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LETTER

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Re-emergence of H5N8 highly pathogenic avian influenza virus in wild birds, China

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ABSTRACT

In mid-November 2020, deaths of whooper swan were reported in the Yellow River Reservoir Area, China. In the present study, we describe the genetic characterizations and phylogenetic relationships of four clade 2.3.4.4b H5N8 highly avian influenza viruses (HPAIVs) identified from a sick whooper swan and environmental samples collected in the Yellow River Reservoir Area in late November 2020. They were closely related to recent H5Nx HPAIVs causing outbreaks in Eurasia in the 2020-2021 influenza season, suggesting these isolates might be imported into China via migratory birds. The newly identified H5N8 HPAIVs possessed Q226 and G228 (H3 numbering), indicating that they prefer to avian-like receptors. However, they had three mutations falling within known antigenic regions, including T144A in antigenic region A, T192I in antigenic region B, and N240D in antigenic region D. Our study highlights the risk of the rapid global spread of H5N8 HPAIVs and the necessity for continuous monitoring of avian influenza viruses in wild birds.

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Wild birds, in particular certain species of waterfowl and shorebirds, are considered the natural reservoirs for avian influenza viruses [1]. Long-distance spread, especially intercontinental spread of AIVs, such as H5N1 [2,3], H5N6 [4], and H5N8 [5] HPAIVs, is closely associated with wild bird migration. More importantly, the hemagglutinin (HA) gene of the H5Nx AIVs has evolved into multiple phylogenetic clades and subclades (https://www.who.int/influenza/gisrs_laboratory/201101h5n1evoconceptualdiagram.pdf?ua=1), some of which have shown propensity of global spread [6,7]. The ongoing surveillance of live bird markets in China revealed that clade 2.3.4.4 H5Nx HPAIVs were first detected in poultry in 2008, and have gradually become dominant both in domestic poultry [8,9] and wild birds [4,5,10] from 2014 onwards. In 2010, the first identification of clade 2.3.4.4 H5N8 HPAIV in poultry was documented in China, and H5N8 HPAIV caused outbreaks in South Korea in early 2014 [11]. In autumn/winter of 2014/2015, clade 2.3.4.4 H5N8 HPAIVs were extensively transmitted among eastern Asia, Europe and North America via the migration of wild birds. In the 2016/2017 influenza season, clade 2.3.4.4 H5Nx HPAIVs, particularly the H5N8 and H5N6 subtypes, repeatedly invaded Europe, causing numerous outbreaks in poultry and wild birds [11].

To date, clade 2.3.4.4 H5Nx AIVs have further diversified into eight subclades, namely clades 2.3.4.4a to 2.3.4.4h [12]. Clade 2.3.4.4 H5N8 HPAIVs

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re-emerged and have caused >640 outbreaks in wild birds and domestic poultry in ~20 European and Asian countries including China during 2019–2021 (https://www.oie.int/en/disease/avian-influenza/). In this study, we described the genetic characterizations of clade 2.3.4.4 H5N8 HPAIVs causing an outbreak in whooper swan (*Cygnus cygnus*) in November 2020, China.

On 18 November 2020, two dead whooper swans were found in the Yellow River Wetland of Pinglu (https://news.cgtn.com/news/2020-12-03/H5N8-bird-flu-found-among-wild-swans-in-N-China-VV8nR4RhSM/index.html). The Yellow River Wetland of Pinglu is adjacent to the Sanmenxia Reservoir Area, both of which are located along the East Asian-Australasian (EA) flyway [13–15]. There are more than 200 bird species wintering or stopping over at the Yellow River Reservoir Area (including the wetland of Pinglu and Sanmenxia Reservoir Area), including whooper swan, Pochard, Crested Pochard, Red Duck, and so on. Generally, these birds arrive at the wetland in November from Mongolia and Siberia [14] and leave in next May. In January 2015, clade 2.3.2.1c H5N1 HPAIVs circulating in wild birds among Eurasia and Africa [2] were reported to kill tens of whooper swans [14] in the Sanmenxia Reservoir Area. However, in recent years, the number of wintering whooper swan in the Yellow River Reservoir Area has been gradually increasing, and has reached ~10,000 in the winter of 2020.

On 28 November 2020, one dying whooper swan was found in the Yellow River Reservoir Area (Supplementary figure 1). The visible clinical signs included weakness, cloudy eyes, and shallow breathing. Oropharyngeal and cloacal swabs from the bird and 23 feces from the environment were collected for pathogen identification. The samples were maintained at 4°C in the viral-transport medium before use. Total viral RNA was extracted from each swab and fecal sample according to the instructions of the MagaBio plus Virus RNA Purification Kit (BIOER, China), then was tested by qRT-PCR kit for influenza A virus (Mabsky Biotech Co., Ltd.). Two swabs from the sick whooper swan and eleven fecal samples from environment were tested positive. Full-length AIV genome sequences of the qRT-PCR positive samples were obtained using both Sanger and Next Generation Sequencing (NGS) [9]. The bird species of the fecal samples was further confirmed through nested PCR and Sanger sequencing of the cytochrome oxidase I (COI) gene as described previously [16]. The partial COI sequences of the environmental samples CAS002-F17 and CAS002-F18 were identical and they shared the highest nucleotide identity (99.85%) with the whooper swan (*Cygnus cygnus*) COI gene across the aligned regions (680 bp). The partial sequences of the COI gene of CAS002-F17 have been deposited into China National Microbiology Data Center (NMDC; https://nmdc.cn/coronavirus; accession No. NMDCN0000Q29).

A total of four genomes of H5N8 HPAIVs were obtained, including two from the same sick bird (A/whooper swan/Henan/CAS001-G/2020(H5N8) (CAS001-G), A/whooper swan/Henan/CAS001-K/2020(H5N8) (CAS001-K)), and two from environmental feces (A/whooper swan/Henan/CAS002-F17/2020(H5N8) (CAS002-F17) and A/whooper swan/Henan/CAS002-F18/2020(H5N8) (CAS002-F18)), respectively. The four genomes have been deposited into NMDC (accession Nos. NMDCN0000IP1-NMDCN0000IS8) and the Global Initiative on Sharing Avian Influenza Data (GISAID) database (https://www.gisaid.org; accession Nos. EPI1843651-EPI1843682).

High nucleotide identities between the four strains resolved in this study and other Autumn/Winter 2020 H5N8 HPAIV genomes were revealed (>99.5% in all eight gene segments). The HA gene of the Asian strain (A/mallard/Korea/WA820/2020(H5N8) (Korea-WA820), the NA, PB2, PB1, NP and NS genes of A/whooper swan/Inner Mongolia/w1-1/2020(H5N8) (IM-w1-1)), the PA gene of the European strains (A/chicken/Omsk/0119/2020(H5N8) (EU-0119), and the M gene of A/chicken/Kostroma/304-10/2020(H5N8) (EU-304-10)) shared the highest nucleotide identities with CAS001 described in this study (Supplementary Table 1).

To better understand the evolution of these H5N8 AIVs, complete genomes of 1625 H5N8 strains were phylogenetically analysed, including the four H5N8 viruses sequenced in this study and all global H5N8 viruses ($n = 1621$) available in GISAID and GenBank databases. In the HA phylogeny (Figure 1 and Supplementary Figure 2), 65.5% of the H5N8 strains ($n = 1065$) fell within clade 2.3.4.4b, mainly from the 2016/2017 and 2020/2021 seasons, while 33.0% in clade 2.3.4.4c ($n = 536$) mainly from the 2014/2015 season. In clade 2.3.4.4b, the Eurasian H5N8 viruses during 2020/2021 ($n = 372$) were further divided into two separate groups (Group I and Group II) in the phylogenetic tree and the mean genetic distance between the two subclades was >4.0% (Supplementary Table 2), far greater than 1.5% used to propose a novel clade/subclade of H5Nx AIVs (https://www.who.int/influenza/gisrs_laboratory/201101h5n1evoconceptualdiagram.pdf?ua=1). 28.5% strains ($n = 106$) fell within Group I, including 64 European isolates from Hungary, Poland, Germany and Czech Republic since January 2020 (relating to outbreaks in the Spring of 2020), and 42 Asian isolates circulating in Korea and Japan from October 2020 [17–19]. The four strains in this study belonged to Group II, together with 70.0% H5N8 strains during 2020/2021 ($n = 262$). This group includes a strain from Iraq in May 2020; strains from the Russian Federation from July

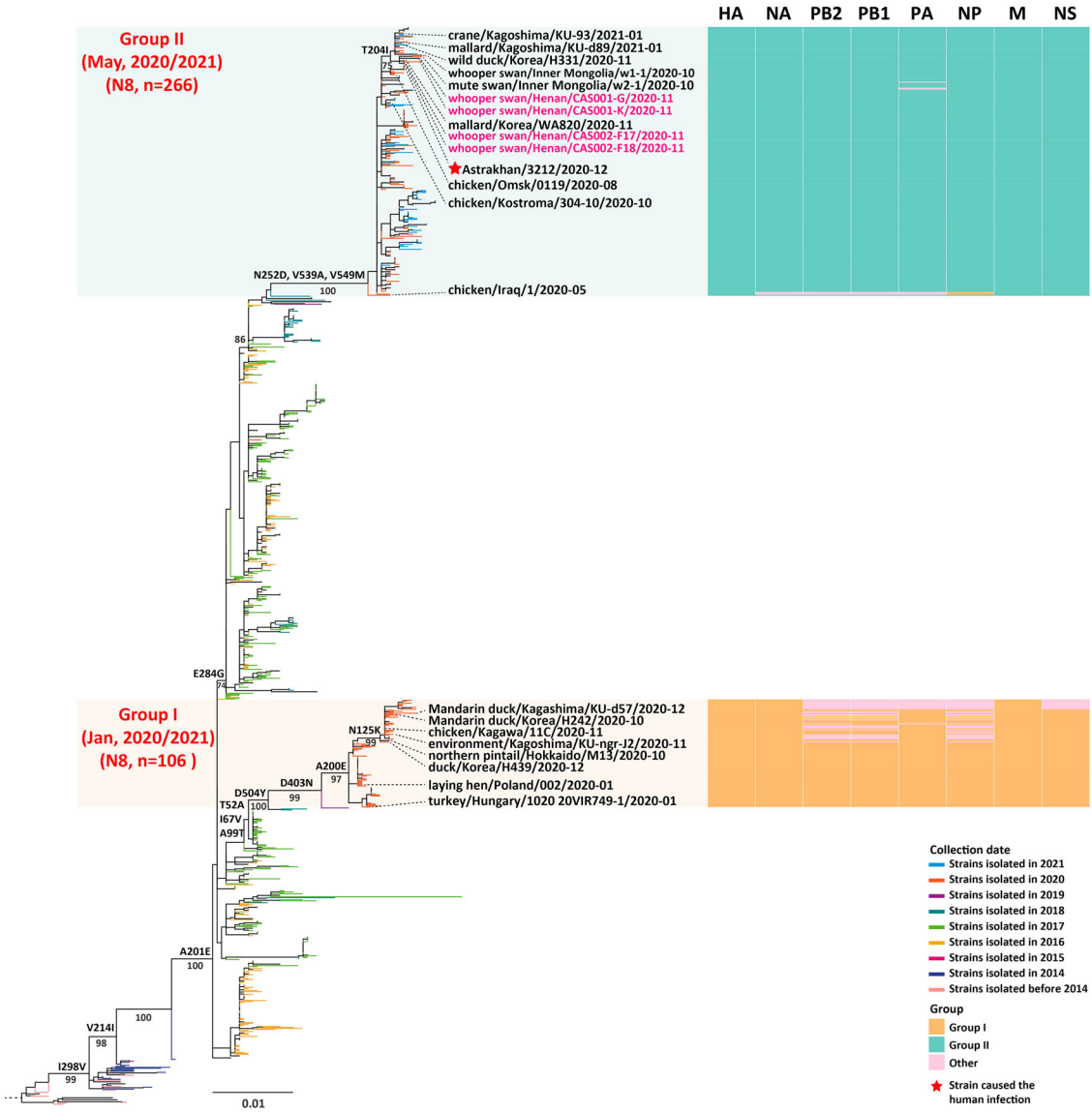

Figure 1. Phylogenetic analysis of H5N8 HPAIVs. Line colour in the topology of the HA phylogenetic tree represents the collection date of all H5N8 strains with whole genomes isolated before 2014, or from 2014 to 2021. Maximum likelihood trees in this study were performed using RAxML (version 8.1.6) under GTRGAMMA nucleotide substitution model with 1000 bootstrap replicates. The four strains in this study were colored in magenta, and the representative Eurasian isolates in 2020/2021 season were colored in black. The red pentagram represents the human-infecting H5N8 strain, A/Astrakhan/3212/2020(H5N8). In the right panel, different colours represent the different classified lineages for the HA, NA, PB2, PB1, PA, NP, MP, and NS genes, which have been shown in the figure.

2020 onwards; Asian strains include those from Inner Mongolia (October 2020), Korea (November 2020–January 2021), Japan (January 2021). European Group II strains include those from Netherlands, Germany and England (October 2020 onwards), and other European countries from November 2020 onwards [20]. Notably, the strain, A/Astrakhan/3212/2020, which caused the first human infection with H5N8 and was identified in Russia in December 2020 [21] was also clustered into Group II.

We further analysed the phylogenetic relationships of the remaining seven genes of the Groups I and II H5N8 AIVs classified according the topology of the HA gene (Supplementary Figures 3–9 and Supplementary Data 1). Generally, most of the Group II strains were clustered together in the trees of the eight genes, except several strains with few internal genes (e.g. PA) presenting a separate source (Figure 1). Likewise, all the four new H5N8 strains fell within Group II in the eight gene trees, respectively (Figure 1). For Group I strains, their HA, NA and M genes were always grouped together. However, for other internal genes, some strains ($n = 29$) in this group originated from other sources, suggesting likely reassortment events. Apart from a few Group II strains ($n = 3$) possessing Group I-like NP gene sequences, no frequent reassortment events between Groups I and II were observed in our study (Figure 1).

The HA protein of the four newly resolved H5N8 HPAIV strains contained a cleavage site motif of REKRRKR↓GLF and Q226 and G228 (H3 numbering) at the receptor binding site, indicating that these

H5N8 HPAIVs prefer to avian-like receptors. However, they also contained the amino acid substitution T160A in the HA protein, which has been reported to enhance the binding capacity to human-like receptors [10]. Amino acid substitutions of the antigenicity-associated amino acids in the HA protein, particularly in the HA1 protein, are considered a major evolutionary force driving antigenic variation of influenza A virus via impairing antibody recognition and prompting escape from immune responses [22–24]. In comparison with A/duck/Jiangsu/k1203/2010(H5N8) (K1203) [25], the four H5N8 HPAIVs described here belonging to Group II (2020–2021) of subclade 2.3.4.4b, possess three amino acid substitutions in the antigenic regions on HA including T156A (144, H3 numbering) in antigenic region A, T204I (192, H3 numbering) in antigenic region B, and N252D (240, H3 numbering) in antigenic region D. Notably, these antigenicity-associated amino acid substitutions were also seen in HA gene sequences of Group II H5N8 of subclade 2.3.4.4b. Therefore, the variations of antigenicity-associated amino acid sites in Group II might indicate the potential antigenic drift of these H5N8 viruses, including our strains.

We further summarized all the key amino acid changes in the four H5N8 strains (Supplementary Data 2). Amino acids Q591, E627 and D701 were observed in the PB2 protein of these strains, suggesting a low replication ability of these H5N8 AIVs in mammals [10]. No drug-resistance-associated mutations (Q136 K, G147 V, H274Y and R292 K in NA, N2 numbering; S31N in M2) were found in these strains, and therefore they may be sensitive to the NA and M2 inhibitors [14,26].

In conclusion, we identified and described the genetic and phylogenetic characteristics of four clade 2.3.4.4b H5N8 HPAIV genomes causing an outbreak in whooper swan in the Yellow River Reservoir Area, China in November 2020. These H5N8 HPAIV strains exhibited close genetic relationships with recent strains circulating in Asia and Europe. In fact, H5Nx HPAIVs, particularly the H5N8 subtype, have swept Eurasia in the 2020–2021 influenza season of the Northern Hemisphere, causing hundreds of outbreaks in tens of countries and more than 20 million domestic poultry have been culled in South Korea and Japan (https://www.oie.int/en/disease/avian-influenza/). Our results once again highlight the probability of rapid global spread of HPAIVs. Due to the antigenicity-associated molecular variations and pandemic potential, continuous monitoring of AIVs is urgently needed both in migratory birds and domestic poultry.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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