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 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
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].

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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).
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 Tian Shan 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 aquaculture 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. (continued to the next page)
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.: EPI154885-6/10).
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].

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

| Protein | Mutation | Function |
|---------|----------|----------|
| HA      | S108P    | Enhanced binding to human-like α2,6-SA receptor |
|         | S137A    |          |
|         | T160A    |          |
|         |          | Highly pathogenic |
| 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 |

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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