that these novel H5N1 viruses are HPAIV.

Virus	TCID ₅₀	IVPI	MLD ₅₀	No. of infected mice/total no. of mice (virus titer in sample [log ₁₀ TCID ₅₀ /ml]) ^a					
				Lung			Brain		
				3	5	7	3	5	7
Ws/HN/SMX4/15	10 ^{-7.5} /ml	2.79	10 ^{3.0} TCID ₅₀	3/3	3/3	3/3	0/3	1/3	3/3
				(2.5, 4.0, 3.5)	(4.25, 4.0, 4.75)	(2.0, 3.75, 2.5)	(—, —, —)	(—, —, <)	(1.5, 2.0, 1.5)

Table 4. Pathogenicity of Ws/Henan/SMX4/2015(H5N1) virus in chickens and mice. —, Virus was not detected in the stock homogenates. <, The virus titer was lower than 1 log₁₀ TCID₅₀. ^aThe mice were infected with 10^{4.5} TCID₅₀ of virus. The brain and lung tissues of the infected mice were collected for virus titration in MDCK cells.

In addition, the novel H5N1 virus was found to cause severe pathological lesions in the non-surviving wild whooper swans and the pochard. Viral encephalitis, severe lung damage with congestion and hemorrhage, and necrotic enteritis can be readily observed from the histopathology results (Fig. 2).

Furthermore, whooper swan/HN/SMX4/2015 was also shown to be highly pathogenic in mice, with a median lethal dose (MLD₅₀) of 10^{3.0} TCID₅₀ (median tissue culture infective dose) (Fig. 3 and Table 4). The virus not only replicated in the lungs during 3–7 d.p.i. with high lung virus titers (LVTs), but was found to be present in the brains starting at 5 d.p.i. Interestingly, LVTs peaked at 5 d.p.i. before gradually decreasing, whereas the peak of virus replication in the brain was at 7 d.p.i. (Table 4). This suggests that the virus first replicated in the target organs (lungs), before bypassing the blood brain barrier to infect the brain.

Receptor binding properties. In light of the highly pathogenic nature of the new H5N1 isolates, as well as the high genetic identities between these viruses and H5N1 of human origin, potential risks of these novel H5N1 viruses to humans was evaluated by a solid-phase receptor binding assay. The new H5N1 isolates were found to bind preferentially to the avian α -2,3-linked (3'SLNLN) receptor over the human α -2,6-linked (6'SLNLN) sialylglycan receptor (Fig. 4), suggesting that these novel isolates currently have a low risk for human infections.

Discussion

Genetic and phylogenetic analyses showed that this outbreak in whooper swans, pochards, and other wild ducks in the Sanmenxia Reservoir Area was caused by a H5N1 virus. These viruses were shown to be novel reassortants, possessing a Clade 2.3.2.1c HA gene and an H9N2-derived PB2 gene, and had the highest genetic homology with Alberta2014 and Tiger2013. The HA protein of these novel isolates also have the N158D and T160A amino acid substitutions, which were associated with at least two confirmed mammalian infections in the past^{23,24} and highlights the potential risk to public health.

The novel isolates were found to be highly pathogenic to chickens and mice, and virus isolated from mouse brains after challenge. It should be noted that meningoencephalitis was also observed in the fatal human case with Alberta2014, an unusual outcome for infections with H5N1 HPAIV in humans²⁰. Neurological symptoms were also noted in the non-surviving tiger infected with Tiger2013, with the heart, liver, spleen, lung, kidney, aquae pericardii, and cerebrospinal fluid all positive for H5N1 virus as detected by real-time RT-PCR²¹. This suggests that the novel Sanmenxia Clade 2.3.2.1c-like H5N1 viruses possesses tropism for the nervous system in several mammal species, and could pose a significant threat to humans if these viruses develop the ability to bind human-type receptors more effectively.

No domestic poultry breeding farms exist within 3 km of the Sanmenxia Reservoir Area and the majority of the Clade 2.3.2.1c strains are isolated from migratory birds (Fig. 1A). Therefore, the Sanmenxia Clade 2.3.2.1c-like H5N1 virus is likely spread by the long-distance migration of wild birds, especially ducks, which might have been infected by the virus from domestic poultry during stopover sites in their migration^{6,10,11,31}. Whooper swans are known to breed in northern Eurasia, and winter in Europe and eastern Asia, such as China, Korea, and Japan³². There are approximately 20 000 whooper swans wintering in China, and more than half of these animals winter in the Sanmenxia Reservoir Area along with various ducks from the East Asian/Australasian and Central Asian flyways³³. These birds arrive in the Sanmenxia Reservoir Area during early October for wintering, and then fly back to Mongolia and Siberia for breeding next spring (Figs 5 and 6).

It is believed that overlapping migratory flyways help circulate H5 HPAIVs amongst different bird species, and allow the spread of the virus across continents⁸. Recently sequential outbreaks of the H5N8 virus in domestic poultry in China, Korea, and Japan during 2010–2015^{34,35}, Europe in 2014–2015³⁶, and North America in 2014–2015^{4,37,38} were considered to have occurred due to waterfowl migration^{36,37,39}. Therefore, the migration of wild birds plays an important role in the transmission and spread of H5 HPAIVs, posing a severe risk to animal and human health. Further investigation is required to monitor the evolution and transmission of these novel Sanmenxia Clade 2.3.2.1c-like H5N1 isolates, in order to

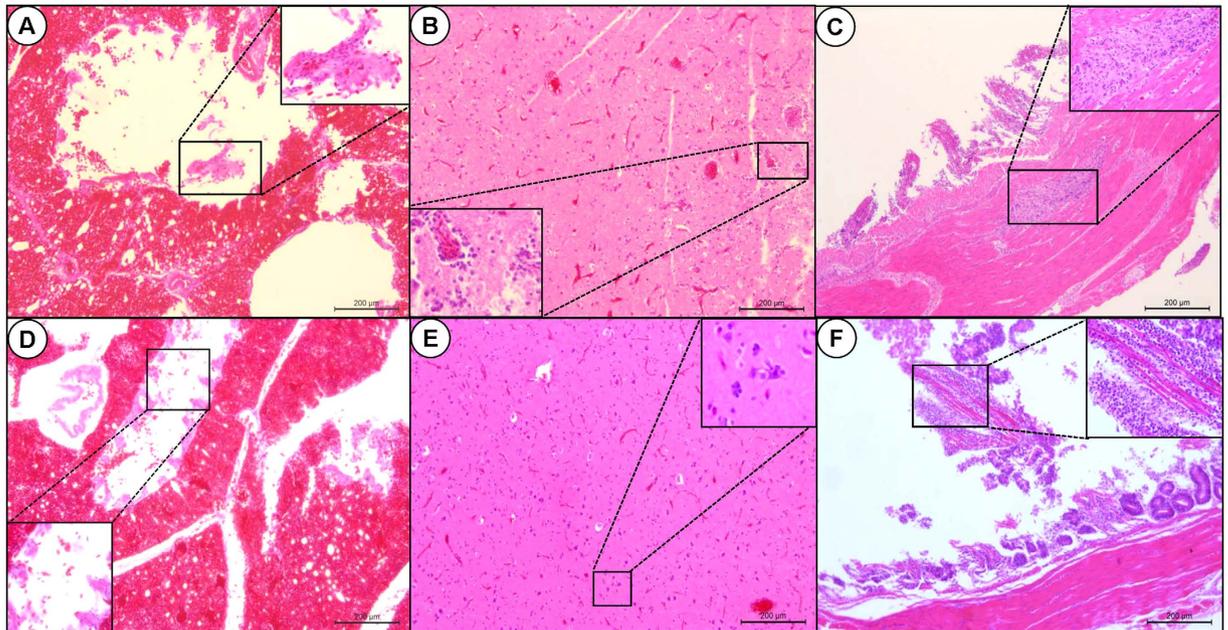


Figure 2. Histopathologic analysis in lung, brain, and small intestine of the dead whooper swan and pochard naturally infected by the H5N1 virus. Representative pictures of the lung (A,D), brain (B,E), and small intestine (C,F) of the whooper swan (A–C) and pochard (D–F) infected by the Sanmenxia Clade 2.3.2.1c H5N1 isolate. Scale bar = 200 μm. Bronchial epithelial cell desquamation, congestion and hemorrhage are found in the lungs (A,D); Inflammatory cells infiltrate around blood vessel and nerve cells are found in the brains (B,E). Intestinal villus desquamation, lymphatic tissue cells neorobiosis and disaggregation under mucosa are found in the small intestines (C,F).

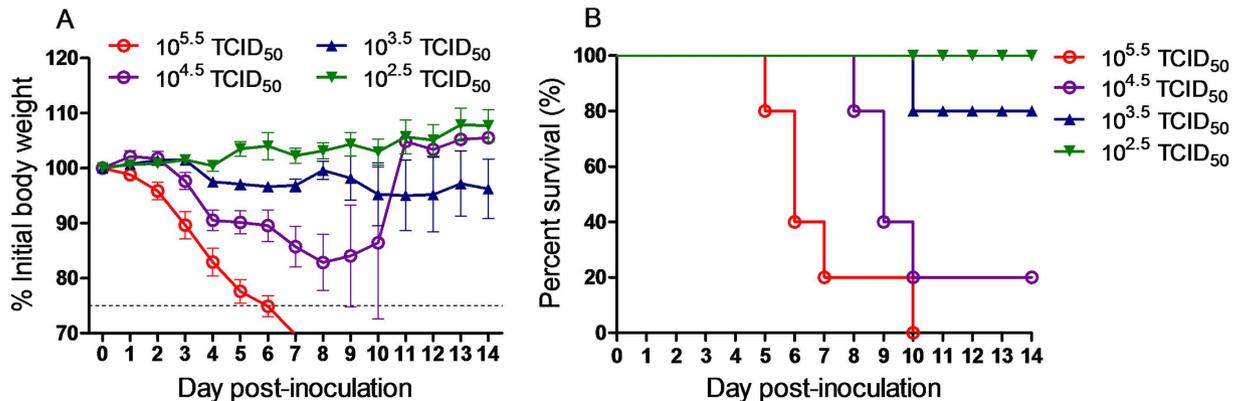


Figure 3. Pathogenicity of the Sanmenxia H5N1 virus in mice. Mice were infected by 10-fold serial dilutions of Ws/HN/SMX4/15(H5N1) virus. (A) Average percentage weight change. (B) Kaplan-Meier survival curve.

prevent their spread to other countries and avoiding a repeat of what had happened in the past with circulating Qinghai-like Clades 2.2 and 2.3.2 H5N1, and Clade 2.3.4.4 H5N8 viruses^{7,9,15,16,19,36,37,40,41}.

Materials and Methods

Virus isolation and gene sequencing. The oropharyngeal and cloacal swabs, tissues and organs of the sick or dead birds, and feces from apparently healthy birds were collected for virus isolation. Swab samples and tissues were maintained at 4 °C in viral-transport medium and sample tubes, respectively. Before inoculation, swab samples with viral-transport medium were thoroughly mixed. The tissues and organs were homogenized using a Qiagen TissueLyser II machine (30 cycles/s; 4 min) in 1 ml of cold PBS under sterile conditions. The solid debris in the swab and tissue samples were pelleted by

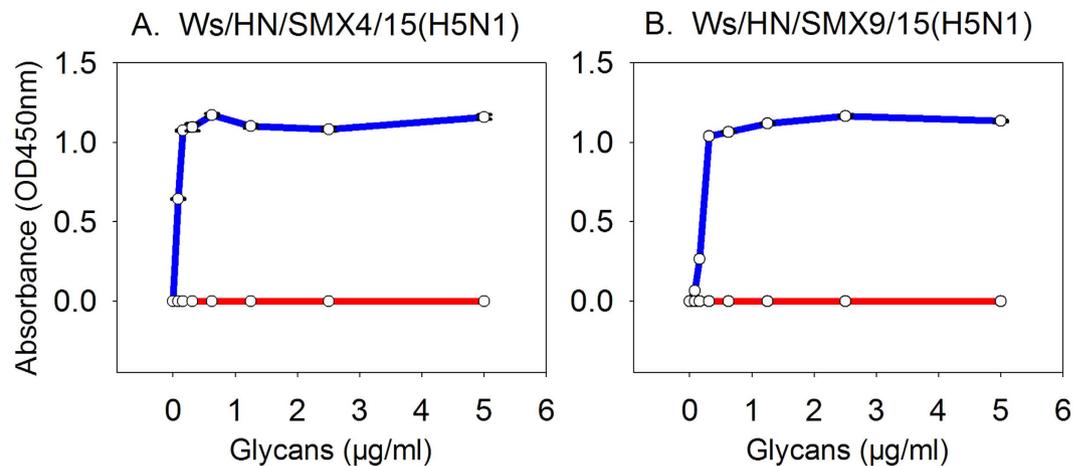


Figure 4. Receptor binding properties of the Sanmenxia H5N1 virus. Solid-phase binding assay for (A) Ws/HN/SMX4/15(H5N1) and (B) Ws/HN/SMX9/15(H5N1) viruses to both α 2,3-linked (3'SLNLN) (colored in blue) and α 2,6-linked sialylglycan receptors (6'SLNLN) (colored in red), respectively. The data presented is the mean \pm standard deviation (S.D.).

centrifugation at $5,000 \times g$ for 10 min. The supernatants were then inoculated in 10-day-old chicken embryos for 72 hours at 37°C .

The hemagglutination (HA) and hemagglutination inhibition (HI) assay with H5N1 AIV and Newcastle disease virus (NDV) antigens and their antisera was performed according to WHO standard protocols⁴². Viral RNA from the HA titer-positive allantoic fluid was extracted using TRIZOL (Invitrogen) according to manufacturer instructions. All gene segments were amplified using *Ex Taq*TM DNA polymerase (Takara) with the improved primers⁴³, and sequenced by the ABI 3730XL automatic DNA analyzer.

Genetic and phylogenetic analysis. The gene and amino acid sequences were edited using DNAMAN (Version 7.0) and BioEdit (Version 7.0.5.2). Referenced sequences were downloaded from the NCBI (<http://www.ncbi.nlm.nih.gov>) and GISAID (www.gisaid.org) databases via the online Blast search. Multiple sequence alignment was performed using Muscle⁴⁴. Maximum-likelihood phylogenetic trees were inferred with the software RAxML⁴⁵ under the GAMMAGTR model with 1000 bootstraps.

Histopathologic analysis. The brain, lung, small intestine, spleen, kidney, and liver of non-surviving wild birds were fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut into 5-mm-thick serial sections, and stained with hematoxylin and eosin (H&E) for histopathological evaluation as previously described⁴⁶.

Studies in chickens. To determine the pathogenicity of these novel virus isolates, the IVPI was determined according to the recommendations of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2014⁴⁷. Fresh infective allantoic fluid with a HA titer of 128 was diluted 1/10 in sterile isotonic saline. 0.1 ml of the diluted virus was used for an intravenous challenge in ten 6-week-old SPF White Leghorn chickens.

Birds were examined at 24-hour intervals for 10 days. At each observation, each bird was scored 0 if normal, 1 if sick, 2 if severely sick, and 3 if dead. The judgment of sick and severely sick birds is a subjective clinical assessment. Typically, 'sick' birds demonstrate one of the following signs and those that are 'severely sick' demonstrate more than one of the following signs: respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. The IVPI is the mean score per bird per observation over the 10-day period.

Studies in mice. Fourteen 6- to 8-week-old (~17 g) female BALB/c mice (Vital River Laboratory, Beijing, China) from each group were anesthetized with carbon dioxide (CO_2) and inoculated intranasally (i.n.) with $50 \mu\text{l}$ of the 10-fold dilutions of virus in phosphate-buffered saline (PBS). Each group contains five mice and the MLD_{50} value was determined by the Reed and Muench method⁴⁸, and expressed as the TCID_{50} value corresponding to 1 MLD_{50} .

Nine mice were inoculated i.n. with $50 \mu\text{l}$ of $10^{4.5}$ TCID_{50} virus diluted in phosphate-buffered saline (PBS). Mock-infected control animals were inoculated i.n. with $50 \mu\text{l}$ PBS. Three mice from each group were euthanized at 3, 5, and 7 d.p.i., in which the brains and lungs were collected and homogenized using a QIAGEN TissueLyser II machine (30 cycles/s, 4 min) in 1 ml of cold PBS under sterile conditions, respectively. Solid debris was then pelleted by centrifugation at $5,000 \times g$ for 10 min, and the homogenates were used for virus titrations in MDCK cells.

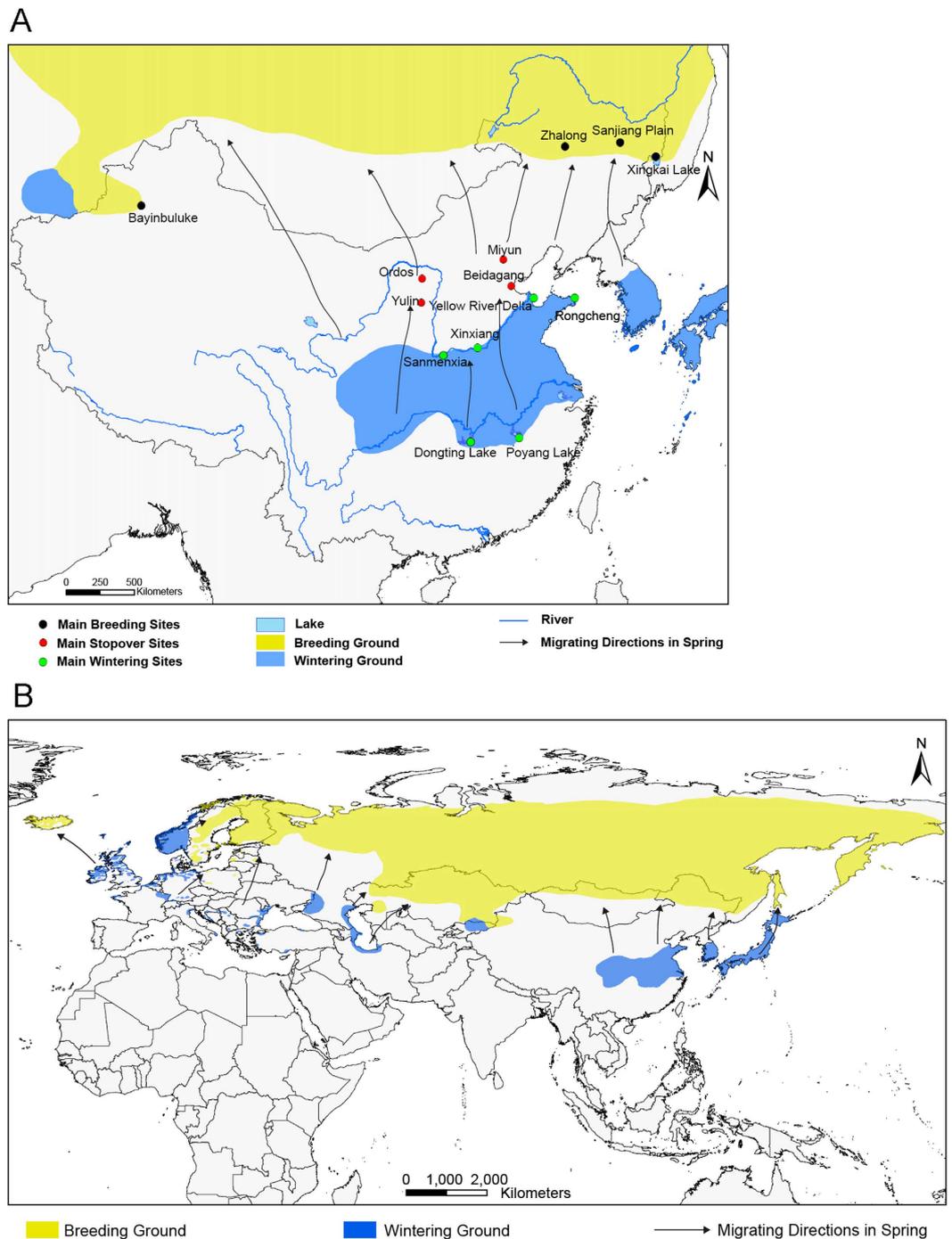


Figure 5. The migration routes of whooper swan (*Cygnus cygnus*). The migratory routes of whooper swan in China (A) and worldwide (B) were mapped by the ArcGIS Desktop 10.2 software.

All of the mice were monitored daily for general behavior and clinical signs, including food intake, body weight, inactivity, and mortality for 14 days. Any mouse that lost $\geq 25\%$ of its preinoculation body weight was euthanized.

Receptor-binding assay. Viral receptor-binding specificity was determined using the solid-phase direct binding assay as described previously⁴⁹. Briefly, 96-well microtiter plates were coated by biotinylated glycans 3'SLNLN (NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-SpNH-LC-LC-Biotin) and 6'SLNLN (NeuAc α 2-6Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-SpNH-LC-LC-Biotin), then the virus dilutions containing 128 HA units with the neuraminidase inhibitors (10 nM of each of Oseltamivir, Zanamivir, and Penmivir) was incubated. The virus-receptor binding reaction was detected with rabbit

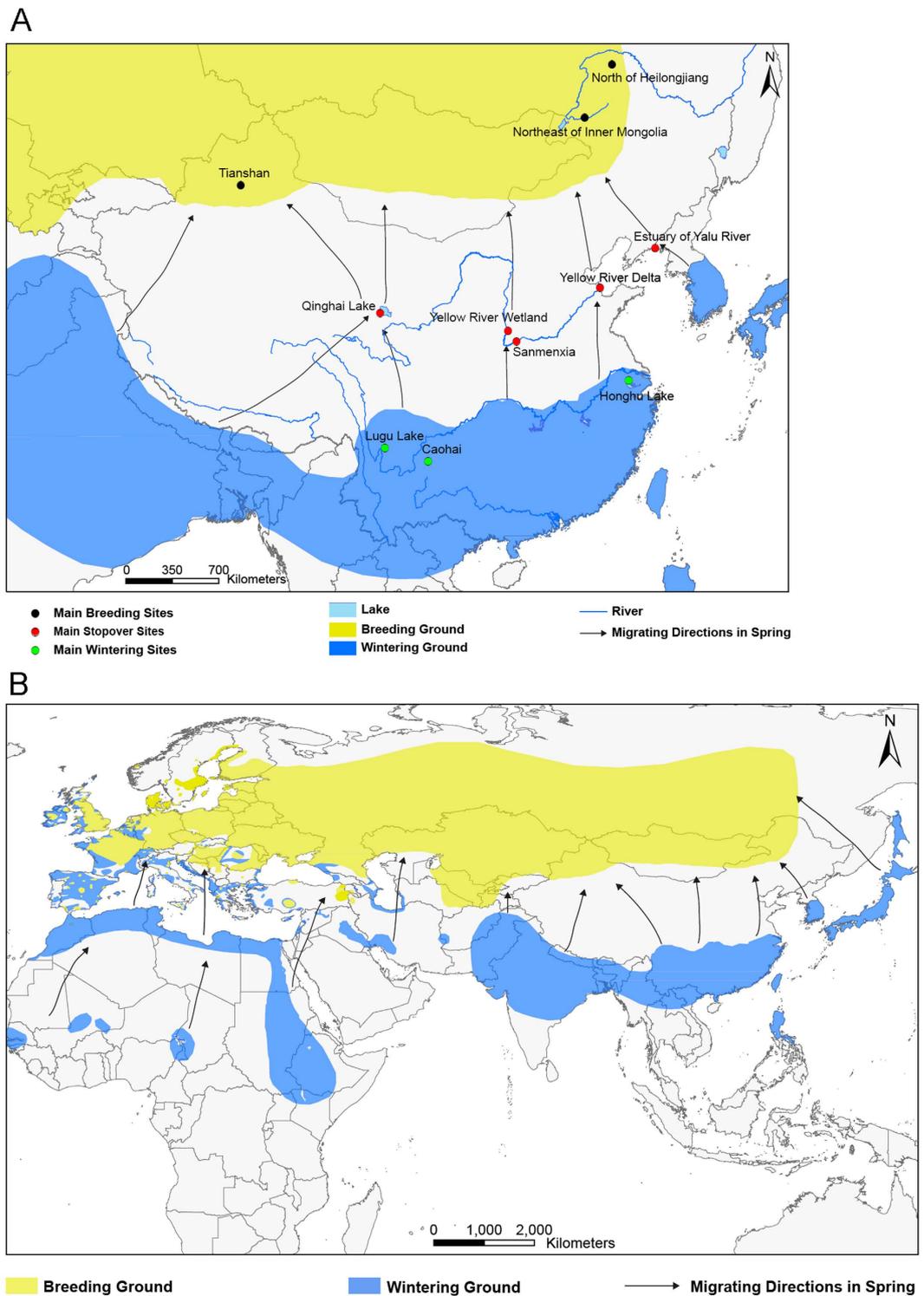


Figure 6. The migration routes of pochard (*Aythya ferina*). The migratory routes of pochard in China (A) and worldwide (B) were mapped by the ArcGIS Desktop 10.2 software.

antisera against the H5N1 virus and HRP-linked goat-anti-rabbit antibody. The results were measured by TMB (tetramethylbenzidine) at 450 nm.

Migration routes of whooper swan and pochard. The migration routes including breeding ground, wintering ground, and the migrating directions of whooper swans (*Cygnus cygnus*) and pochards (*Aythya ferina*) in China and worldwide were mapped by the ArcGIS Desktop 10.2 software according to results of previous studies^{10,50–52}.

Ethics statement. All animal research was approved by the Chinese Academy of Sciences of Research Ethics Committee, under approval number PZIMCAS2013001, and complied with the Beijing Laboratory Animal Welfare and Ethical Guidelines of the Beijing Administration Committee of Laboratory Animals.

Gene sequences. The GISAID accession numbers for the genome sequences of the H5N1 viruses are EPI560202-8, EPI560212, EPI560152-9, EPI559888, EPI559895, EPI559903, EPI559910, EPI559920, EPI559913, EPI559928, EPI559931, EPI559792, EPI559800, EPI559803, EPI559789, EPI559806, EPI559791, EPI559811, EPI559812, EPI559761-2, EPI559764, EPI559770-4, and EPI559724-31.

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Author Contributions

G.F.G., F.L., W.S. and Y.B. are co-senior authors, who conceived and designed the research. Y.B., W.S., Y.Y., X.L., Z.Z. and J.C. performed the experiments. J.H., F.L., Y.Li., W.R., R.G., D.L., W.L. and H.W. performed sample collections. Y.B., G.W. and W.S. prepared and revised the manuscript. W.G., Y.Liu., and B.Z. provided helpful suggestions about the study. All authors read and approved the final manuscript. Y.B. (Ph.D. & D.V.M) is the Director of Technology Platform for Influenza Virus Research, Institute of Microbiology, Chinese Academy of Sciences.

Additional Information

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