



Genetic and antigenic characterization of H5 and H7 avian influenza viruses isolated from migratory waterfowl in Mongolia from 2017 to 2019

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Abstract

The circulation of highly pathogenic avian influenza viruses (HPAIVs) of various subtypes (e.g., H5N1, H5N6, H5N8, and H7N9) in poultry remains a global concern for animal and public health. Migratory waterfowls play important roles in the transmission of these viruses across countries. To monitor virus spread by wild birds, active surveillance for avian influenza in migratory waterfowl was conducted in Mongolia from 2015 to 2019. In total, 5000 fecal samples were collected from lakesides in central Mongolia, and 167 influenza A viruses were isolated. Two H5N3, four H7N3, and two H7N7 viruses were characterized in this study. The amino acid sequence at hemagglutinin (HA) cleavage site of those isolates suggested low pathogenicity in chickens. Phylogenetic analysis revealed that all H5 and H7 viruses were closely related to recent H5 and H7 low pathogenic avian influenza viruses (LPAIVs) isolated from wild birds in Asia and Europe. Antigenicity of H7Nx was similar to those of typical non-pathogenic avian influenza viruses (AIVs). While HPAIVs or A/Anhui/1/2013 (H7N9)-related LPAIVs were not detected in migratory waterfowl in Mongolia, sporadic introductions of AIVs including H5 and H7 viruses into Mongolia through the wild bird migration were identified. Thus, continued monitoring of H5 and H7 AIVs in both domestic and wild birds is needed for the early detection of HPAIVs spread into the country.

Keywords Avian influenza · Characterization · Migratory waterfowl · Mongolia · Surveillance

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Introduction

Surveillance of avian influenza in wild birds has increased substantially worldwide in recent years because of the spread of H5 highly pathogenic avian influenza viruses (HPAIVs) among domestic poultry and wild birds in Asia, Europe, and Africa [1, 2]. Since the emergence of H5N1 HPAIVs in Asia

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[3], numerous global efforts have focused on elucidating the relative roles of wild bird and poultry movement in virus dissemination. To better understand the ecology of avian influenza viruses (AIVs) in wild birds, the data from wild bird surveillance studies are used to identify factors correlated with AIV detection in wild birds, such as reservoir species, bird health status, age, season, and location [4].

Each of the known subtype (H1–H16 and N1–N9) of influenza A virus (IAV) has been isolated from waterfowl, especially migratory wild ducks, that are infected with the viruses via waterborne transmission at their nesting lakes close to the Arctic Circle in Siberia, Alaska, and Canada during their breeding season in summer. These viruses replicate in columnar epithelial cells, forming crypts in the colon, and they are excreted in feces [5]. The infections of these AIVs do not cause illness in birds; however, current H5 HPAIVs that have been isolated in Asia, Europe, and Africa have caused death in several wild bird species [6].

Mongolia is located on three flyways of wild birds, namely the East Asian–Australasian, Central Asian, and East African–West Asian flyways, through which wild birds migrate from their northern territory in Siberia to the southern regions. In this context, intensive surveillance of AIVs in Mongolia has been conducted since autumn 1996 [7, 8]. Accordingly, the surveillance for migratory waterfowl in central Mongolia is essential for monitoring AIVs that were maintained in nesting lakes in Siberia and that spread southward with their migration, especially if recent H5 HPAIVs in Asia and H7N9 AIVs in China were brought to the north.

In the present study, to monitor AIVs among wild bird populations in Mongolia, fresh duck fecal samples were collected during autumn surveillance from 2015 to 2019. In total, 167 AIVs were isolated from 5,000 fecal samples, and two H5N3, four H7N3, and two H7N7 viruses isolated in 2017 and 2019 were characterized. This study aimed to analyze H5 and H7 AIVs in Mongolia, genetically and antigenically to clarify relations with recent H5 and H7 AIVs, especially HPAIVs and A/Anhui/1/2013 (H7N9)-related low pathogenic avian influenza viruses (LPAIVs).

Materials and methods

Isolation and identification of viruses

In each year, 1000 duck fecal samples were collected in the central region of Mongolia, including the Arkhangai Province (Ugii nuur, 47° 76' N, 102° 74' E; Doitiin tsagaan nuur, 47° 37' N, 102° 31' E; Duruu tsagaan nuur, 49° 00' N, 101° 12' E; Tsagaan nuur, 48° 23' N, 102° 35' E; Alagzegstei nuur, 47° 37' N, 102° 32' E) and Bulgan Province (Khunt nuur 48° 25' N, 102° 34' E; Khunt rashaan nuur, 48° 27' N, 102° 32' E; Sharga nuur, 48° 55' N, 101° 56' E),

annually from 2015 to 2019. Fecal samples were stored at a temperature of less than 10 °C and mixed with transport medium [minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10,000 U/ml penicillin G, 10 mg/ml streptomycin, 0.3 mg/ml gentamicin, and 0.5% bovine serum albumin] before virus isolation using embryonated chicken eggs. At first, all samples were inoculated into the allantoic cavity of 10-day-old chicken embryos and incubated for 48 h at 35 °C. After incubation, the infectious allantoic fluid was harvested, and the hemagglutination titer was determined using 0.5% chicken red blood cells. For further characterization, the subtypes of influenza viruses were identified via hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using the reference antisera of AIVs [7].

Sequencing and phylogenetic analysis

For the genetic analysis, viral RNA was extracted from the allantoic fluid of infected chicken embryos using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA). Viral RNA was reverse-transcribed using the Uni12 primer [9] and M-MLV reverse transcriptase (Life Technologies), then the full-length hemagglutinin (HA) and other internal gene segments were amplified via polymerase chain reaction using gene-specific primer sets [9]. Direct sequencing of gene segments was performed using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic Analyzer (Life Technologies). For sequencing some isolates, next-generation sequencing was applied as follows. MiSeq libraries were prepared using KAPA RNA Hyper Prep Kit (Illumina, Inc., San Diego, CA, USA) and KAPA Dual-Indexed Adapter Kit (Roche, Basel, Switzerland). The prepared MiSeq libraries were sequenced on a MiSeq by using MiSeq Reagent kit v3 (Illumina, Inc.) with 2 × 300 bp paired-end read length. The sequencing data were analyzed using GENE-TYX Network version 12 (Genetyx Co., Tokyo, Japan), GeneStudio (<https://genestudio.com/>), or CLC Genomics Workbench 12 (Qiagen, Hilden, Germany). The gene sequences obtained in the present study have been registered at GenBank (Supplemental Table 1).

Phylogenetic analysis of H5 or H7 HA gene was performed by the maximum likelihood (ML) method using the Tamura–Nei model and bootstrap analysis ($n = 1000$) using MEGA7.0 software with default parameters [10]. The sequence data from two H5 HA and six H7 HA genes were compared with the reference sequences obtained from public databases, respectively. For the reference sequences, the nucleotide sequences of classical and recent H5 and H7 viruses were downloaded from GenBank/EMBL/DDBJ and Global Initiative on Sharing All Influenza Data (GISAID).

Table 1 Influenza viruses isolated from migratory waterfowls in Mongolia during the surveillance in autumn between 2015 and 2019

Location	Subtypes of influenza virus				
	2015 (40/1000 samples)	2016 (10/1000 samples)	2017 (21/1000 samples)	2018 (73/1000 samples)	2019 (23/1000 samples)
Arkhangai province	H1N1 (1)	H3N8 (1)	H2N2 (1)	H2N3 (8)	H3N6 (2)
	H1N2 (1)	H4N6 (6)	H3N8 (4)	H2N4 (3)	H3N8 (2)
	H3N8 (15)		H4N6 (9)	H3N6 (3)	H4N2 (3)
	H4N6 (3)		H10N9 (1)	H3N8 (24)	H4N6 (7)
	H6N2 (1)			H4N1 (1)	H5N3 (1)
	H10N2 (2)			H4N6 (17)	H7N7 (2)
	H10N3 (2)			H12N5 (2)	
Bulgan province	H1N1 (1)	H3N8 (1)	H3N8 (2)	H3N1 (1)	H3N7 (1)
	H2N3 (2)	H4N6 (2)	H7N3 (4)	H3N8 (10)	H3N8 (2)
	H3N6 (1)			H12N5 (4)	H4N2 (1)
	H3N8 (4)				H4N6 (1)
	H4N6 (2)				H5N3 (1)
	H10N3 (1)				
	H10N7 (4)				

H5 and H7 viruses are shown in bold

The number of isolates of each antigenic subtype is shown in parenthesis

Antigenic analysis

The antigenic properties of representative H7Nx isolates were determined via the cross-HI test using chicken polyclonal antisera. Chicken polyclonal antisera against representative influenza virus strains were prepared, and HI tests were performed as previously described [11]. To visualize the antigenic character of the H7 viruses, the antigenic map was built using the web-based software (<https://acmacs-web.antigenic-cartography.org/>), and the test result containing cross-HI titers were uploaded to obtain x/y coordinates of each antiserum and antigen.

Results

Isolation of IAIVs from the fecal samples of migratory waterfowl

The surveillance was targeted migratory waterfowl in four main lakes located in the Arkhangai and Bulgan Province of Mongolia. In total, 167 influenza viruses were isolated from 5000 fecal samples of migratory waterfowl (Table 1 and Supplemental Table 1); respectively, 40, 10, 21, 73, and 23 AIVs were isolated each autumn from 2015 to 2019. The isolation rate of AIVs for each autumn over 5 years was 1.0–7.3%, and the highest number of isolated viruses was observed in the Arkhangai Province. Among each sampling site, Doitiin Tsagaan nuur in the Arkhangai Province and Khunt nuur in the Bulgan Province were found to be the

highest percentage of isolated virus during the overall surveillance time. The majority of HA subtypes of all isolates was H3 and H4.

In the present study, further characterization was conducted using two H5N3 and six H7Nx isolates. The two H5N3 isolates were A/duck/Mongolia/419/2019 and A/duck/Mongolia/926/2019 from the Arkhangai and Bulgan Provinces. Four H7N3 isolates were A/duck/Mongolia/652/2017, A/duck/Mongolia/751/2017, A/duck/Mongolia/782/2017, and A/duck/Mongolia/786/2017 collected in Bulgan Province. Two H7N7 isolates, namely A/duck/Mongolia/1/2019 and A/duck/Mongolia/6/2019, were collected in Arkhangai Province.

Genetic and phylogenetic analysis of H5 and H7 subtype viruses

The full-length HA gene sequences of the H5 and H7 isolates were characterized. The deduced amino acid sequences of the HA cleavage site of the two H5N3 isolates were PQRETR/GLF and PQREIR/GLF, indicating that they were LPAIVs. The HA receptor-binding site of the H5N3 isolates was analyzed, and residues at positions 226 and 228 were identified as Q and G, respectively, suggesting avian-type receptor specificity. None of the H5N3 subtype viruses contained amino acid substitutions E627K in PB2, which is the molecular marker for mammalian adaptation of AIVs and indicates increasing viral pathogenicity to mammals. Furthermore, the HA cleavage sites of the four H7N3 and two H7N7 isolates were analyzed, and the determined sequence

was PELPKGR/GLF and PEIPKGR/GLF, respectively, suggesting that the viruses were LPAIVs. The HA receptor-binding site of H7Nx isolates was also analyzed, and HA proteins of isolates had residues of 226Q and 228G, indicating avian-type receptor specificity. Similarly, H7Nx LPAIVs did not involve a mutation for potential mammalian adaptation in PB2 protein. Taken together, these data indicate that all H5 and H7 isolates were LPAIVs without mammalian adaptation markers.

The HA gene of these isolates was phylogenetically analyzed by the ML method along with reference strains of HPAIVs and LPAIVs (Figs. 1 and 2). In this phylogenetic analysis, H5 HA genes were divided into two lineages: Eurasian and North American. The Eurasian lineage was

clustered into two sub-lineages: Gs/Gd-like and non-Gs/Gd [12]. All H5N3 isolates from migratory waterfowl in Mongolia in 2019 belonged to the non-Gs/Gd sub-lineage that clustered with H5N3 subtype viruses recently reported in Asia and were distinct from Gs/Gd-like viruses. For other gene segments, each segment was classified together with multivariable subtype viruses circulating among wild birds (Supplemental Figs. S1–S5, S7, S8). Moreover, the H7 HA genes were phylogenetically divided into several lineages: Eurasian, Australian, Historical Europe, and North American. Eurasian lineage was further clustered into three sub-lineages: European–Asian, Far Eastern, and Chinese H7N9 [7]. All H7Nx isolates isolated in Mongolia in 2017 and 2019 belonged to the European–Asian

Fig. 1 Phylogenetic tree for H5 HA genes of AIVs. Full-length sequences of HA genes of two H5 subtype viruses were analyzed by the ML method along with those of reference strains using MEGA7.0 software. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1000 replications. The numbers below or above the node indicate bootstrap values $\geq 60\%$. The viruses isolated in this study are highlighted in gray. The previous isolates in our surveillance are indicated with black triangle symbol. HPAIVs are indicated in bold

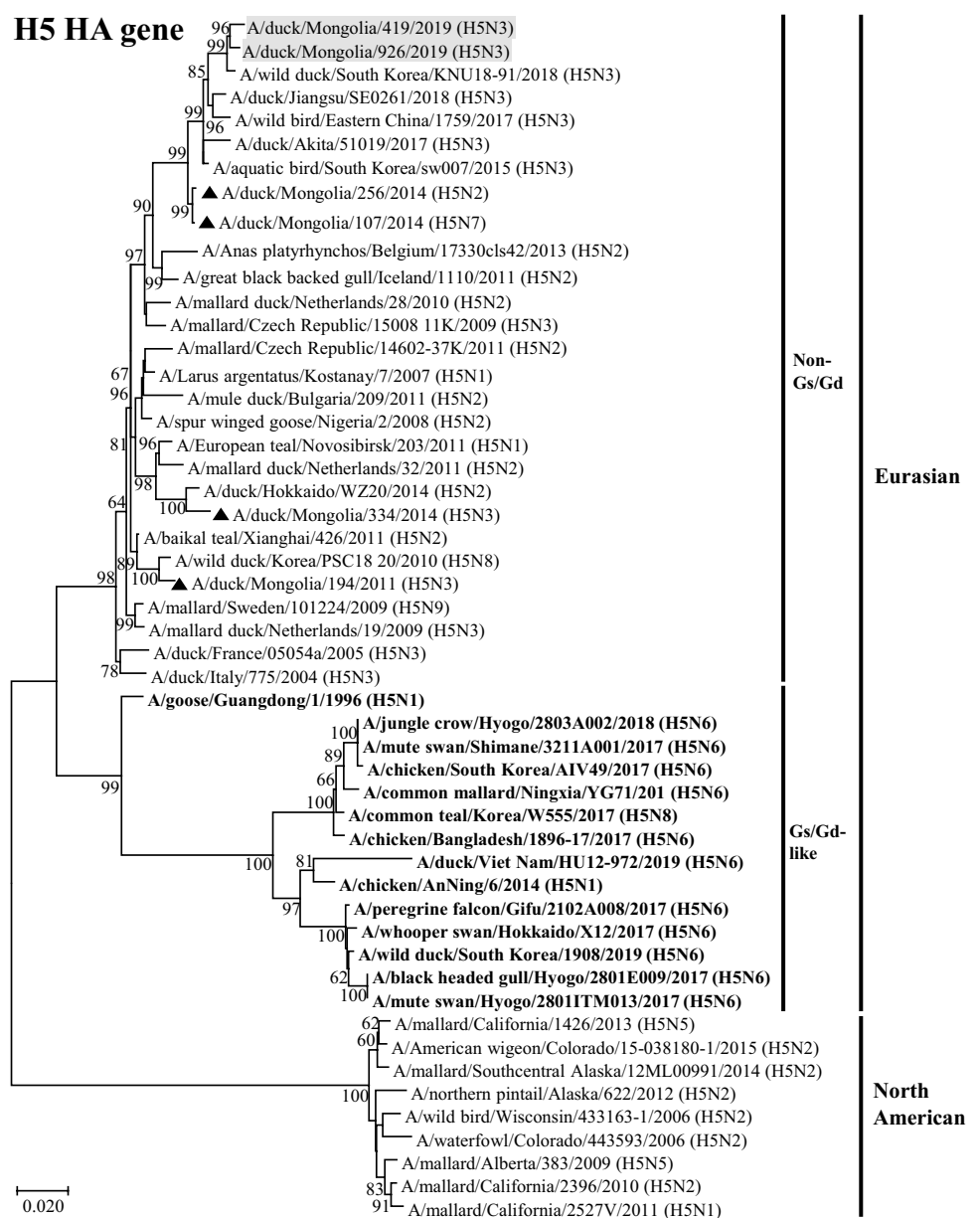
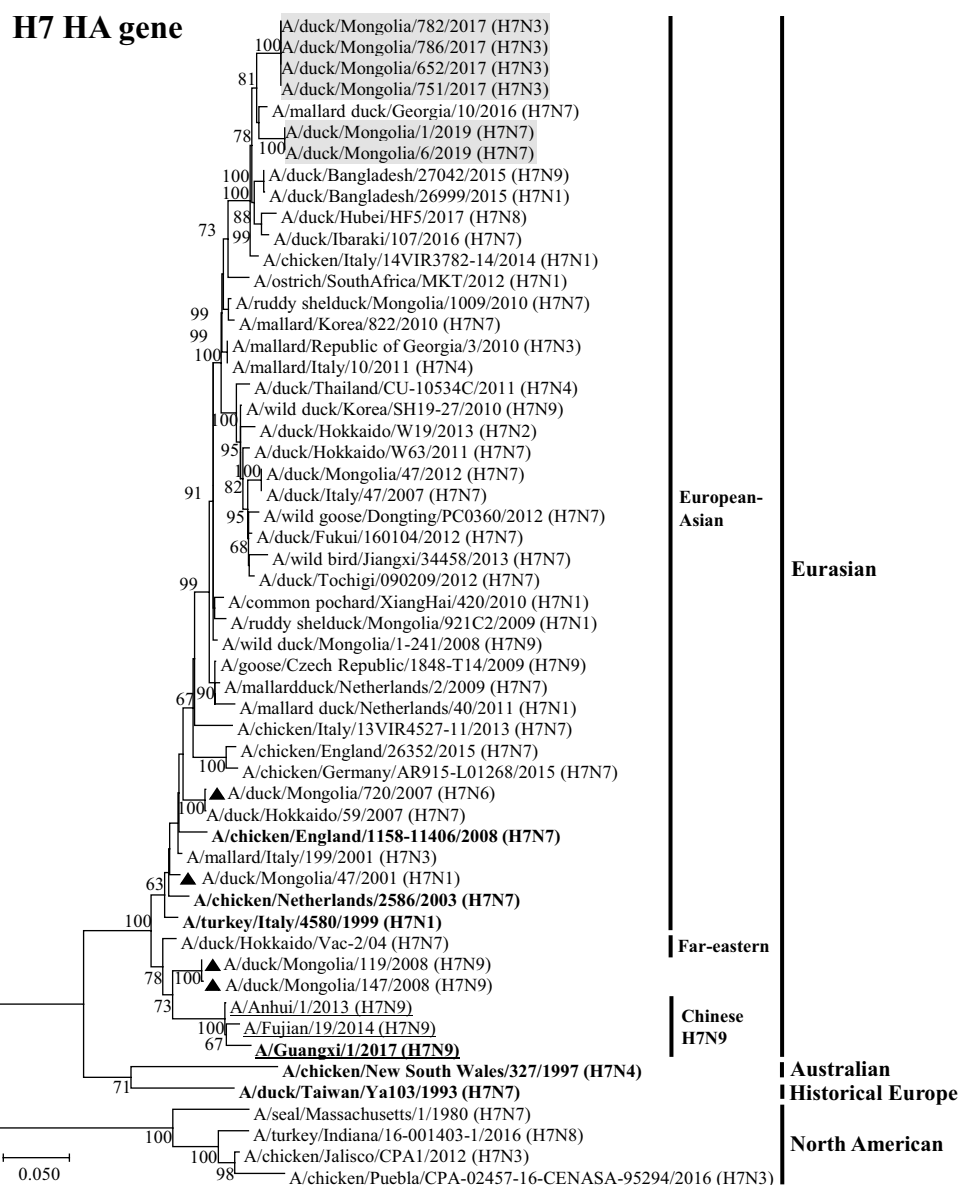


Fig. 2 Phylogenetic tree for the H7 HA genes of IAVs. Full-length sequences of HA genes of six H7 subtype viruses were analyzed by the ML method along with those of reference strains using MEGA7.0 software. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1000 replications. The numbers below or above the node indicate bootstrap values $\geq 60\%$. The viruses isolated in this study are highlighted in gray. The previous isolates in our surveillance are indicated with black triangle symbol. Highly pathogenic IAVs are indicated in bold and Chinese H7N9 viruses are underlined



sub-lineage. Reference strains of Chinese H7N9 viruses were classified together with the Far Eastern sub-lineage; therefore, H7N3 and H7N7 isolates of migratory waterfowls had a different ancestor than that of Chinese H7N9 viruses. Other gene segments of H7N3 and H7N7 viruses were also classified with typical LPAIVs circulating in wild birds (Supplemental Figs. S1–S8) [13].

In phylogenetic analysis, the N3 and N7 neuraminidase (NA) genes were classified with Eurasian lineage viruses and had a genetic relationship with other variable N3 and N7 subtype viruses including AIVs isolated in Mongolia in 2010 and 2015. All other internal genes of H7N3 and H7N7 were phylogenetically closely related to recent H7 subtype viruses isolated from wild birds in Asia and Europe.

Antigenic analysis of H7Nx subtype viruses

Representative strains of H7 viruses from Mongolia and the panel of H7 viruses belonging to Eurasian and North American lineage were antigenically analyzed by the cross-HI test using their corresponding antisera (Table 2). HI titers of each antiserum against A/duck/Mongolia/652/2017 (H7N3) and A/duck/Mongolia/1/2019 (H7N7) were closely identical to those against previously isolated H7N2 viruses classified in the European–Asian sub-lineage [7]. The antigenic cartography was built from the cross-HI test which revealed that the present H7N3 and H7N7 strains were antigenically closely related to recent H7 viruses isolated from wild birds. These belonged to the major antigenic group and were distinct from H7 HPAIVs (Supplemental Fig. S9).

Table 2 Cross HI test of H7 influenza viruses with polyclonal antibodies

Lineage	Viruses	Subtype	HI titers of the antiserum						
			Dk/Hok/ W19/13	Ty/ Italy/4580/99	Dk/Hok/ Vac-2/04	Anhui/1/13	Ck/ NSW/327/97	Dk/TW/ Ya103/93	SI/Mass/1/80
Eurasian									
European– Asian	A/duck/Mongolia/652/2017	H7N3	10,240	1280	5120	1280	5120	640	320
	A/duck/Mongolia/1/2019	H7N7	10,240	1280	5120	1280	5120	1280	1280
	A/duck/Hokkaido/W19/2013	H7N2	2560	1280	5120	1280	2560	320	320
	A/turkey/Italy/4580/1999	H7N1	320	<i>1280</i>	160	80	320	80	80
Far Eastern	A/duck/Hokkaido/Vac-2/2004	H7N7	20,480	2560	<i>20,480</i>	2560	10,240	1280	1280
Chinese H7N9	A/Anhui/1/2013	H7N9	5120	1280	5120	<i>2560</i>	5120	320	320
Australian	A/chicken/New South Wales/327/1997	H7N4	5120	1280	1280	640	<i>5120</i>	320	320
Historical Europe	A/duck/Taiwan/Ya103/1993	H7N7	320	80	160	160	160	<i>2560</i>	40
North American	A/seal/Massachusetts/1/1980	H7N7	10,240	2560	20,480	2560	10,240	320	<i>2560</i>

Viruses isolated in this study are shown in bold

Homologous titers are indicated with Italics

Dk duck, *Ty* turkey, *Ck* chicken, *SI* seal, *Hok* Hokkaido, *NSW* New South Wales, *TW* Taiwan, *Mass* Massachusetts

Discussion

H5 or H7 HPAIVs as well as LPAIVs are a global threat to livestock production, distribution systems, and human health. However, sustained, comprehensive, coordinated global efforts to monitor the continually changing genetic diversity of AIVs circulating in nature remain insufficient to predict the emergence of novel HPAIVs and human infections of AIVs. Wild aquatic birds have played a role in the evolution of H5 HPAIVs by reassortment with LPAIVs. These factors have contributed to the spread of these viruses to parts of Asia, Europe, Africa, and North America after an outbreak in wild birds at Qinghai Lake in China in 2005 [14]. Circulations of H7 LPAIVs in wild birds and poultry have been reported throughout the world [7, 13, 15–20]. In addition, H7N9 HPAIVs and LPAIVs are at risk to spread by wild bird and human-caused movements [21, 22]. In our surveillance conducted over the past 5 years, targeting migratory waterfowl in four different lakes in the central region of Mongolia, 167 viruses were isolated. No HPAIVs were isolated from the fecal samples of migratory waterfowl in the present study, indicating that the circulation of HPAIVs in the northern nesting lake is still limited. However, these do not fully represent the country state in terms of the infection of IAV in wild birds.

Two AIVs are currently pandemic threats; H5 HPAIVs have spilled over repeatedly to humans since their first identification in 1997, and H7N9 AIVs, initially detected in March 2013, have caused serious human infections across China [23]. In terms of H7N9 virus infection in humans, a total of 1568 laboratory-confirmed human cases have been reported since early 2013 [24]. A novel H7N9 HPAIV variant possessing multiple basic amino acids at the cleavage site of the HA protein was first reported in two cases of human infection in January 2017 [25]. H5N3 LPAIVs were detected from migratory waterfowl in Mongolia in 2019, and HA gene and other gene segments of those H5 isolates were phylogenetically classified in the Eurasian lineage, sharing identical genetic characteristics with recent H5 LPAIVs isolated from wild birds in Asia. Phylogenetic analysis of H7N3 and H7N7 isolates revealed a close relation to H7 LPAIVs belonging to the European–Asian sub-lineage, but no relation to Chinese H7N9 viruses.

Antigenicity monitoring is important for diagnosis and control measures for HPAIVs and LPAIVs. In the present study, representative H7 viruses were analyzed for antigenic characterization. Kim et al. reported the antigenic difference of viruses in Eurasian group I (referred to as the Far Eastern sub-lineage in this study) and group II (Eurasian–Asian sub-lineage) [15]. Our antigenic analyses revealed that the

antigenicity of H7 viruses isolated from migratory waterfowl was similar to each other regardless of genetic diversity, and antigenic diversity may emerge after the adaptation of H7 viruses to poultry, same as has been reported for North American H7 viruses [19].

Because of the recent increased numbers of zoonotic infections in poultry and human infections in China, influenza A virus (H7N9) has remained a public health threat. It is possible that wild birds infected with Chinese H7N9 viruses transmit them to surrounding countries, as occurs with H5 HP AIVs. Continued circulation of H7 influenza viruses among poultry and wild birds risks widespread dissemination of these viruses. Accordingly, it is important that H5 and H7 viruses are continually monitored among wild waterfowl.

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Author contributions AU and EB performed genetic analyses and antigenic analyses. AU and EB prepared this manuscript. AU, EB, TH, KB, TA, JT, TU, KhB, KM, TH, TK, MS, YT, ST, MI, KO, TS, NK, YK, JM, MI, and AT conducted sampling of feces from migratory wild birds in Mongolia and performed virus isolation and subtyping. MO, KM, AT, HK, DB, and YS provided laboratory management support and manuscript editing.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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