Reassortant highly pathogenic avian influenza H5N6 virus originating from the wildbird-origin H5N6 and the poultry H9N2/H7N9 viruses in Xinjiang, China*

QIAN ZHANG¹, XINDI MEI¹, CHENG ZHANG^{1,2}, FEI DU¹, NANA CHANG¹,
DILIHUM AJI¹, JUAN LI³, WEIFENG SHI³, YUHAI BI^{1,2}, ZHENGHAI MA¹

¹Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi, 830046, China

²CAS Key Laboratory of Pathogenic Microbiology and Immunology, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Disease, Institute of Microbiology, Center for Influenza Research and Early-Warning (CASCIRE), University of the Chinese Academy of Science, Beijing, 100101, China

³Key Laboratory of Etiology and Emerging Infectious Disease in Shandong First Medical University, Taian, 271016, China

Received 31.12.2020

Accepted 24.02.2021

Zhang Q., Mei X., Zhang C., Du F., Chang N., Aji D., Li J., Shi W., Bi Y., Ma Z.

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Summary

The Asian lineage of H5, H7, and H9 avian influenza viruses (AIVs) is the most prevalent in the world, and the co-existence of H5, H7, and H9 viruses provide an opportunity to generate some novel reassortants. Herein we report our isolation of two H5N6 virus strains (named XJ-H5N6) from domestic geese in live poultry markets (LPMs) in Urumqi during the winter of 2016-2017, and analysis of the viral genetic mutations, pathogenicity, reassortant, and origin. BLAST analysis revealed that the *HA*, *NA*, *MP*, *NP*, and *NS* of XJ-H5N6 had the highest sequence identity (98.5-99.5%) with those 5 genes from H5N6 highly pathogenic avian influenza virus (HPAIV) isolated from wild birds in Ningxia (NX-WB-H5N6); the viral *PA* and *PB1* had the highest sequence identity (98.6% and 98.9%) with those 2 genes from HPAIV strain A/Environment/Chongqing/45373/2015 (H5N6) (CQ-EN-H5N6); while the viral *PB2* had the highest sequence identity (99.0%) with this gene from low pathogenic avian influenza virus (LPAIV) strain A/environment/Jiangsu/01.20 TCCX004/2015 (H9N2). Except for the *PB2*, phylogenetic analysis showed that the other 7 viral genes were clustered together with NX-WB-H5N6 and CQ-EN-H5N6, while the viral *PB2* was clustered together with the poultry H9N2/H7N9 viruses. The viral HA protein contained multiple basic amino acid residues (RRRKR/GL) at the cleavage site which represents HPAIV. Additionally, the viral multiple mutations could enhance the viral virulence and the capacity of cross-species transmission. Our data indicate that XJ-H5N6 has been generated by the reassortant of H5N6 HPAIV from wild birds in Ningxia and H9N2/H7N9 LPAIV in poultry. The results of this study may help us understand the viral adaptation and transmission patterns from wild birds to poultry.

Keywords: avian influenza virus, H5N6, poultry, wild birds, reassortant

Since the emergence of H5N1 (A/goose/Guangdong/1/1996) in China in 1996, the viral *HA* has evolved into multiple distinct phylogenetic clades, subclades, and lineages around the globe. In 2014, the clade 2.3.4.4 H5Nx HPAIVs spread rapidly via migratory birds and evolved into four groups (A-D). A group C H5N6 outbreak was initially reported in Laos in 2013, then spread rapidly to Vietnam, Japan, South Korea, and China (26). In China, H5N6 viruses have widely

circulated in wild waterfowl and poultry (1). A previous study showed that the H5N6 AIVs in poultry can transmit among ferrets via direct contact (23). H5N6 HPAIVs with the same genotype to human-origin strains have also been found in apparently healthy wild birds (2). So the virus can be transmitted through migratory birds, and almost all H5N6 patients have documented exposure to poultry and/or LPMs (1, 8). Therefore, surveillance for AIVs is needed in commercial poultry.

Urumqi is located in the northern Tianshan Mountain, which is an important habitat for migratory

* This work was supported by Xinjiang Uygur Autonomous Region Regional Cooperative Innovation Project (2017E01022) and the National Science Technology Major Project (2018ZX10101004).

birds (13). In this area, a lot of reservoirs have been formed by the construction of a dam in the narrow mouth of a ravine or a river originating from melting snow (27). These reservoirs are an important habitat for wild birds, and are also used as waterfowl farms. There is a high probability of contact between wild birds and waterfowls. In fact, HPAIVs outbreaks in Xinjiang have been repeatedly documented in recent years, and there have been several outbreaks of H5N6 HPAIV in Xinjiang between 2019 and 2020. In the current study, we isolated the H5N6 HPAIVs in poultry from LPMs in Urumqi, and analyzed the origin and molecular characteristics of the virus.

Material and methods

Samples collection and storage. From October 2016 to April 2017, a total of 354 oropharyngeal and cloacal swabs from apparently healthy poultry (chickens, ducks, and geese) in LPMs in Urumqi, China were collected and placed into tubes containing viral transport medium DMEM (1,000 µ/L penicillin, 500 µg/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 before transport to the laboratory, and then immediately stored at -80°C .

Virus isolation and Whole-Genome Sequencing. The solid debris in the swabs was pelleted by centrifugation at $7000 \times g$ for 5 min. The supernatants were inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Beijing Merial Ltd). After incubation at 37°C for 48-72 h, the allantois fluids of the inoculated eggs were collected and tested by Hemagglutination assay, the positive samples were further confirmed by RT-PCR using the universal influenza A primers target the PB1 gene (24).

The whole-genome sequences of AIV isolates were determined by Next-generation sequencing (NGS), the viral RNAs were quantified using the 2100 Bioanalyzer System (Agilent Technologies). RT-PCR and cDNA synthesis were conducted using the PrimeScript One Step RT-PCR kit (Takara) with influenza A-specific primers MBTuni-12 and MBTuni-13 (1, 29). The sequencing libraries with an insert size of 200 bp were prepared by end-repairing, dA-tailing, adaptor ligation, and PCR amplification, all according to the instructions provided by the manufacturer (Illumina). The libraries were sequenced on an Illumina HiSeq 4000 Sequencer by 101 bp paired-end sequencing, and sequencing depth for AIV isolates was 0.2 G per sample.

Phylogenetic analysis and molecular characterization. The sequences of AIVs were obtained from the Global Initiative on Sharing All Influenza Data (GISAID) and NCBI. The sequences were then aligned using the Clustal W program implemented in MEGA 7.0, and then Phylogenetic trees were constructed using MrBayes v3.2.6. The generalized time-reversible model parameters and priors were incorporated into the nexus file for execution in MrBayes, utiliz-

ing subtree pruning and redrafting branch swapping and the GTR+ Gamma model of nucleotide substitution for each of the viral genes. The programs PAUP version 4.0, jModel-Test were used. Monte Carlo Markov chains were run for 1 million iterations, with a sampling frequency of 10,000 steps, the tree was constructed after discharging the first 10% burn-in. Finally, the convergence standard deviation was assessed by estimating split frequent (< 0.01), the tree was visualized using FigTree v1.4.2. MegAlign in Laser-gene 7 (DNASTAR) was used to determine the nucleotide sequence similarity percentages and mutation sites.

Results and discussion

Virus detection, isolation, and subtyping. Through the regular AIV surveillance in Urumqi LPMs from October 2016 to April 2017, 9 strains of AIV were isolated (7 as H5N8 viruses and 2 as H5N6 viruses) from 354 samples (Supplementary Tab. 1). 2 strains of H5N6 virus were isolated from two domestic geese on December 18, 2016, one from a cloaca swab and another from a throat swab. The 2 viruses were the focus of this paper. The sequence alignment analysis showed the viral 8 genes (including *HA*, *NA*, *PB1*, *PB2*, *NS*, *PA*, *NP*, and *MP*) of the 2 isolates shared very high nucleotide identity ($> 99.9\%$) respectively, the isolates were named A/Goose/Xinjiang/008/2016 (H5N6) and A/Goose/Xinjiang/009/2016 (H5N6), or XJ-H5N6 for short. The full sequences of XJ-H5N6 viruses were submitted to GISAID (accession nos.: EPI1548859-1548874).

Nucleotide identity analysis. BLAST analysis was performed for each gene fragment of the isolates (Tab. 1). The *HA* and *NP* had the highest sequence identity with those 2 genes from A/Shoveller/Ningxia/483-28/2015 (H5N6) respectively. The *NA* has the highest sequence identity with this gene from A/Common Teal/Ningxia/488-53/2015 (H5N6) (NX488-53). The *MP* and *NS* had the highest sequence identity with those 2 genes from A/White-eyed Pochard/Ningxia/486-33/2015 (H5N6), respectively, hence all the 5 genes had the highest sequence identity with those genes from NX-WB-H5N6. The *PA* and *PB1* had the highest sequence identity with those 2 genes from A/Environment/Chongqing/45373/2015 (H5N6) (CQ-EN-H5N6), respectively. Additionally, the *PB2* has the highest sequence identity with

Tab. 1. The highest nucleotide identity with the gene of XJ-H5N6 virus

Gene	Viruses with the highest nucleotide identity	Accession no.	Identity (%)
HA	A/Shoveller/Ningxia/483-28/2015 (H5N6)	EPI1159893	98.5
NA	A/Common Teal/Ningxia/488-53/2015 (H5N6)	EPI1159965	98.8
MP	A/Ruddy Shelduck/Ningxia/486-33/2015 (H5N6)	EPI1159753	99.0
NP	A/Shoveller/Ningxia/483-28/2015 (H5N6)	EPI1159954	99.1
NS	A/Ruddy Shelduck/Ningxia/486-33/2015 (H5N6)	EPI1159954	99.5
PA	A/Environment/Chongqing/45373/2015 (H5N6)	EPI749549	98.9
PB1	A/Environment/Chongqing/45373/2015 (H5N6)	EPI749551	98.6
PB2	A/environment/Jiangsu/01.20 TCCX004/2015(H9N2)	EPI660256	99.0

Fig. 1. Phylogenetic analysis of HA, NA, MP, NP, NS, PA, PB1, PB2 of XJ-H5N6. The H5N6 viruses isolated in this study are colored in red

this gene from A/environment/Jiangsu/01.20TCCX004/2015 (H9N2).

Phylogenetic analysis. Phylogenetic analyses were performed on each gene of XJ-H5N6 (Fig. 1). HA of XJ-H5N6 was clustered together with NX-WB-H5N6 and CQ-EN-H5N6, and belonged to Group C of clade 2.3.4.4. The NA, MP, NP, NS, PA, and PB1 were also clustered together with NX-WB-H5N6 and CQ-EN-H5N6 viruses. Nevertheless, the PB2 was clustered together with A/environment/Jiangsu/TCCX004/2015 (H9N2) and A/chicken/Jiangsu/SD012/2015 (H7N9), and not together with H7N9 isolated from Xinjiang including A/chicken/Xinjiang/SD033/2014 (H7N9), A/chicken/Xinjiang/SD016/2014 (H7N9), and A/Environment/Xinjiang/73033/2013 (H7N9). In brief, the viral PB2 originated from the poultry H9N2/H7N9 viruses in Jiangsu, and the other 7 viral segments originated from NX-WB-H5N6 or CQ-EN-H5N6. Moreover, all the viral genes also had high sequence identity (98.0-99.2%) and a small genetic distance with A/duck/Hunan/12.07 YYGK113-P/2013 (H5N6) (HN113-P). HN113-P was thought to be the origin of NX-WB-H5N6, except for the PB1, while the 7 genes of NX-WB-H5N6 originated from HN113-P and share the highest nucleotide identity with EN-CQ-45373 (12). Therefore, XJ-H5N6 is a reassortant virus that may originate from NX-WB-H5N6 and the poultry H9N2/H7N9 viruses in Jiangsu.

The avian-origin H9N2 viruses widely exist in wild waterfowls and poultry and under certain circumstances can be transmitted to humans, usually causing mild infections. It is also known that several new emerging zoonotic AIVs such as H7N9, H5N1, H10N8, and H5N6 contain the PB2 from the H9N2 viruses (5), and this virus can frequently donate the internal genes to generate

reassortant viruses for adaption in new hosts (25). For example, the H5N1 HPAIVs from a human in America is a reassortant with the *PB2* from an H9N2 virus (18), and the internal genes of the novel human-pathogenic H7N9 AIVs are also derived from H9N2 viruses circulating in Eastern China (7). Influenza virus adaptive mutations in *PB2* usually cause enhanced polymerase activity and promote high virulence (22). And our data indicate that H9N2/H7N9 LPAIV in poultry donate *PB2* to generate the reassortant viruses XJ-H5N6, which could adapt in poultry.

Molecular characteristics. *HA*, *NA*, *PB2*, and *NS1* are known as major determinants of the host range restriction and pathogenicity of influenza viruses (11, 17).

In our study, the multiple mutations of the isolates could help us understand the viral risk, as well as adaptation and transmission patterns from wild bird to poultry (Tab. 2). XJ-H5N6 carried the poly-basic amino acids (RRRKR/GL) at the cleavage site in *HA*, which represents HPAIV. The receptor-binding site contains Q226 and G228 (H3 numbering), suggesting an avian-like α 2,3-sialic acid (α 2,3-SA) receptor-binding preference, and the S128P, S137A and T160A mutations in the *HA* could enhance binding capacity to a human-like α -2,6-SA receptor (21). These 3 mutations were also found in A/Yunnan/0127/2015 (H5N6) from human infection and NX488-53, and it has been reported that NX488-53 virus can infect BALB/c mice and transmit among guinea pigs via direct contact or through aerosol (12, 28).

Here we found that the *NA* stalk of XJ-H5N6 possessed an 11-amino-acid deletion (59-69 deletion) (Tab. 2), which might enable wild bird-origin AIVs to adapt to domestic poultry and influence the viral virulence. Given previous reports that the short-stalk *NA* of H7N9 (3) and H5N1 (14) AIVs could increase virulence in mice, and also are characteristic of the adaptation of influenza viruses from poultry (16). Additionally, the adaptation of AIV with short-stalk *NA* can cross from wild aquatic birds to poultry (4).

In the current study, several mutations in *PB2* (Tab. 2), including L89V, G309D, R477G, I495V, I504V, D391E, and A661E, could play essential roles in enhancing the viral virulence. Our findings are also in agreement with previous findings in that the L89V, G309D, R477G, and I495V mutations increased the viral replication activity in mammalian cells and increase viral virulence in mice (9), the I504V mutation enhanced viral polymerase activity and virulence

Tab. 2. The mutation of XJ-H5N6 virus

Protein	Mutation	Function	Reference
HA	S128P	Enhancing binding to human-like α 2,6-SA receptor	(22)
	S137A		(22)
	T160A		(22)
	Q226L	Avian-like α 2,3-SA receptor	
	G228S		
	Cleavage site RERRRKR/G	Highly pathogenic	
NA	59-69 del.	Increasing virulence in mice; adapted to poultry	(3), (4), (15), (17)
PB2	L89V	Enhancing viral virulence and replication in mice	(10)
	G309D		(10)
	R477G		(10)
	I495V		(10)
	I504V		(20)
	D391E	Increasing viral replication and transmission in mammals	(16)
	A661E		(16)
NS1	D92E	Highly pathogenic and increasing the virulence in mice	(21)
	P42S	Increasing viral replication and virulence in mice	(6)

in mice (19), and the D391E and A661E mutations induced favorable conditions for the viral replication and transmission in mammalian hosts by affecting the *PB2* structure (15).

With respect to *NS1* that plays an important role in virus replication, virulence, and inhibition of the host antiviral immune response, it is known that certain *NS1* mutations are responsible for alterations in virus pathogenicity (10). Indeed, 2 among those *NS1* mutations, D92E and P42S, have drawn a great deal of attention. In particular, the D92E mutation is in association with the resistance to the antiviral effects of cytokines and a high fatality rate (20), and the P42S mutation plays an important role in regulating the host IFN response by blocking the activation of IRF3 and thus facilitates virus replication (6). Therefore, the D92E and P42S mutations in the *NS1* (Tab. 2) could enhance the viral virulence.

In summary, our data indicate that XJ-H5N6 is a novel reassortant HPAIV and has originated from H5N6 HPAIV in wild birds and H9N2/H7N9 LPAIV in poultry. The multiple mutations in the virus could help us to understand the viral adaptation and transmission patterns from wild bird to poultry, and assess the risk of an influenza pandemic and cross-species infection.

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Corresponding author: Prof. Zhenghai Ma, College of Life Science and Technology, Xinjiang University, Shengli road 666, Urumqi, Xinjiang, 830046, China; e-mail: mzhxju@126.com