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Letter

Emergence of H5N8 avian influenza virus in domestic geese in a wild bird habitat, Yishui Lake, north central China

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Dear Editor,

China is located in the eastern part of the Eurasian continent, with a large north–south range, resulting in a large temperature difference. Wild birds migrate two times annually along with the north–south range, including eastern, central and western routes in China. Wild birds are reported to carry influenza viruses from multiple sources, causing the virus to spread across a wide range of regions, which present great challenges for the prevention and control of avian influenza viruses (AIVs) (He et al., 2021; Shi and Gao, 2021).

In the peak period of winter migration, wetlands or lakes are hot spots for bird gathering, and they are also hot spots for the spread of avian influenza viruses. Hebei is located in the core of the Central Plains in China and is a shared destination for wild birds in the east and central migrating routes (Tian et al., 2015). However, niche studies on the influenza epidemic have not been conducted in Hebei Province for many years, and long-term monitoring of migratory birds in Hebei Province is imperative.

AIV belongs to the family *Orthomyxoviridae* and genus *Influenza virus A*. It is a single-stranded negative-sense RNA virus. AIV contains eight genes encoding ten proteins, such as surface proteins hemagglutinin (HA) and neuraminidase (NA). Combination of HA number and NA number determines the virus sub-type. To date, 16 HA and nine NA subtypes of AIVs have been reported in wild birds and poultry of the globe. Besides, AIV is divided into the highly pathogenic avian influenza virus (HPAIV) and the low pathogenic avian influenza virus (LPAIV) based on its pathogenicity in chickens (Bi et al., 2020).

Recently, studies have reported the widespread occurrence of highly pathogenic avian influenza viruses (HPAIVs), including H5N1, H5N6, and H5N8, which may be due to wild birds, known as natural reservoirs for AIVs (Bi et al., 2016a; Bi et al., 2016b; Chen et al., 2019). Meanwhile, wild birds infected with HPAIVs may come in contact with domestic poultry. Previous studies on AIVs in China revealed that clade 2.3.4.4 (H5Nx) HPAIVs have become dominant in both wild birds and domestic animals since the first poultry-infected case emerged in 2008 (Bi et al., 2020).

In January 2021, A/goose/hebei/HG12/2021 (H5N8) (HG12) was isolated from cloacal swabs of domestic geese (no symptoms) near Yishui Lake, Baoding City, Hebei Province, China (Fig. 1A). Swab samples from domestic geese were inoculated into 10-day-old specific pathogen-free (SPF) chicken embryos and cultured at 37 °C for 48 h. The isolated virus with positive HA test was subcultured 3 times in 10-day-old SPF chicken embryos. Allantoic fluid from chicken embryos was collected and stored at −80 °C. Viral RNA was extracted from allantoic fluid for specific reverse transcriptional PCR and sequencing of the influenza virus genome was completed as previously described (Hoffmann et al., 2001). Then the genome sequences were submitted to Genebank with the accession numbers OM868039 to OM868040, ON878716 to ON878721. Cluster W was used for comparison between reference sequences and the isolated strain in this study. The GTRGAMMA nucleotide substitution model in PhyML 3.1 software was used, and 1000 bootstrap replicates were run to evaluate the maximum likelihood (ML) phylogenies. Phylogenetic trees were constructed and visualized using Figtree V 1.4.3 software.

According to the phylogenetic analysis of the complete genome, the isolate HG12 belongs to the branch 2.3.4.4b of HPAIV (H5N8) (Fig. 1B). As shown in Fig. 1C, the *HA* and *NA* genes of A/goose/Hebei/HG12/2021(H5N8) had high homology to those isolates from wild birds in Russia, Korea, Kazakhstan and provinces of China, including Inner

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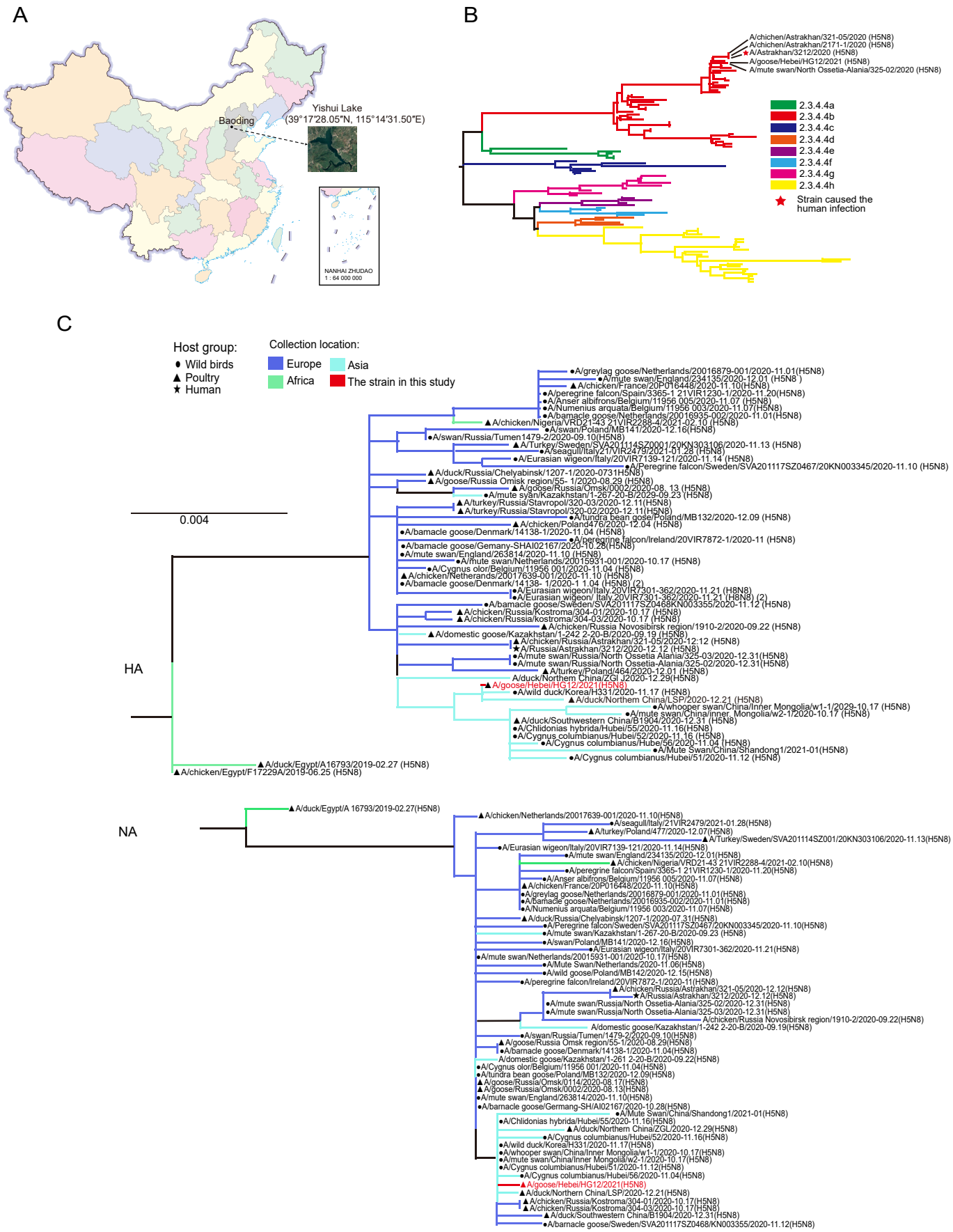
A
Baoding
Yishui Lake
(39°17′28.05″N, 115°14′31.50″E)
NANHAI ZHUDAO
1 : 64 000 000
B
A/chichen/Astrakhan/321-05/2020 (H5N8)
A/chichen/Astrakhan/2171-1/2020 (H5N8)
A/Astrakhan/3212/2020 (H5N8)
A/goose/Hebei/HG12/2021 (H5N8)
A/mute swan/North Ossetia-Alania/325-02/2020 (H5N8)
2.3.4.4a
2.3.4.4b
2.3.4.4c
2.3.4.4d
2.3.4.4e
2.3.4.4f
2.3.4.4g
2.3.4.4h
Strain caused the human infection
C
Host group:
Wild birds
Poultry
Human
Collection location:
Europe
Asia
Africa
The strain in this study
0.004
HA
A/greylag goose/Netherlands/20016879-001/2020-11.01(H5N8)
A/mute swan/England/234135/2020-12.01 (H5N8)
A/chicken/France/20P016448/2020-11.10(H5N8)
A/peregrine falcon/Spain/3365-1 21VIR1230-1/2020-11.20(H5N8)
A/Anser albifrons/Belgium/11956 005/2020-11.07 (H5N8)
A/Numenius arquata/Belgium/11956 003/2020-11.07(H5N8)
A/bamacle goose/Netherlands/20016935-002/2020-11.01(H5N8)
A/chicken/Nigeria/VRD21-43 21VIR2288-4/2021-02.10 (H5N8)
A/swan/Poland/MB141/2020-12.16(H5N8)
A/swan/Russia/Tumen1479-2/2020-09.10(H5N8)
A/Turkey/Sweden/SVA201114SZ0001/20KN303106/2020-11.13 (H5N8)
A/seagull/Italy21VIR2479/2021-01.28 (H5N8)
A/Eurasian wigeon/Italy/20VIR7139-121/2020-11.14 (H5N8)
A/Peregrine falcon/Sweden/SVA201117SZ0467/20KN003345/2020-11.10 (H5N8)
A/duck/Russia/Chelyabinsk/1207-1/2020-0731H5N8)
A/goose/Russia Omsk region/55- 1/2020-08.29 (H5N8)
A/goose/Russia/Omsk/0002/2020-08. 13 (H5N8)
A/mute swan/Kazakhstan/1-267-20-B/2029-09.23 (H5N8)
A/turkey/Russia/Stavropol/320-03/2020-12.11(H5N8)
A/turkey/Russia/Stavropol/320-02/2020-12.11(H5N8)
A/tundra bean gose/Poland/MB132/2020-12.09 (H5N8)
A/chicken/Poland476/2020-12.04 (H5N8)
A/bamacle goose/Denmark/14138-1/2020-11.04 (H5N8)
A/peregrine falcon/Ireland/20VIR7872-1/2020-11 (H5N8)
A/bamacle goose/Gemany-SHAI02167/2020-10.28(H5N8)
A/mute swan/England/263814/2020-11.10 (H5N8)
A/mute swan/Netherlands/20015931-001/2020-10.17 (H5N8)
A/Cygnus olor/Belgium/11956 001/2020-11.04 (H5N8)
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A/bamacle goose/Denmark/14138- 1/2020-1 1.04 (H5N8) (2)
A/Eurasian wigeon/Italy 20VIR7301-362/2020-11.21 (H8N8)
A/Eurasian wigeon/ Italy 20VIR7301-362/2020-11.21 (H8N8) (2)
A/bamacle goose/Sweden/SVA201117SZ0468KN003355/2020-11.12 (H5N8)
A/chicken/Russia/kostroma/304-01/2020-10.17 (H5N8)
A/chicken/Russia/kostroma/304-03/2020-10.17 (H5N8)
A/chicken/Russia Novosibirsk region/1910-2/2020-09.22 (H5N8)
A/domestic goose/Kazakhstan/1-242 2-20-B/2020-09.19 (H5N8)
A/chicken/Russia/Astrakhan/321-05/2020-12:12 (H5N8)
A/Russia/Astrakhan/3212/2020-12.12 (H5N8)
A/mute swan/Russia/North Ossetia Alania/325-03/2020-12.31(H5N8)
A/mute swan/Russia/North Ossetia-Alania/325-02/2020-12.31(H5N8)
A/turkey/Poland/464/2020-12.01 (H5N8)
A/duck/Northern China/ZGL J2020-12.29(H5N8)
A/goose/Hebei/HG12/2021(H5N8)
A/wild duck/Korea/H331/2020-11.17 (H5N8)
A/duck/Northern China/LSP/2020-12.21 (H5N8)
A/whooper swan/China/Inner Mongolia/w1-1/2029-10.17 (H5N8)
A/mute swan/China/inner. Mongolia/w2-1/2020-10.17 (H5N8)
A/duck/Southwestern China/B1904/2020-12.31 (H5N8)
A/Chlidonias hybrida/Hubei/55/2020-11.16(H5N8)
A/Cygnus columbianus/Hubei/52/2020-11.16 (H5N8)
A/Cygnus columbianus/Hube/56/2020-11.04 (H5N8)
A/Mute Swan/China/Shandong1/2021-01(H5N8)
A/Cygnus columbianus/Hubei/51/2020-11.12 (H5N8)
A/duck/Egypt/A16793/2019-02.27 (H5N8)
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NA
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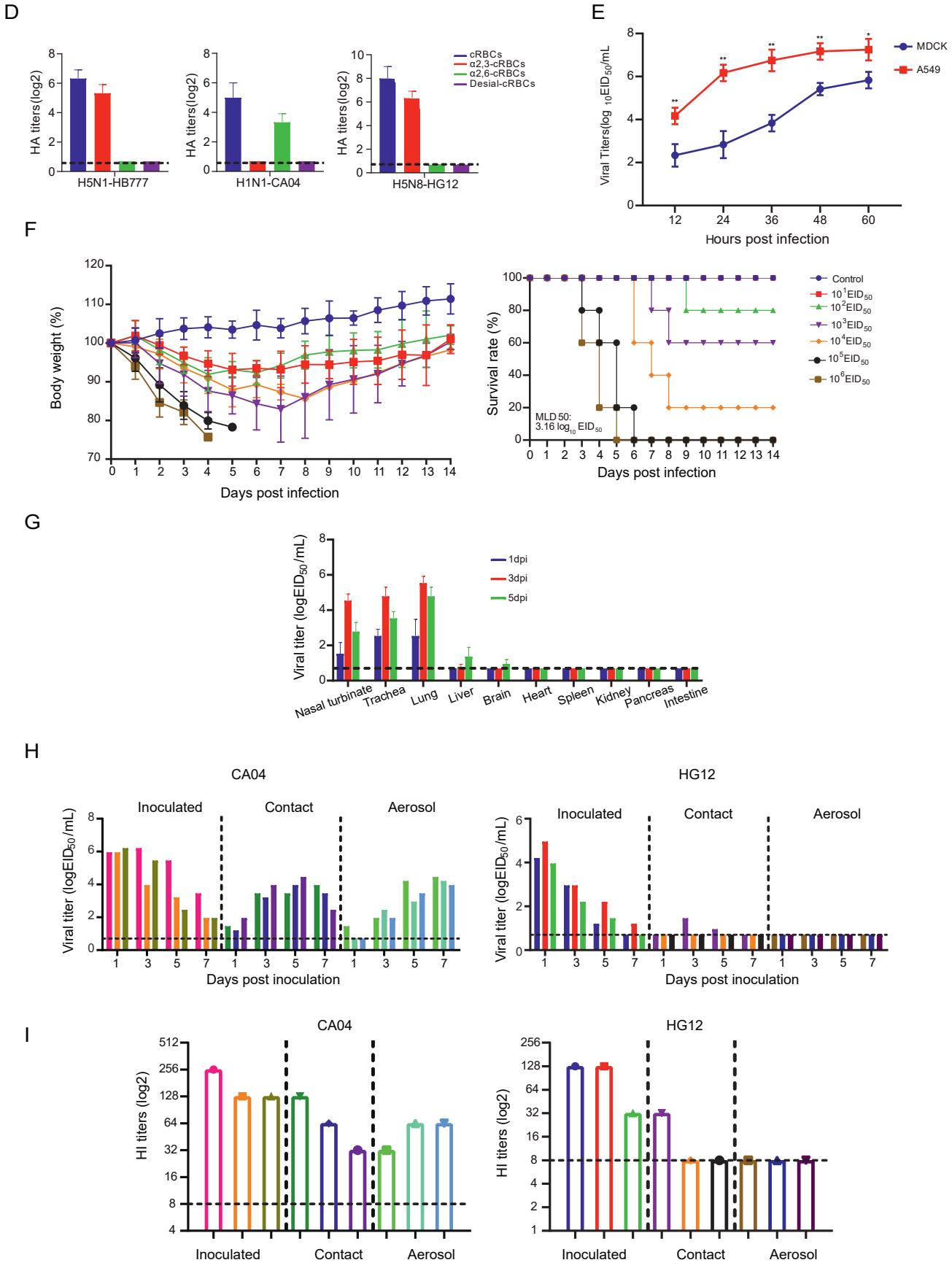

Fig. 1. *(continued)*

Mongolia, Hubei and Shandong. The high homology of *HA* and *NA* genes between HG12 from domestic geese and other isolates from wild birds could be due to the same reassortant transmitted by the wild birds or different reassortants but the same *HA/NA* genes with those from wild birds. Meanwhile, the highly homologous strains came from a variety of wild birds (mute swan, wild duck, whooper swan, Chlidonias hybrida, *Cygnus columbianus*, etc.) and poultry (chicken, domestic goose, duck, etc.), suggesting that the virus might have the ability to spread between wild and domestic birds. Importantly, this isolate was more closely related to another strain, A/Russia/Astrakhan/3212/2020-12.12 (H5N8), which was isolated from humans in Russia (Pyankova et al., 2021). The homology of eight genes between HG12 and the isolate in Russian was 99.2%–99.8%. Meanwhile, the genetic evolution analysis of the *HA* gene showed that the two strains were located in the same evolutionary branch, indicating that the two strains were closely related. Interestingly, the homology of HG12 and strains isolated in Turkey and Poland suggested that transportation of poultry or wild animal migration may also contribute to the transregional transmission of influenza (Normile, 2006; Dalby and Iqbal, 2015).

The receptor binding specificity of the virus was determined by HA analysis with 1% chicken red blood cells (CRBCs). The poultry isolate A/chicken/Hebei/HB777/2006 (H5N1) and human isolate A/California/04/2009 (H1N1) viruses were used as controls for preferential binding to avian-type SAα-2,3-Gal and human-type SAα-2,6-Gal, respectively. As shown in Fig. 1D, HG12 preferentially bound avian-type SAα-2,3-Gal. In addition, the growth kinetics of HG12 virus in A549 and Madin-Darby canine kidney (MDCK) cells were compared. HG12 had better replication capacity in A549 cells than in MDCK cells (Fig. 1E), which suggested its potential of infection in humans. Then the pathogenicity of HG12 virus to mammals was studied in BALB/c mice model. Groups of five 6-week-old female BALB/c mice (Beijing Weitonglihua Laboratory Animal Technology Co., LTD.) were anesthetized with isoflurane and inoculated intranasally with 50 μL suspension containing 10^1–10^6 50% egg infectious dose (EID_{50}) virus, respectively. As shown in Fig. 1F, HG12 was highly pathogenic to mice, and the virus was distributed in turbinate bone, trachea, and lung (Fig. 1G). The survival rate of mice at 10^3 EID_{50} dose was 60%. In this study, the mouse lethal dose 50% (MLD_{50}) of the HG12 strain was calculated to be $10^{3.16}$ EID_{50}, which is approximately 100–1000 times lower than those in previous studies on H5N8 (Kim et al., 2014; Pulit-Penaloza et al., 2015; Yu et al., 2018). A portion of the lung was fixed at 3 days post infection (dpi, the infection dose was $10^{5.0}$ EID_{50}) for histopathological examination. As shown in the Supplementary Figure S1, the pulmonary arterioles and alveolar wall capillaries of mice infected with HD12 were filled with a large number of red blood cells and were severely congested. The alveolar wall of infected mice was infiltrated with lymphocytes. Some epithelial cells of the bronchiole degenerated, and there were exfoliated epithelial cells and mucous material in the bronchioles.

Nine Hartley strain albino female guinea pigs (300–350 g) purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd. were randomly allocated into three groups for studying the transmission of HG12 virus in mammals: the inoculated group, the contact group and the aerosol group (n = 3 per group) (Zhang et al., 2021). The human isolate A/California/04/2009 (H1N1) was used as the control group. Three guinea pigs in each group were inoculated intranasally with 200 μL of 10^5 EID_{50} virus. At 24 hpi, three additional unvaccinated guinea pigs were placed in the same isolation unit for direct contact transmission studies, and three additional unvaccinated guinea pigs from each group were placed in adjacent cages to monitor aerosol transmission. Four nasal rinses (1, 3, 5, and 7 dpi) were collected from all animals and titrated in SPF embryo eggs. Nasal wash samples were then collected using 1 mL sterile PBS (supplemented with 1% penicillin/streptomycin). Serum was collected from all guinea pigs at 21 dpi, and hemagglutination inhibition tests were performed as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Stear, 2005). As shown in Fig. 1H and I, HG12 had much lower contact transmissibility than CA04 strain, with a 33.3% transmission rate (one of three guinea pigs in the direct contact group was positive), and no aerosol transmissibility among mammals was detected.

In this study, the HPAIV (H5N8) of branch 2.3.4.4b was isolated for the first time from domestic geese near Yishui Lake in Hebei Province in 2021. At present, China's ecological niche for influenza monitoring is mainly located in Qinghai Lake, Poyang Lake, Dongting Lake, Shanghai Chongming Island. In our study, we found that Yishui Lake may be another important ecological niche warranting continuous monitoring.

Based on the phylogenetic analysis of the virus and the migration patterns of wild birds, we speculate that the H5N8 virus in this study may be transmitted by migratory birds from Siberia to central China and infected domestic geese through the ecological niche of Yishui Lake. In MDCK and A549 cells, HG12 virus showed strong growth characteristics *in vitro*, which suggest that it may have strong pathogenicity to mammals.

It is worth noting that HG12 virus showed enhanced pathogenicity in mice. The MLD_{50} of A/goose/Hebei/HG12/2021 was nearly 100–1000 times lower than that of the previously circulating H5N8 strain. The MLD_{50} of A/Gyrfalcon/Washington/41088-6/2014, A/mallard duck/Korea/W452/2014/MDk/W452 (H5N8) and A/mallard/Shanghai/SH-9/2013 (H5N8) in mice were $10^{6.4}$ EID_{50}, $10^{5.5}$ EID_{50} and $10^{5.75}$ EID_{50} (Kim et al., 2014; Pulit-Penaloza et al., 2015; Yu et al., 2018). Given the ability of the H5N8 virus to spread rapidly in birds and produce new strains through recombination, continuous and close surveillance of the avian influenza virus in migratory birds as well as poultry is necessary. The homology between HG12 and A/Russia/Astrakhan/3212/2020-12.12(H5N8) (Pyankova et al., 2021) was very high, and we should be vigilant for human epidemic or pandemic caused by H5N8.

There is increasing concern about H5N8 AIV due to its widespread distribution among a variety of poultry and wild animals around the world. The prevalence and evolution of H5N8 were influenced by the long-distance migration of wild birds, the recombination ability of AIVs and changes in receptor binding ability. Although the HG12 strain in this study was found during routine surveillance (from healthy domestic geese) and did not cause a large-scale infection, we still need to pay constant attention to it. At the peak of winter bird migration, wetlands or lakes are hot spots for birds to gather and spread the virus. Therefore, the detection of avian influenza virus needs to be strengthened to provide a

Fig. 1. Geographical location, genetic evolution and pathological characteristics of the isolate A/goose/Hebei/HG12/2021(H5N8). **A** The location where the swab sample was collected. **B** Phylogenetic analysis of the branch of genetic evolution based on the genome of HG12. **C** Phylogenetic tree based on *HA* and *NA* genes of HG12. **D** Agglutination activities of viruses for various red blood cells. Blue: chicken red blood cells (cRBCs); Red: a-2,3 cRBCs (treated with VCNA and resialylated with a-2,3 glycans); Green: a-2,6 cRBCs (treated with VCNA and resialylated with a-2,6 glycans); Purple: desialylated (Desial) cRBCs (treated with VCNA). All assays were performed in at least three independent experiments. The error bars represent the standard deviation. **E** Growth dynamics of HG12 in A549 and Madin-Darby canine kidney (MDCK) cells. Viruses were collected from the supernatant of cells at 12, 24, 36, 48 and 60 hours post infection, and the viral titer was determined in 9-day-old SPF embryonated chicken eggs. **F** Body weight changes of mice (left) and the survival percentage of mice (right) infected with different amounts of HG12 virus (n = 5). **G** The virus titers in different tissues of BALB/c mice at 1, 3 and 5 dpi after infection with HG12 virus were detected in chicken embryos (n = 5). Dotted lines indicate the lowest detection limit. **H** Comparison of contact and aerosol transmissibility of CA04 and HG12 viruses. Three guinea pigs were included in each group. Nasal wash of each pig was collected to detect the virus titer at 1, 3, 5, 7 dpi. **I** The hemagglutination inhibition (HI) antibody titers of serums from each guinea pig in (**H**) were determined at 21 days post infection.

strong guarantee for public health safety and zoonosis prevention and control. What's more, monitoring of HPAIVs should be strengthened. When significant antigen variation is found, the vaccine should be updated in time to build an immune barrier. In addition, reducing small-scale, home-based poultry farming, and increasing large-scale, high-standard modern poultry farming with high-level biosafety management and personal protection measures will help reduce HPAIVs transmission and potential human infection.

Footnotes

Experimental processes were approved by the Animal Care and Use Committee of Changchun Veterinary Research Institute (approval number: SCXK, 20210099). All experiments with the influenza A (H5N8) virus were performed in an animal biosafety level-3 laboratory (ABSL-3). The authors declare that they have no conflicts of interest. This study was supported by the National Natural Science Foundation of China (82150202), the Beijing Nova Program (Z211100002121064) and the Key Research Projects in Hebei Province (18227517D).

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