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Prevalence of avian influenza virus in wild birds before and after the HPAI H5N8 outbreak in 2014 in South Korea[§]

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Since 2003, highly pathogenic avian influenza (HPAI) virus outbreaks have occurred five times in Korea, with four HPAI H5N1 outbreaks and one HPAI H5N8 outbreak. Migratory birds have been suggested to be the first source of HPAI in Korea. Here, we surveyed migratory wild birds for the presence of AI and compared regional AI prevalence in wild birds from September 2012 to April 2014 for birds having migratory pathways in South Korea. Finally, we investigated the prevalence of AI in migratory birds before and after HPAI H5N8 outbreaks. Overall, we captured 1617 migratory wild birds, while 18,817 feces samples and 74 dead birds were collected from major wild bird habitats. A total of 21 HPAI viruses were isolated from dead birds, and 86 low pathogenic AI (LPAI) viruses were isolated from captured birds and from feces samples. Spatiotemporal distribution analysis revealed that AI viruses were spread southward until December, but tended to shift north after January, consistent with the movement of migratory birds in South Korea. Furthermore, we found that LPAI virus prevalences within wild birds were notably higher in 2013–2014 than the previous prevalence during the northward migration season. The data from our study demonstrate the importance of the surveillance of AI in wild birds. Future studies including in-depth genetic analysis in combination with evaluation of the movement and ecology of migratory birds might help us to bridge the gaps in our knowledge and better explain, predict, and ultimately prevent future HPAI outbreaks.

Keywords: avian influenza, HPAI, H5N8, migration

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Introduction

Avian influenza (AI) viruses are type A viruses belonging to the family *Orthomyxoviridae*. The influenza virus has been isolated from various mammals, including humans, pigs, horses, minks, and marine mammals as well as from domestic and wild birds; the latter (e.g., waterfowl and shorebirds) are thought to be the source of influenza A viral infections in other animal species. To date, 16 hemagglutinin (HA) and 9 neuraminidase (NA) antigenic subtypes have been isolated from wild birds (Stallknecht and Shane, 1988). Based on their genetic sequences and their ability to cause disease in birds, these viruses are classified as either highly pathogenic (HPAI) or low pathogenic (LPAI) AIs (Webster *et al.*, 1992).

HPAI viruses had been sporadically observed in wild birds prior to 2005; these observations were thought to be associated with the infection of domestic farm birds, and high mortality rates were noted (Gilbert *et al.*, 2012). However, in April 2005, this situation changed after over 6000 migratory birds were found to be infected with the HPAI H5N1 strain in Qinghai Lake in northern China (Chen *et al.*, 2005, 2006; Liu *et al.*, 2005). Since the outbreak in Qinghai, infections in migratory birds in Asia, Europe, and Africa have increased dramatically. The geographic spread of HPAI H5N1 and the concomitant mortality of various wild birds raised concerns about the introduction and propagation of HPAI by specific species of wild birds along their migratory routes (Kilpatrick *et al.*, 2006; Whitworth *et al.*, 2007; Gilbert *et al.*, 2012).

Since 2003, HPAI virus outbreaks have occurred five times in Korea, with four HPAI H5N1 outbreaks and one HPAI H5N8 outbreak (Kim *et al.*, 2014). Migratory birds were suggested to be the initial source of the HPAI in Korea (Jeong *et al.*, 2014). HPAI H5N1 in wild magpie (*Pica pica*) was first reported near poultry farms in 2003 (Kwon *et al.*, 2005). In 2010–2011, 20 cases of HPAI H5N1 infection have been detected in wild birds such as the mallard, baikal teal, mandarin duck, whooper swan, Eurasian eagle owl, white-fronted goose, spot-billed duck, Eurasian sparrow hawk, and common kestrel (Kim *et al.*, 2012). In 2014, a variety of wild birds including the baikal teal, have also been shown to be infected with HPAI H5N8, and the detection of HPAI in wild birds has increased significantly rather than 2010–2011. Furthermore, in both 2010 and 2014, HPAI was even detected in healthy wild birds, emphasizing the importance of surveillance of AI in wild birds (Kim *et al.*, 2011; Jeong *et al.*, 2014).

In this study, we report some of our survey results with the two objectives; first, a comparison of AI virus prevalence in wild birds with migratory pathways in the South Korean peninsula with the sites and times of AI outbreaks, and second, a comparison of AI virus prevalence in migratory birds

before and after the 2014 HPAI H5N8 outbreak, which represented the first case of Korean HPAI identified with this viral strain.

Materials and Methods

Wild bird capture and sampling

Wild birds were lured by scattering rice grains near the rivers of the study area and on the farmlands where they usually feed. Ducks and geese were captured using a cannon net. Doves were captured using a mist-net on the flock breeding grounds. Approximately 1 ml blood was collected from each bird using a 1 or 3 ml sterile syringe from the brachial wing vein depending on the size of the bird. Swab samples were collected from the cloaca and oropharynx using sterile cotton swabs and immediately transported to the laboratory. Tissue samples (from the trachea, kidney, and cecal tonsil) were collected from doves. Sampling and transport were carried out in accordance with standard sampling and transport techniques described by the United Nations Food and Agriculture Organization (FAO) (Whitworth *et al.*, 2007).

Preparation of wild bird feces and dead bird samples

Fecal and swab samples were diluted 10% (v/v) in 1× phosphate-buffered saline (PBS; Gibco BRL), pH 7.4, gently vortexed, and then centrifuged at 1,000 × *g* (1580R; LaboGene). The organs, including the trachea, kidney, and cecal tonsil, were collected from dead wild birds for laboratory testing for the presence of AI. The organ samples were mixed 10% (v/v) in 1× PBS (pH 7.2; Gibco BRL) and then the mixed samples were ground in a sterilized mortar with a small amount of silica sand, and centrifuged at 2,100 × *g* for 10 min.

AI virus detection by reverse transcription-polymerase chain reaction (RT-PCR)

Virus RNA was extracted from 200 µl supernatant from fecal and allantoic fluid using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's recommendations. In order to detect the matrix (M) and nucleo-protein (NP) genes in the extracted RNA obtained from AI viruses, one-step RT-PCR was performed. This was carried out using the AccuPower RocketScript RT-PCR PreMix (Bioneer) and the M52C/M253R (10 pM/µl) M- and NP-1200F/NP-1529R (10 pM/µl) NP-gene specific primers, as previously described (Fouchier *et al.*, 2000; Lee *et al.*, 2001). Cleavage sites of H5 and H7 were also analyzed using one-step RT-PCR. The H5 subtype (Gall *et al.*, 2008) was detected using H5-kha-1/-3 primers (10 pM/µl), and the H7 subtype (Gall *et al.*, 2008; Sidoti *et al.*, 2010) was detected using G.K 7.3/7.4 (20 pM/µl) and H7-F/-R (20 pM/µl) primer sets as described.

Isolation of AI virus

The supernatant from fecal, swab and organ samples treated with streptomycin (2 mg/ml), penicillin (2,000 IU), and kanamycin (0.25 mg) were then inoculated into 11-day-old SPF eggs via the allantoic cavity route and then the eggs were incubated at 37°C for 5 days. This procedure was carried out twice for each sample in eggs. After harvesting the allantoic fluid, a hemagglutination (HA) test and RT-PCR for AI detection and subtyping were conducted.

Serological tests for migratory birds

All sera were tested for the presence of antibodies against the AI virus using a FlockChek* AI MultiS-Screen Antibody Test Kit ELISA kit (IDEXX Laboratories, Inc.) according to the manufacturer's instructions. The hemagglutination inhibition (HI) test for the presence of antibodies against the H5 and H7 subtypes was performed was based on the common β-procedure micro method (Allan *et al.*, 1978).

AI virus subtyping by RT-PCR

RNA from the isolated AI viruses was extracted from 200 µl allantoic fluid from embryonated eggs using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's recommendations. To determine the HA and NA subtypes, one step RT-PCR was performed using gene-specific primer sets. PCR was carried out as described by Hoffmann *et al.* (2001), with modifications. Briefly, cDNA synthesis was performed at 42°C for 20 min, and predenaturation was then carried out at 94°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 45°C for 30 sec, and extension at 72°C for 7 min. An additional extension step was carried out at 72°C for 10 min.

Results

Monitoring the prevalence of AI in South Korea from 2012 to 2014

All surveillance was conducted at migratory bird habitats and major streams. For active surveillance, swab and blood samples were collected from 1,617 individual birds includ-

Table 1. Summary of AI virus surveillance in South Korea from 2012 to 2014

	2012–2013				2013–2014			
	Number of samples	AIV prevalence (%)			Number of samples	AIV prevalence (%)		
		Total	H5	H7		Total	H5	H7
Live birds[a]	804	47.6	0.6	0.0	813	24.0	0.5	0.2
Feces[b]	7,940	2.8	0.1	0.0	10,877	6.2	0.2	0.04
Dead birds[c]	2[d]	0.0	0.0	0.0	72[e]	37.5	29.17	0.0
Total	8,746				11,762			

AI prevalence data were analyzed using serology tests[a] or RT-PCR[b,c]; [d]carcasses of the Eurasian spoonbill (*Platalea leucorodia*); [e]most samples were of ducks and geese that were found dead during the HPAI outbreak. AI, avian influenza; RT-PCR, reverse transcription-polymerase chain reaction; HPAI, highly pathogenic AI.

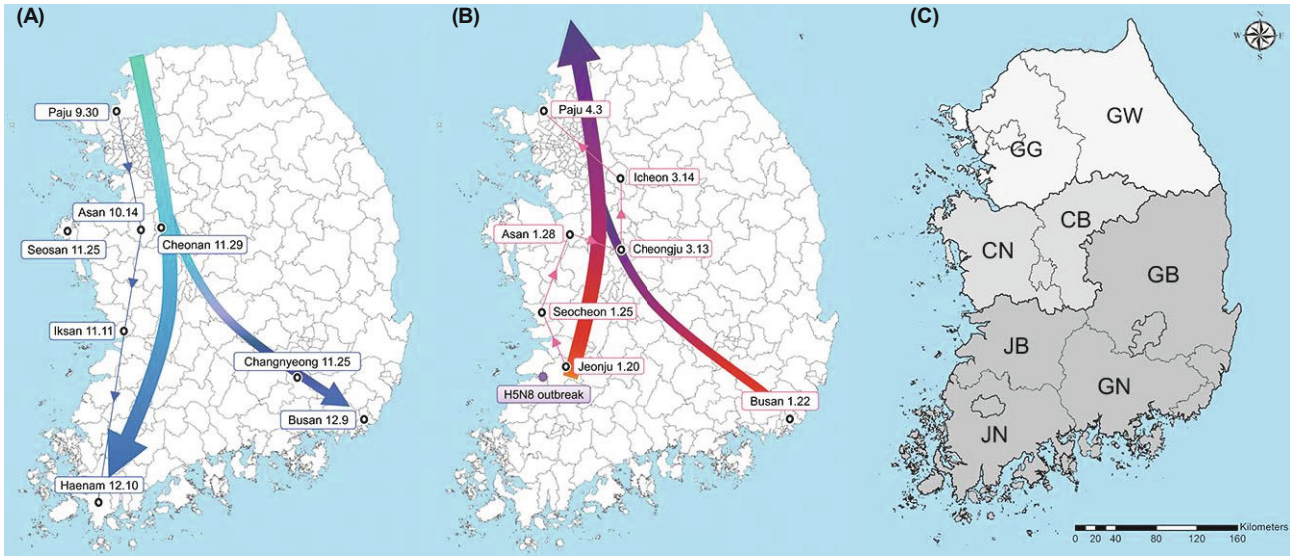

Fig. 1. Spatiotemporal distribution of isolated LPAI viruses from 2013 to 2014. Circles indicate sampling sites of virus isolation. (A) Virus isolation sites from September to December 2013. (B) Virus isolation sites from January to April 2014. (C) South Korea provinces: GG, Gyeonggi; GW, Gangwon; CN, Chungnam; CB, Chungbuk; JB, Jeonbuk; JN, Jeonnam; GB, Gyeongbuk; GN, Gyeongnam. LPAI, low pathogenic avian influenza.

ing ducks and geese (winter birds) and oriental turtle doves (permanent residents). A total of 18,817 environmental fecal samples were collected in the wintering season (from September to April) from 2012 to 2014 (Table 1). The results of AI antibody tests in wild birds confirmed the high prevalence of AI in ducks and geese (35.7%). In contrast, AI antibodies were not detected in pigeons.

In 2012–2013, 7940 environmental fecal samples of wild

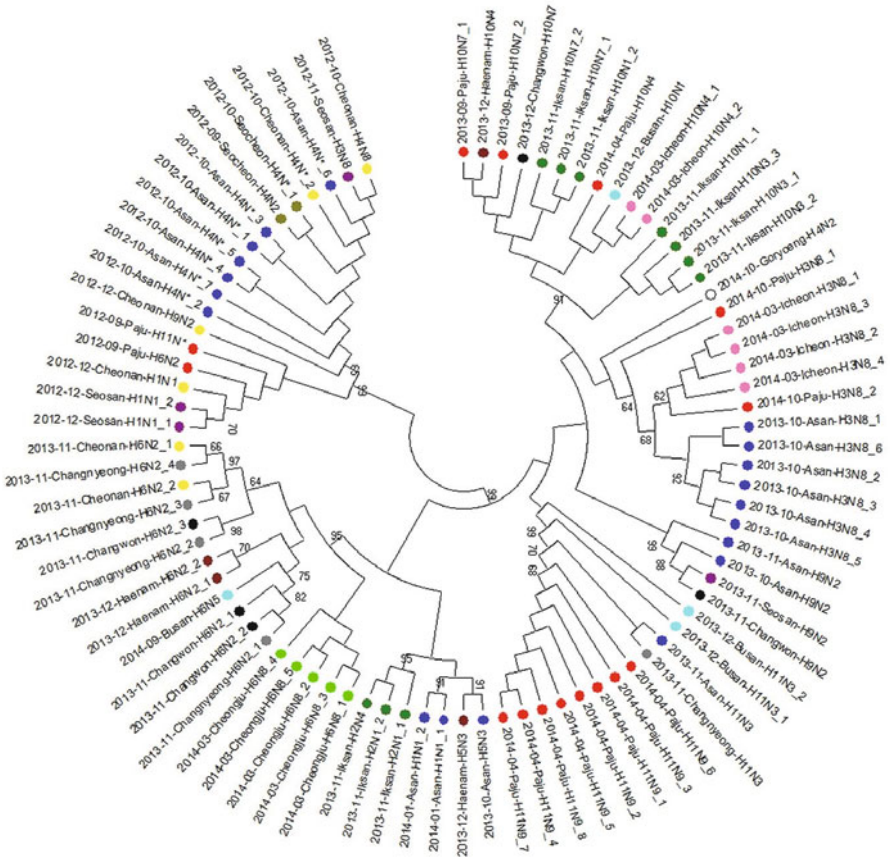

Fig. 2. Distribution of isolated viruses. Neighbor-joining tree based on HA genes showing distribution of 86 isolates from the swab and fecal samples from 2012 to 2014. A distance model was generated using the program MEGA5 (CEMI). Colored circles indicate the province or origin. HA, hemagglutinin.

Table 2. Monthly AI virus prevalence in wild bird feces, 2012–2014

Month	Sample		AI positive[a]		Prevalence (%)	
	2012–2013	2013–2014	2012–2013	2013–2014	2012–2013	2013–2014
9	100	250	12	23	12.0	9.0
10	750	850	47	105	6.0	12.0
11	490	1,800	42	261	8.6	14.5
12	500	1,000	49	25	9.8	2.5
1	1,800	3,662	46	88	2.6	2.4
2	2,000	1,725	7	95	0.4	5.5
3	1,700	950	9	46	0.5	4.8
4	600	640	10	27	1.7	4.2
	7,940	10,877	222	670	2.8	6.2

AI prevalence data were analyzed using RT-PCR[a]. AI, avian influenza; RT-PCR, reverse transcription-polymerase chain reaction

birds were collected. The annual AI prevalence in fecal samples was 2.8% (222/7940). From January to April, the average prevalence was 1.2%, while that from September to December was 8.2%. In particular, September had the highest prevalence (12.0%). In 2014, the average prevalence was 3.7% from January to April, while that from September to December was 10.6%. HPAI viruses were not found; however, a total of 86 AI viruses were isolated from wild birds and were confirmed to be LPAI viruses by genetic analysis.

For passive surveillance, a total of 72 wild bird carcasses were collected during January and February 2014, compared to the 2 recovered in 2013 (Table 1). Carcasses from baikal teals, common coots, and bean geese were collected in Gochang county, Geumgangho, and Seocheon city. Forty percent of the 2014 carcasses (29/72) were positive for AI on serology testing; in particular, 68.4% (26/38) of samples from Donglim reservoir in Gochang county were positive. In RT-PCR, positivity for the M and NP genes was noted in 37.5% (27/72) of cases and among them 29.2% (21/72) of AI viruses were isolated by inoculation into embryonated eggs. All isolated AI viruses were HPAI H5N8.

The distributions of isolated viruses from fecal samples coincided with migratory routes

Viruses isolated from environmental fecal samples were analyzed to determine the spatiotemporal distribution from 2013 to 2014 (Fig. 1). In 2013, AI virus was first isolated from Paju on September 30. On October 14, November 4, and November 25, AI virus was isolated from Asan. Additionally, AI virus was isolated from Seosan and Changnyeong on November 25 and then again from Changnyeong on December 5. AI viruses were also isolated from Iksan (November 11), Busan (December 9), and Haenam (December 10), as outlined in Fig. 1A. In 2014, LPAI viruses were isolated from Jeonju on January 20, Busan on January 22, Seocheon on January 25, and Asan on January 28. No viruses were isolated in February. Subsequently, AI viruses were isolated from Cheongju on March 13 and Incheon on March 14. Finally, AI viruses were isolated from Paju on April 3, the last reported location of AI virus isolation during the 2013–2014 wintering season. Generally, AI viruses appeared to spread south until December, after which they tended to shift in a northern direction beginning in January. The types of viruses isolated depended on the time or region in which the virus was isolated (Fig. 2). In 2012, the Chungnam H4 subtype was the predominantly isolated strain. In 2013 and 2014, the H3 and H6 subtypes were most frequently isolated from Chungcheong and Gyeongsang, respectively. In addition, most of the separated H6 subtypes of AI viruses showed high homology (98%) with the A/muscovy duck/Vietnam/LBM455/2013 (H6N2) strain, previously isolated in Southeast Asia. H6 subtype viruses were also isolated separately in spot-billed ducks captured in Cheonan. H10 subtypes isolated from Gyeonggi, Jeolla, Gyeongsang, and Chungcheong showed 99% homology with the viruses isolated from Jangxi (A/migratory duck/Jiangxi/33238/2013 [H10N7]; A/duck/Jiangxi/6648/2013 [H10N8]; A/migratory duck/Jiangxi/30246/2013 [H10N5]; and A/chicken/Jiangxi/JXA131916/2013 [mix]) in 2013 in China.

Differences in the changes of AI virus prevalence in environmental fecal samples

The AI virus prevalence in fecal samples is displayed by month in Table 2. The prevalence was measured by RT-PCR, and all of the AI viruses identified from environmental fecal samples were LPAI viruses. In 2012–2013, the fecal prevalence of AI virus decreased from December (9.8%) to March (0.5%) and then increased again in April (1.7%), whereas in 2013–2014, it decreased from November to January, and

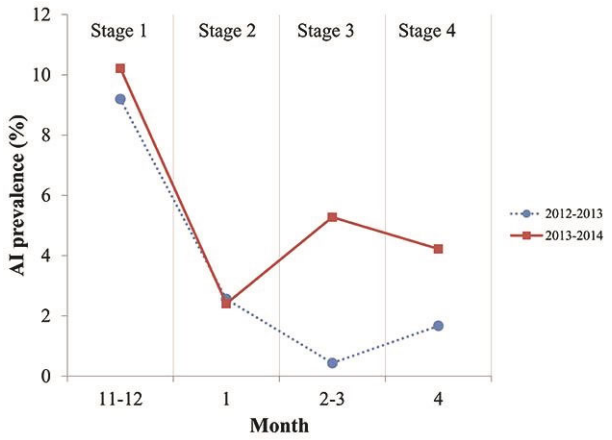

Fig. 3. AI prevalence in fecal samples by migratory stage. AI, avian influenza.

then increased in February (Table 2). The fecal AI virus prevalences during 2012–2013 were consistent with the seasonal movement of migratory birds (Supplementary data Fig. S1). However, 2013–2014 differed, in that the prevalence of AI virus detection was increased to 10 times that of February from the previous year (Fig. 3).

Discussion

Analysis of the geographic distributions of HPAI outbreaks and the mortality rates of wild birds has revealed that the AI virus prevalence is related to the migratory route (Takekawa *et al*., 2013). In Korea, waterfowl (winter birds) are the primary migratory birds, and winter birds are mainly associated with the occurrence of AI (Lee *et al*., 2008). According to National Institute of Biological Resources of "A study on the migration route and the status of wintering and stop over sites of migratory birds", the period of waterfowl migration in South Korea suggested into four stages. Stage 1 is from October to December, which represents the migration season of waterfowl from the north; stage 2 is in January, the wintering period; stage 3 is from February to March, when the winter birds in South Korea migrate north; and stage 4 is in April, when passing migratory birds (those wintering farther south than South Korea) migrate to the north. According to the reports of the National Institute of Environmental Research of "Surveillance and monitoring of wildlife diseases in Korea, 2012", the fecal prevalence of AI virus increases during stages 1 and 4, when migratory birds pass through South Korea, while the prevalence of AI during stages 2 and 3 depends on virus circulation within the flock. Circulation of the AI virus in migratory bird populations occurs frequently in South Korea. For example, three isolates of H9N2 virus were identified from Asan, Seosan, and Changwon on different collection dates, respectively. However, their genetic origins were the same.

In 2012–2013, our data indicated that the prevalence of AI virus was highest in stage 1, when birds began to migrate north, and then decreased during stage 3 in 2013. The prevalence then increased again in stage 4 (Fig. 3). However, the prevalences of the AI virus during 2013–2014 were different. During 2014, it increased in stage 3 rather than in stage 2. Interestingly, the viruses isolated in our study were all LPAI viruses, and not HPAI viruses. In other words, infection with LPAI viruses caused a high prevalence of AI during stage 3 in 2014. Furthermore, RT-PCR revealed that the fecal prevalence of AI virus during stage 3 was 5.15%, which was higher than that in the previous year (Table 2). These results, therefore, suggest that high levels of LPAI virus circulate within a flock and/or that LPAI viruses exhibit rapid or strong transmission ability among migratory bird flocks.

Molecular genetic research into HPAI virus and the cause of its pathogenicity has identified such findings as the cleavage site, and underlying genetic reassortments and rearrangements (Subbarao *et al*., 1998; Fouchier *et al*., 2004). However, the circumstances under which viruses change from a low to a highly pathogenic state have not been identified. In our study, genetic relationships between isolated viruses and HPAI H5N8 have not yet been detected, but we did note that the LPAI virus prevalences in 2013–2014 winter seasons were notably higher than those prevalences in 2011–2013 during the northward migration season within wild birds. Concomitant with an increased LPAI concentration, persistent infection and/or multiple AI virus infections in wild birds would also be potentially higher. In addition, the opportunity for viruses to be in contact with each other would also increase. Along these lines, there have been some reports regarding the relationship between LPAI virus circulation and HPAI reassortants in poultry. For example, a case report of the LPAI H9N2 circulation within Egypt and Bangladesh suggested the existence of genetic reassortment of HPAI H5N1 (Monne *et al*., 2013a; Marinova-Petkova *et al*., 2014), and from Bangladesh, evidence has been provided of reassortant HPAI H5N1 viruses with the PB1 gene of H9N2 (Monne *et al*., 2013b). In addition, some HPAI virus clusters were found to have been already present within several individual viral populations from the beginning of the LPAI H7N1 outbreak (Monne *et al*., 2014). From this perspective, increased LPAI virus concentrations might affect the recombination of viruses, and also provide increased opportunity for the development of highly pathogenic strains in wild birds. Therefore, this data might provide important insights into the occurrence of HPAI.

In summary, our study demonstrates the importance of surveillance of AI virus in wild birds. We found that migratory birds contributed to the introduction and propagation of AI. The data from our study demonstrate the importance of the surveillance of AI in wild birds. Future studies including in depth genetic analysis in combination with evaluation of the movement and ecology of migratory birds might help us to bridge the gaps in our knowledge and better explain, predict, and ultimately prevent future HPAI outbreaks.

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