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Novel reassortant 2.3.4.4B H5N6 highly pathogenic avian influenza viruses circulating among wild, domestic birds in Xinjiang, Northwest China

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ABSTRACT

Background: The H5 avian influenza viruses (AIVs) of clade 2.3.4.4 circulate in wild and domestic birds worldwide. In 2017, nine strains of H5N6 AIVs were isolated from aquatic poultry in Xinjiang, Northwest China.
Objectives: This study aimed to analyze the origin, reassortment, and mutations of the AIV isolates.
Methods: AIVs were isolated from oropharyngeal and cloacal swabs of poultry. Identification was accomplished by inoculating isolates into embryonated chicken eggs and performing hemagglutination tests and reverse transcription polymerase chain reaction (RT-PCR). The viral genomes were amplified with RT-PCR and then sequenced. The sequence alignment, phylogenetic, and molecular characteristic analyses were performed by using bioinformatic software.
Results: Nine isolates originated from the same ancestor. The viral *HA* gene belonged to clade 2.3.4.4B, while the *NA* gene had a close phylogenetic relationship with the 2.3.4.4C H5N6 highly pathogenic avian influenza viruses (HPAIVs) isolated from shoveler ducks in Ningxia in 2015. The *NP* gene was grouped into an independent subcluster within the 2.3.4.4B H5N8 AIVs, and the remaining six genes all had close phylogenetic relationships with the 2.3.4.4B H5N8 HPAIVs isolated from the wild birds in China, Egypt, Uganda, Cameroon, and India in 2016–2017, Multiple basic amino acid residues associated with HPAIVs were located adjacent to the cleavage site of the HA protein. The nine isolates comprised reassortant 2.3.4.4B HPAIVs originating from 2.3.4.4B H5N8 and 2.3.4.4C H5N6 viruses in wild birds.
Conclusions: These results suggest that the Northern Tianshan Mountain wetlands in Xinjiang may have a key role in AIVs disseminating from Central China to the Eurasian continent and East African.

Keywords: Highly pathogenic avian influenza virus; H5N6; reassortant; poultry; wild bird

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Conflict of Interest
The authors declare no conflicts of interest.

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INTRODUCTION

Since 2014, highly pathogenic avian influenza viruses (HPAIVs) of clade 2.3.4.4 (H5) have been monitored in poultry and wild birds in European and Asian countries, and the viruses of that clade have evolved into eight groups (A-H) [1,2]. Group A includes H5N8 avian influenza viruses (AIVs) that were identified in Asia, Europe, and North America [1]. In 2016, a novel H5N8 AIV lineage initially emerged in the wild birds in Qinghai Lake, spread to Mongolia, Siberia, and Europe, and was subsequently identified in China and Korea; this new lineage was classified into clade 2.3.4.4B [3]. On the other hand, group C H5N6 AIVs were first reported in Laos in 2013 and gradually became the main source of sporadic AIV infections in poultry in Southern China [4]. Group D comprises H5N6 viruses mainly identified in China and Vietnam. Subsequent studies have reported that H5N6 viruses of groups 2.3.4.4E, 2.3.4.4F, 2.3.4.4G, and 2.3.4.4H have been isolated from poultry and wild birds [2].

Outbreaks of 2.3.4.4B H5N8 AIVs were reported in South Korea in 2014 [5]. The viruses were subsequently disseminated via migratory birds and have since been detected in wild and domestic birds worldwide [1,2,6-8]. Almost simultaneously, 2.3.4.4B H5N6 HPAIVs were undergoing global transmission and have been detected in wild and domestic birds [8-13]. The cocirculation of H5N6 and H5N8 viruses among wild and domestic birds could increase the probability of reassorting novel viruses [9-11]; indeed, reassortants derived from the H5N6 and H5N8 HPAIVs have been detected in various wild birds and poultry [8,9,14,15]. Moreover, it has been reported that 46% of all 2.3.4.4B H5N6 viruses in domestic poultry were derived from wild birds [9]. Thus, reassortant viruses could pose potential threats to poultry farming and human public health. In this study, nine H5N6 AIVs were isolated from aquatic poultry in Xinjiang Uyghur Autonomous Region, China, and all were grouped into clade 2.3.4.4B. This study aimed to analyze the origin of the isolates.

MATERIALS AND METHODS

Sample collection

From March 2017 to December 2018, a total of 354 oropharyngeal and cloacal swabs from apparently healthy poultry (chickens, ducks, and geese) in live poultry markets (LPMs) in Urumqi, China were collected and placed in tubes containing viral transport medium DMEM (1,000 u/L penicillin, 500 ug/L streptomycin, 100 mg/L Nystatin, 100 mg/L Polymyxin B sulfate salt, 1000 mg/L Sulfamethoxazole, 0.05 g/L Ofloxacin). These tubes were kept in an icebox at −20°C before transport to the laboratory and then immediately stored at −80°C.

The animal experiment portion of this study was approved by the Committee on the Ethics of Animal Experiments of Xinjiang Key Laboratory of Biological Resources and Genetic Engineering (BRGE-AE001) and carried out under the guidelines of the Animal Care and Use Committee of the College of Life Science and Technology, Xinjiang University.

Virus isolation and identification

The swab samples were vortexed and centrifuged, and the supernatants inoculated into 10-day-old specific-pathogen-free embryonated chicken eggs. Seventy-two hours after inoculation, allantois fluid was harvested, and hemagglutinin (HA) activity was assayed using 1% chicken red blood cells [14]. All HA-positive samples underwent reverse transcription polymerase chain reaction (RT-PCR) using universal primers targeting the PB1 gene [15].

Viral RNA extraction (Bioer) and RT-PCR (TOYOBO) were performed according to the manufacturers' instructions.

Routine surveillance samples were processed in a biosafety level 2 (BSL-2) laboratory of the Center for Influenza Research and Early-Warning (CASCIRE), Chinese Academy of Science, while experiments with live H5N6 viruses were conducted in a CASCIRE biosafety level 3 (BSL-3) laboratory. Coveralls, gloves, and N95 masks were used during the work at the BSL-2 and BSL-3 laboratories, and all wastes were autoclaved.

Whole-genome sequencing

Next-generation sequencing was used to determine the whole-genome sequences of the AIV isolates. The viral RNA samples were quantified using the 2100 Bioanalyzer System (Agilent Technologies). The RT-PCR and cDNA synthesis procedures were conducted using PrimeScript One-Step RT-PCR Kit Ver.2 (RR055A Takara) and influenza A-specific primers MBTuni-12 and MBTuni-13. Sequencing libraries with an insert size of 200bp were prepared by end-repairing, dA-tailing, adaptor ligation, and PCR amplification, all performed according to the standard manufacturer's instructions (Illumina, USA). The libraries were sequenced on an Illumina HiSeq4000 platform (Illumina) [4,16].

Phylogenetic analysis

Phylogenetic trees were constructed for each gene segment of the nine AIV isolates to investigate their evolutionary relationships. The AIV reference sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and GISAID (http://www.gisaid.org) via the online Basic Local Alignment Search Tool (BLAST). The datasets for phylogenetic analysis were generated by aligning with reference sequences closely related to the viral sequences of the isolates and removing sequences with the same strain name and those without a clear subtype or collection date. The final sequence numbers of each gene segment are PB2 37, PB1 28, PA 32, HA 35, NP 33, NA 24, MP 27, and NS 33 (**Supplementary Table 1**). The sequences were aligned with ClustalW using MEGA 7.0. The general time-reversible nucleotide substitution model with invariant sites (I) and the gamma rate of heterogeneity (GTR +Γ) results were selected as providing the best fit for all datasets. Maximum clade credibility (MCC) phylogenetic trees were generated by applying maximum likelihood analysis with 1000 bootstrap replicates in the MEGA-X program and visualized/annotated using Fig tree 1.4.3 software [4].

RESULTS

Virus isolation

Nine strains of H5N6 AIV were isolated between July and November 2017, one from a duck swab and the rest from goose swabs. The eight gene segments of the 9 isolates shared very high nucleotide homology (> 99.9%); thus, the isolates originated from the same ancestor. The strains have been designated as A/goose/Xinjiang/011-015/2017(H5N6), A/duck/Xinjiang/016/2017 (H5N6), and A/goose/Xinjiang/017-019/2017(H5N6), or XJ-H5N6/2017 for short. The complete sequences of the isolates have been submitted to NCBI (accession numbers: MW109029-MW109036, MW110101-MW110108, and MW110205-MW110260).

Table 1. Rates of nucleotide homology among eight genes of XJ-H5N6/2017

Gene	Viruses with the highest nucleotide homology	Accession No.	Homology (%)
PB2	A/Whooper Swan/Sanmenxia/01/2016 (H5N8)	EPI1550911	99.3
PB1	A/Whooper Swan/Sanmenxia/01/2016 (H5N8)	EPI155091	99.3
PA	A/Bar-headed Goose/Qinghai/BTY9-LU/2016 (H5N8)	EPI774258	99.6
HA	A/Whooper Swan/Sanmenxia/01/2016 (H5N8)	EPI1550914	99.4
NP	A/green-winged teal/Egypt/877/2016 (H5N8)	EPI1010491	99.4
NA	A/Shoveler/Ningxia/488-53/2015 (H5N6)	EPI159965	99.1
	A/Environment/Chongqing/45373/2015 (H5N6)	EPI749552	99.1
MP	A/Whooper Swan/Sanmenxia/01/2016 (H5N8)	EPI1550917	99.6
NS	A/duck/India/10CA01/2016 (H5N8)	EPI858839	99.1

Sequence and phylogenetic analysis

Homology BLAST and phylogenetic analyses were performed on eight genes of the viral isolates (**Table 1** and **Fig. 1**). The viral *NA* gene exhibited the highest sequence homology (99.1%), and the sequence clustered together with those of H5N6 AIVs isolated from shoveler ducks sampled in Ningxia (NX488-53) and from the environment of Chongqing in 2015; those sequences were designated as 2.3.4.4C H5N6 AIVs. The viral *NP* gene segments shared the highest sequence homology (99.4%) with 2.3.4.4B H5N8 AIVs isolated from the green-winged teal in Egypt in 2016 and were grouped into an independent subcluster within the phylogenetic tree. The remaining six genes had the highest sequence homologies (99.1%–99.6%) and relatively close genetic relationships in the phylogenetic tree with the 2.3.4.4B H5N8 AIVs. Five of those genes, *HA*, *PB2*, *NS*, *PA*, and *MP*, clustered together with 2.3.4.4B H5N8 AIVs isolated from migratory swans in Sanmenxia, Hubei, and Shanxi in 2016, and this sixth gene, *PB1*, clustered together with H5N8 AIVs isolated from grey-headed gulls sampled in Uganda in 2017. The above results suggest that XJ-H5N6/2017 is a novel reassortant 2.3.4.4B H5N6 AIV derived from 2.3.4.4B H5N8 and 2.3.4.4C H5N6 viruses present in wild birds.

Molecular characteristics of the H5N6 virus isolates

Assessment of the multiple amino acid mutations present in the nine isolates could help elucidate H5N6 virulence. Multiple basic amino acids (KEKRRKR↓GLF) were observed at cleavage sites in the HA protein of the H5N6 isolates (**Table 2**), suggesting that the isolates were HPAIVs. The viral receptor-binding sites all contained Q226 and G228 (H3 numbering), indicative of an avian-like α2,3-sialic acid (α2,3-SA) receptor-binding preference; however, the S128P, S137A, and T160A mutations in HA could enhance the capacity to bind to a human-like α-2,6-SA receptor [17]. In all nine isolates, 11 amino acid deletions (59–69) in the NA stalk were observed, suggesting the isolates could have different adaptive and virulence characteristics in poultry and mammals [18]. In addition, the L89V, G309D, R477G, I495V [19], and I504V [20] mutations in the PB2 protein and the P42S and D92E mutations in the NS1 were retained in all nine isolates; notably, those mutations can enhance viral virulence and replication activity in mammals [21]. Moreover, the M2-F38L mutation has been associated with antiviral resistance (amantadine and rimantadine) [22].

DISCUSSION

The Xinjiang region is located in northwest China within the interior of the Eurasian Continent. It comprises an overlapping bird migration region between the Central Asian Flyway and the West Asian–East African Flyway. The Northern Tianshan Mountain (NTM) region in Xinjiang includes many complex mountain-oasis-desert systems, and several water reservoirs have been formed by dam construction in narrow mouths of ravines or rivers,

with much of the water coming from snowmelt [23]. Many wild birds migrate along the NTM region every year, and the reservoirs in the region have become key stopover areas for migratory birds from Eurasia and Africa. Moreover, these reservoirs are also used during aquatic poultry farming, thereby increasing the odds of contact between aquatic poultry and wild birds, including direct contact with infected wild birds or indirect contact with related materials (e.g., wild-bird feces), which could result in the introduction of wild-bird origin AIVs into aquatic poultry [8,24]. The majority of H5 HPAIVs detections in wild and domestic birds have been associated with migratory flyways and wild-bird aggregation areas [8,25]. This wild-domestic bird interface has had an important role in the spread, reassortment, evolution, and epidemiology of AIVs [8]; in particular, 2.3.4.4 H5 HPAIVs that have emerged since 2013. Such HPAIVs are continuing to reassort, evolve, and spread and have been detected in wild and domestic birds around the world [26]. Since 2016, HPAIVs outbreaks in

Fig. 1. Phylogenetic analysis of eight viral genes of XJ-H5N6/2017. Multiple alignments were constructed using ClustalW. Phylogenetic trees were generated using maximum likelihood analysis, MEGA-X software with 1000 bootstrap replicates. Bootstrap values (≥ 70%) are shown near the nodes. The viruses of XJ-H5N6/2017 are shown in blue. Scale bar indicates nucleotide substitutions per site.
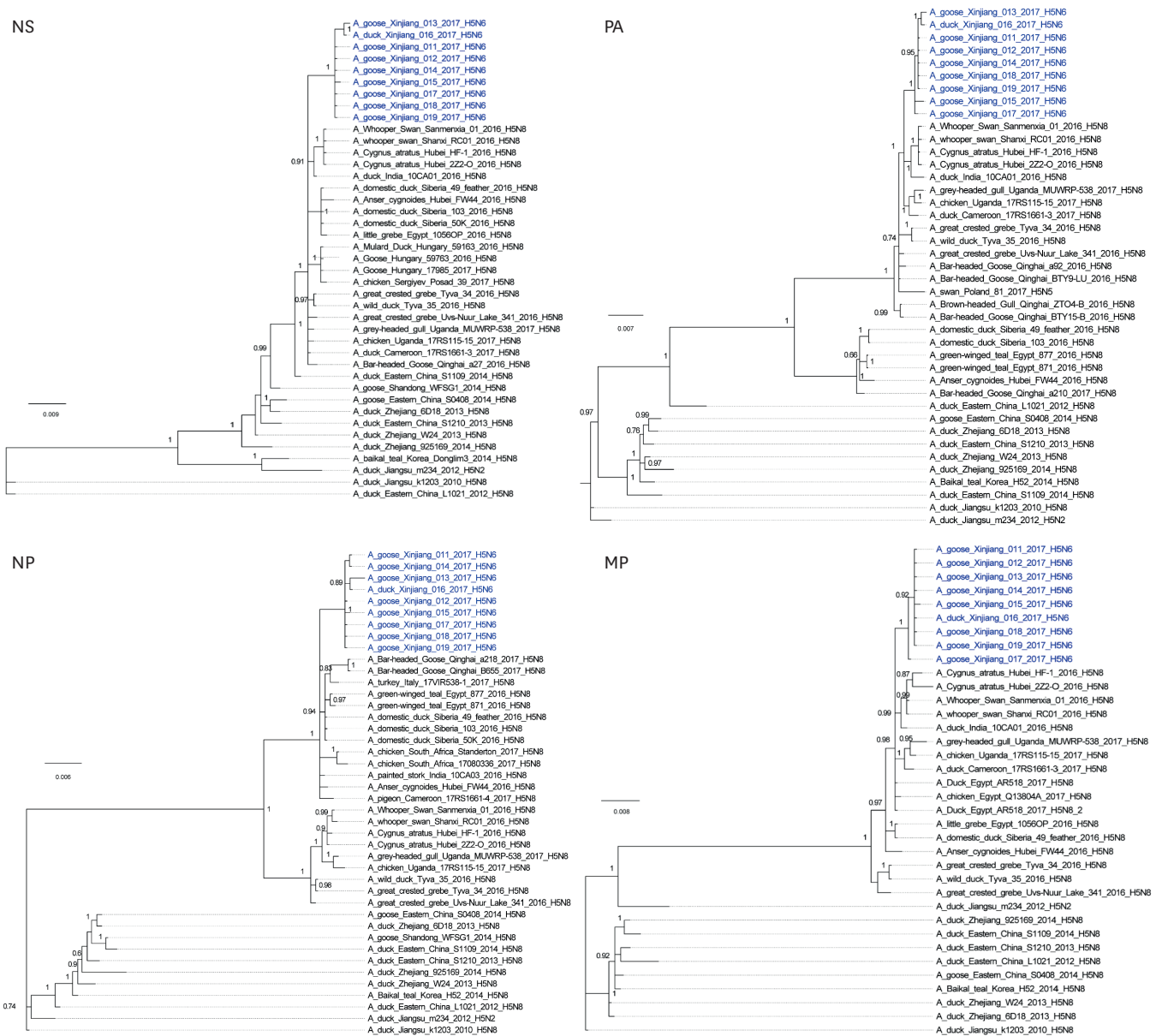
Fig. 1. (Continued) Phylogenetic analysis of eight viral genes of XJ-H5N6/2017. Multiple alignments were constructed using ClustalW. Phylogenetic trees were generated using maximum likelihood analysis, MEGA-X software with 1000 bootstrap replicates. Bootstrap values (≥ 70%) are shown near the nodes. The viruses of XJ-H5N6/2017 are shown in blue. Scale bar indicates nucleotide substitutions per site.

Xinjiang have occurred repeatedly, and there have been several outbreaks of H5N6 HPAIVs (including 2.3.4.4B and 2.3.4.4H) in poultry and migratory birds sampled in Xinjiang [27,28]. In this study, the 2.3.4.4B HPAIV isolates from the aquatic poultry in the LPMs of Urumqi were shown to be novel reassortant viruses derived from 2.3.4.4B H5N8 and 2.3.4.4C H5N6 AIVs of wild birds, suggesting that 2.3.4.4 clade AIVs are circulating in both wild and domestic birds in Xinjiang.

In this study, the *NA* gene of XJ-H5N6/2017 had a close relationship with the wild-bird origin 2.3.4.4C H5N6 AIVs identified from Chongqing and Ningxia, China. Our previous study reported a reassortant 2.3.4.4C H5N6 AIV in Xinjiang (GISAID accession nos.: EPI1548859-

Table 2. Protein mutations associated with viral virulence in XJ-H5N6/2017

Protein	Mutation	Function
HA	S128P	Enhanced binding to human-like α2,6-SA receptor
	S137A	
	T160A	
	Cleavage site	Highly pathogenic
	RERRRKR/G	
NA	59-69 del.	Increased virulence in mice; adapted to poultry
PB2	L89V	Enhanced viral virulence, replication activity in mice
	G309D	
	R477G	
	I495V	
	I504V	Increased viral replication, transmission in mammals
NS1	D92E	Highly pathogenic, increased virulence in mice
	P42S	Increased virulence in mice
M2	F38L	Antiviral resistance

1548874), which originated from the NX488-53 virus isolated in December 2016 [29]. The *NP* gene sequences of the isolates in this study were grouped into an independent subcluster within the 2.3.4.4B H5N8 AIVs, indicating that *NP* has continued to evolve in Xinjiang. The *PB1* gene was most closely related to the 2.3.4.4B H5N8 AIVs from the migratory wild birds sampled in Africa in 2017. In addition, it had a close relationship with 2.3.4.4B H5N8 AIVs from the migratory swans sampled in Central China in 2016. The remaining five genes had close relationships with 2.3.4.4B H5N8 AIVs identified from the migratory swans sampled in Central China in 2016 and bar-headed geese sampled in Qinghai in 2017. The Sanmenxia wetland of the Yellow River is an important wintering ground for migratory swans, with the swans arriving at the wetland in late October each year, departing the wetland during the late February to late March period of the following year, and migrating northwest directly to Siberia and northwest China (e.g., Qinghai wetlands) for breeding and molting [30]. An outbreak of H5N1 HPAIV has occurred at the Sanmenxia wetland, and migrating swans carrying the H5N1 HPAIVs have spread it over long distances [31,32]. Also, H5N8 HPAIVs from wild birds in Hubei, China have been reported to cause the death of migratory swans, and the H5N8 viruses have been isolated in samples from Qinghai Lake and Europe. Thus, the wetlands and lakes in Central China may have a key role in spreading H5N8 viruses in the East Asian-Australasian and Central Asian flyways [12]. These observations suggest that the isolated 2.3.4.4B H5N8 AIVs could have spread into Xinjiang by infected migratory wild birds present in Central China in 2016 and 2017 and spreading into Africa in 2017. Furthermore, these 2.3.4.4B H5N8 AIVs might have reassorted with the NX488-53 virus to generate the novel 2.3.4.4B virus of XJ-H5N6/2017, which could then circulate within aquatic poultry in the water reservoirs of NTM, subsequently spreading into the LPMs of Urumqi via the sale of live poultry. In January 2020, 2.3.4.4H H5N6 HPAIVs [SW/XJ/1/2020(H5N6)] were identified from migratory whooper and mute swans in Xinjiang [28], and the low level of similarity between the isolates in this study and those of SW/XJ/1/2020(H5N6) suggests that these viruses spread into Xinjiang independently. Based on the above observations, the wetlands in Xinjiang may have a key role in the AIVs circulating among the migratory birds and aquatic poultry in Xinjiang and in disseminating the viruses from Central China to the Eurasian continent and East African via the Central Asian Flyway and the West Asian–East African Flyway.

In our study, the multiple mutations detected in the isolates may be associated with viral virulence, adaptation, and transmission (**Table 2**). The viral HA protein included cleavage sites containing multiple basic amino acids associated with HPAIVs; moreover, the S128P, S137A, and T160A mutations can enhance binding capacity to a human-like α-2,6-SA receptor [17].

These three mutations were also present in the NX488-53 virus, which can infect BALB/c mice and transmit among guinea pigs via direct contact or aerosol routes [33,34]. The 11 amino acid deletion in the stalk region of the NA protein has been associated with the adaptation of wild-bird origin AIVs to gallinaceous poultry [35], increasing viral virulence [36], and enhancing viral transmission in ducks [37]. It has also been reported that the NA short-stalk region in H7N9 [36] and H5N1 [38] AIVs can increase virulence in mice, and it is characteristic of the viral adaptation for chickens [35]. Five amino acid substitutions in the PB2 protein (L89V, G309D, R477G, I495V, and I504V) may increase viral replication activity in mammalian cells and viral virulence in mice [19,20]. Two amino acid substitutions in the NS1 protein (D92E and P42S) could be associated with high viral fatality rates and replication efficiency [21,39,40].

In summary, our data indicate that XJ-H5N6/2017 is a novel reassortant 2.3.4.4B HPAIV originating from the 2.3.4.4B H5N8 and 2.3.4.4C H5N6 viruses present in wild birds. The multiple mutations in the amino acids of the novel reassortant are associated with its pathogenicity. To detect novel reassortant HPAIVs in a timely manner, long-term, risk-based surveillance and analysis of AIVs in poultry and wild birds is essential in Xinjiang, China.

SUPPLEMENTARY MATERIAL

Supplementary Table 1
Reference sequences of avian influenza virus strains

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REFERENCES

1. Lee DH, Bertran K, Kwon JH, Swayne DE. Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. J Vet Sci. 2017;18 Suppl 1:269-280.
   PUBMED | **CROSSREF**

2. World Health Organization. Antigenic, Genetic Characteristics of Zoonotic Influenza A Viruses, Development of Candidate Vaccine Viruses for Pandemic Preparedness [Internet]. https://www.who.int/influenza/vaccines/virus/202103_zoonotic_vaccinevirusupdate.pdf. Updated 2021. Accessed 2021 Mar.

3. Du Y, Chen M, Yang J, Jia Y, Han S, Holmes EC, et al. Molecular evolution and emergence of H5N6 avian influenza virus in Central China. J Virol. 2017;91(12):e00143-e17.
   PUBMED | **CROSSREF**

4. Bi Y, Chen Q, Wang Q, Chen J, Jin T, Wong G, et al. Genesis, evolution and prevalence of H5N6 avian influenza viruses in China. Cell Host Microbe. 2016;20(6):810-821.
   PUBMED | **CROSSREF**

5. Lee YJ, Kang HM, Lee EK, Song BM, Jeong J, Kwon YK, et al. Novel reassortant influenza A(H5N8) viruses, South Korea, 2014. Emerg Infect Dis. 2014;20(6):1087-1089.
   PUBMED | **CROSSREF**

6. Ndumu D, Zecchin B, Fusaro A, Arinaitwe E, Erechu R, Kidega E, et al. Highly pathogenic avian influenza H5N8 Clade 2.3.4.4B virus in Uganda, 2017. Infect Genet Evol. 2018;66:269-271.
   PUBMED | **CROSSREF**

7. Adlhoch C, Brown IH, Angelova SG, Bálint Á, Bouwstra R, Buda S, et al. Highly pathogenic avian influenza A(H5N8) outbreaks: protection and management of exposed people in Europe, 2014/15 and 2016. Euro Surveill. 2016;21(49):30419.
   PUBMED | **CROSSREF**

8. Verhagen JH, Fouchier RA, Lewis N. Highly pathogenic avian influenza viruses at the wild-domestic bird interface in Europe: future directions for research and surveillance. Viruses. 2021;13(2):212.
   PUBMED | **CROSSREF**

9. Zhang J, Chen Y, Nan S, Wang X, Lin S, Ma K, et al. Genetic diversity, phylogeography,, evolutionary dynamics of highly pathogenic avian influenza a(h5n6) viruses. Virus Evol. 2020;6(2):veaa079.
    PUBMED | CROSSREF

10. Poen MJ, Venkatesh D, Bestebroer TM, Vuong O, Scheuer RD, Oude Munnink BB, et al. Co-circulation of genetically distinct highly pathogenic avian influenza A clade 2.3.4.4 (H5N6) viruses in wild waterfowl and poultry in Europe and East Asia, 2017–18. Virus Evol. 2019;5(1):vez004.
    PUBMED | CROSSREF

11. Pohlmann A, Hoffmann D, Grund C, Koethe S, Hüssy D, Meier SM, et al. Genetic characterization and zoonotic potential of highly pathogenic avian influenza virus A(H5N6/H5N5), Germany, 2017–2018. Emerg Infect Dis. 2019;25(10):1973-1976.
    PUBMED | CROSSREF

12. Ma L, Jin T, Wang H, Liu H, Wang R, Li Y, et al. Two reassortant types of highly pathogenic H5N8 avian influenza virus from wild birds in Central China in 2016. Emerg Microbes Infect. 2018;7(1):14.
    PUBMED | CROSSREF

13. Kim YI, Si YJ, Kwon HI, Kim EH, Park SJ, Robles NJ, et al. Pathogenicity and genetic characterisation of a novel reassortant, highly pathogenic avian influenza (HPAI) H5N6 virus isolated in Korea, 2017. Euro Surveill. 2018;23(7):18-00045.
    PUBMED | CROSSREF

14. Killian ML. Hemagglutination assay for the avian influenza virus. Methods Mol Biol. 2008;436:47-52.
    PUBMED | CROSSREF

15. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, et al. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A. 2012;109(11):4269-4274.
    PUBMED | CROSSREF

16. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, et al. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. J Virol. 2009;83(19):10309-10313.
    PUBMED | CROSSREF

17. Shen YY, Ke CW, Li Q, Yuan RY, Xiang D, Jia WX, et al. Novel reassortant avian influenza A(H5N6) viruses in humans, Guangdong, China, 2015. Emerg Infect Dis. 2016;22(8):1507-1509.
    PUBMED | CROSSREF

18. Stech O, Veits J, Abdelwhab EM, Wessels U, Mettenleiter TC, Stech J. The neuraminidase stalk deletion serves as major virulence determinant of H5N1 highly pathogenic avian influenza viruses in chicken. Sci Rep. 2015;5:13493.
    PUBMED | CROSSREF

19. Li J, Ishaq M, Prudence M, Xi X, Hu T, Liu Q, et al. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 avian influenza virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res. 2009;144(1-2):123-129.
    PUBMED | CROSSREF

20. Rolling T, Koerner I, Zimmermann P, Holz K, Haller O, Staeheli P, et al. Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. J Virol. 2009;83(13):6673-6680.
    PUBMED | CROSSREF

21. Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, et al. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol. 2008;82(3):1146-1154.
    PUBMED | CROSSREF

22. Chizhmakov IV, Ogden DC, Geraghty FM, Hayhurst A, Skinner A, Betakova T, et al. Differences in conductance of M2 proton channels of two influenza viruses at low and high pH. J Physiol. 2003;546(Pt 2):427-438.
    PUBMED | CROSSREF

23. Ye Z, Liu H, Chen Y, Shu S, Wu Q, Wang S. Analysis of water level variation of lakes and reservoirs in Xinjiang, China using ICESat laser altimetry data (2003–2009). PLoS One. 2017;12(9):e0183800.
    PUBMED | CROSSREF

24. Krammer F, Smith GJ, Fouchier RA, Peiris M, Kedzierska K, Doherty PC, et al. Influenza. Nat Rev Dis Primers. 2018;4(1):3.
    PUBMED | CROSSREF

25. Velkers FC, Manders TT, Vernooij JC, Stahl J, Slaterus R, Stegeman JA. Association of wild bird densities around poultry farms with the risk of highly pathogenic avian influenza virus subtype H5N8 outbreaks in the Netherlands, 2016. Transbound Emerg Dis. 2020;(1):1-12.
    PUBMED | CROSSREF

26. Global Consortium for H5N8 and Related Influenza Viruses. Role for migratory wild birds in the global spread of avian influenza H5N8. Science. 2016;354(6309):213-217.
   PUBMED | CROSSREF

27. Cims. Chinese Influenza Map Surveillance. http://map.flu.org.cn/Default.aspx. Updated 2021. Accessed 2021 Mar.

28. Li Y, Li M, Li Y, Tian J, Bai X, Yang C, et al. Outbreaks of highly pathogenic avian influenza (H5N6) virus subclade 2.3.4.4h in swans, Xinjiang, Western China, 2020. Emerg Infect Dis. 2020;26(12):2956-2960.
   PUBMED | CROSSREF

29. Zhang Q, Mei X, Zhang C, Du F, Chang N, Aji D, et al. A reassortant highly pathogenic avian influenza H5N6 virus originating from the wildbird-origin H5N6, the poultry H9N2/H7N9 viruses in Xinjiang, China. Med Weter. 2021;77(6):6532.
   CROSSREF

30. Jia R, Li S, Meng W, Gao R, Ru W, Li Y, et al. Wintering home range, habitat use of the whooper swans (Cygnus cygnus) in Sanmenxia Wetland, China. Ecol Res. 2019;34(5):637-643.
   CROSSREF

31. Bi Y, Zhang Z, Liu W, Yin Y, Hong J, Li X, et al. Highly pathogenic avian influenza A(H5N1) virus struck migratory birds in China in 2015. Sci Rep. 2015;5(1):12986.
   PUBMED | CROSSREF

32. Li S, Meng W, Liu D, Yang Q, Chen L, Dai Q, et al. Migratory whooper swans *Cygnus cygnus* transmit H5N1 virus between China and Mongolia: combination evidence from satellite tracking and phylogenetics analysis. Sci Rep. 2018;8(1):7049.
   PUBMED | CROSSREF

33. Lu S, Zhao Z, Zhang J, Wang W, He X, Yu M, et al. Genetics, pathogenicity and transmissibility of novel reassortant H5N6 highly pathogenic avian influenza viruses first isolated from migratory birds in western China. Emerg Microbes Infect. 2018;7(1):6.
   PUBMED | CROSSREF

34. Zhang C, Zhao Z, Guo Z, Zhang J, Li J, Yang Y, et al. Amino acid substitutions associated with avian H5N6 influenza A virus adaptation to mice. Front Microbiol. 2017;8:1763.
   PUBMED | CROSSREF

35. Munier S, Larcher T, Cormier-Aline F, Soubieux D, Su B, Guigand L, et al. A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. J Virol. 2010;84(2):940-952.
   PUBMED | CROSSREF

36. Bi Y, Xiao H, Chen Q, Wu Y, Fu L, Quan C, et al. Changes in the length of the neuraminidase stalk region impact H7N9 virulence in mice. J Virol. 2015;90(4):2142-2149.
   PUBMED | CROSSREF

37. Chen S, Quan K, Wang D, Du Y, Qin T, Peng D, et al. Truncation or deglycosylation of the neuraminidase stalk enhances the pathogenicity of the H5N1 subtype avian influenza virus in mallard ducks. Front Microbiol. 2020;11:583588.
   PUBMED | CROSSREF

38. Matsuoka Y, Swayne DE, Thomas C, Rameix-Welti MA, Naffakh N, Warnes C, et al. Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. J Virol. 2009;83(9):4704-4708.
   PUBMED | CROSSREF

39. Seo SH, Hoffmann E, Webster RG. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med. 2002;8(9):950-954.
   PUBMED | CROSSREF

40. Cheng J, Zhang C, Tao J, Li B, Shi Y, Liu H. Effects of the S42 residue of the H1N1 swine influenza virus NS1 protein on interferon responses and virus replication. Virol J. 2018;15(1):57.
   PUBMED | CROSSREF