

Isolation of an H5N8 Highly Pathogenic Avian Influenza Virus Strain from Wild Birds in Seoul, a Highly Urbanized Area in South Korea

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ABSTRACT: Asian-lineage H5 highly pathogenic avian influenza viruses (HPAIV) have caused recurrent outbreaks in poultry and wild birds. In January 2014, H5N8 HPAIV caused outbreaks in South Korea and subsequently spread to East Asia, Europe, and North America. We report the isolation of an H5N8 HPAIV strain from wild birds in Seoul, the most-developed city in South Korea. We analyzed the complete genome sequence of this isolate and estimated its origin using a phylogenetic analysis. The Seoul H5N8 isolate clustered phylogenetically with strains isolated from migratory wild birds but was distinct from Korean poultry isolates. This H5N8 virus was likely introduced into the urbanized city by migratory wild birds. Therefore, wild bird habitats in urbanized areas should be carefully monitored for HPAIV.

Key words: H5N8, highly pathogenic avian influenza virus, South Korea, urbanized city, wild birds.

Asian-lineage H5 highly pathogenic avian influenza viruses (HPAIV) have caused recurrent outbreaks in poultry, infections in wild birds, and clinical and often fatal diseases in humans since they were first detected in China in 1996 (Sonnberg et al. 2013). Between 2005 and 2006, global dissemination of the A/goose/Guangdong/1/1996 lineage virus was observed as clade 2.2 viruses spread along the migratory bird flyway from China to various countries of Asia, Europe, and Africa (Liu et al. 2005). Novel HPAIVs of the H5N8 subtype clade 2.3.4.4, which originated from East China, caused an outbreak in South Korea in January 2014 (Lee et al. 2014). These viruses subsequently spread to various countries in Asia, Europe, and North America via migratory waterfowl (Lee et al. 2015; Verhagen et al. 2015).

The risk of an HPAI outbreak is viewed as relatively low in urban areas owing to the low density of wild waterfowl and domestic poultry (Verhagen et al. 2012). In contrast, HPAIVs are more prevalent in wild birds from key wetland habitats where many of these animals gather. However, the spread of HPAIV via wild birds in urban areas is significant because wild birds could be in close proximity to captive birds and zoo animals and transmit the HPAIV.

On 6 February 2015, 50 fecal samples from wild birds were collected from Jungnangcheon (37°32'48''N, 127°1'53''E), a stream in Seoul (Fig. 1). The stream is a habitat for migratory birds of Seoul and feeds into the Hangang River. The Mallard (*Anas platyrhynchos*), Northern Pintail (*Anas acuta*), Common Pochard (*Aythya ferina*), and Tufted Duck (*Aythya fuligula*) are typically found there in the winter season. Fecal samples were tested for influenza virus by egg inoculation using 9- to 11-d-old specific-pathogen-free chicken embryonated eggs. After a 72-h incubation period, allantoic fluids were harvested from an axenic medium, and undiluted allantoic fluids were tested for hemagglutinin (HA) activity. For the definitive diagnosis of influenza virus, RNA was extracted from HA-positive allantoic fluid using an RNeasy Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The extracted RNA was tested for the influenza virus matrix (M) gene by real-time reverse-transcription PCR as previously described (Spackman et al. 2003). Complete genome sequencing was performed using the reverse-transcription-PCR amplicons of all eight gene segments by next-

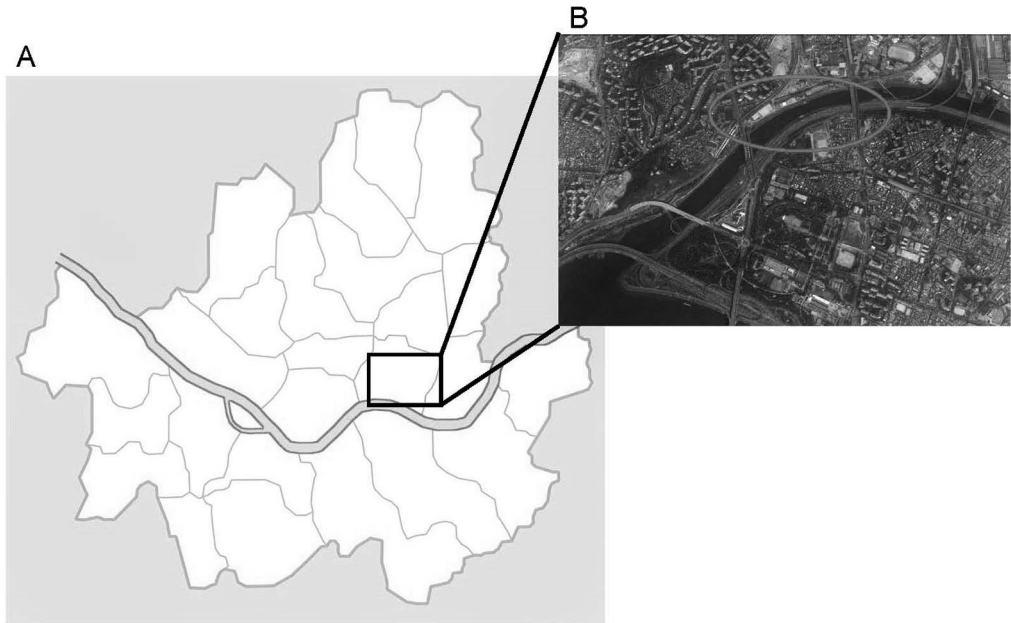


FIGURE 1. A map of (A) Seoul and (B) the Jungnang-cheon stream. The circle indicates the sampling site for the Mallard (*Anas platyrhynchos*) that yielded the Seoul H5N8 avian influenza isolate (A/mallard/Korea/N15-99/2015).

generation sequencing using the Ion Torrent Personal Genome Machine system (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

For the phylogenetic analysis, a maximum likelihood tree was constructed using MEGA 6 (Tamura 2013) with the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) of nucleotide substitutions and γ -distributed rate variation among sites with four rate categories. The statistical analysis of the phylogenetic tree was performed by a bootstrap analysis with 1,000 replicates. Based on previous phylogenetic analyses of H5N8 viruses (Lee et al. 2015; Kwon et al. 2016), the sequence of clade 2.3.4.4 H5 HPAI viruses representing each subgroup was selected as a reference for phylogenetic tree construction.

One of the fecal samples was positive for influenza A virus. The virus was subtyped as H5N8 using BLAST in GenBank (National Center for Biotechnology Information 2016); in addition, the multibasic cleavage site of the HA gene (PLRERRRKR/GLF) was detected. The host of the positive fecal sample was

identified as a Mallard by DNA barcoding techniques as previously described (Lee et al. 2010). Mallards are migratory birds of South Korea and migrate between Russia and China. This species is usually found in Jungnang-cheon in the winter season. Previous studies have indicated that Mallards can be asymptotically infected with HPAI viruses (Keawcharoen et al. 2008; Kwon et al. 2010). The Mallard may be subclinically infected by H5N8 HPAIV because no wild bird carcasses were found at the sampling site.

The nucleotide sequence of A/mallard/Korea/N15-99/2015 (H5N8), hereafter Seoul H5N8, was deposited in the EpiFlu database (<http://www.gisaid.org>; isolate ID: EPI_ISL_181082). Based on data available in the EpiFlu database, the Seoul H5N8 isolate showed the highest similarity to clade 2.3.4.4 H5N8 viruses isolated from wild birds in South Korea and Japan (Table 1).

Previous studies suggested that H5N8 HPAIV originated from East Asia and, since 2014, has evolved into three distinct subgroups: icA1 (Europe/Japan), icA2 (North

TABLE 1. Influenza viruses from the GenBank (National Center for Biotechnology Information 2016) and EpiFlu databases (<http://www.gisaid.org>) with the highest nucleotide identity to each eight-gene segment of an H5N8 avian influenza isolate (A/mallard/Korea/N15-99/2015) made in 2015 from a Mallard (*Anas platyrhynchos*) from Jungnang-cheon stream in Seoul, South Korea.

Gene	Closest strain	Identity (%)	Subgroup	Accession no. (EpiFlu)
PB2	A/chicken/Miyazaki/7/2014 (H5N8)	99.52	icA3 ^a	EPI553340
PB1	A/common teal/Korea/KU-12/2015 (H5N8)	99.65	icA3	EPI595057
PA	A/common teal/Korea/KU-12/2015 (H5N8)	99.59	icA3	EPI595058
HA	A/mallard/Korea/H2003/2014 (H5N8)	99.60	icA3	EPI573238
NP	A/mallard/Korea/KU3-2/2015 (H5N8)	99.74	icA3	EPI595067
NA	A/mallard/Korea/KU3-2/2015 (H5N8)	99.58	icA3	EPI595068
M	A/chicken/Miyazaki/7/2014 (H5N8)	99.90	icA3	EPI553346
NS	A/crane/Kagoshima/KU53/2015 (H5N8)	99.65	icA3	EPI573668

^a Subgroup of H5N8 viruses divided according to Lee et al. 2015.

America/Japan), and icA3 (South Korea/Japan) at a breeding site in Eastern Siberia and Beringia (Lee et al. 2015). Wild bird influenza virus isolates from South Korea are genetically distinct from the H5N8 HPAIV isolates that circulated in poultry farms in South Korea in 2014. Further, based on an estimate of the time to the most-recent common ancestor, the origin of the icA3 subgroup dated back to August 2014, coinciding with the breeding season of migratory wild birds (Kwon et al.

2016). A phylogenetic analysis of the HA and neuraminidase (NA) genes showed that the Seoul H5N8 isolate clustered with wild bird isolates belonging to the icA3 subgroup with high bootstrap support (HA 80; NA 71) and was genetically distinct from H5N8 isolates from Korean poultry farms (Fig. 2). A phylogenetic analysis of the other six genes, polymerase basic (PB) 2, PB1, polymerase acidic (PA), nucleoprotein (NP), M, and nonstructural (NS), also showed high boot-

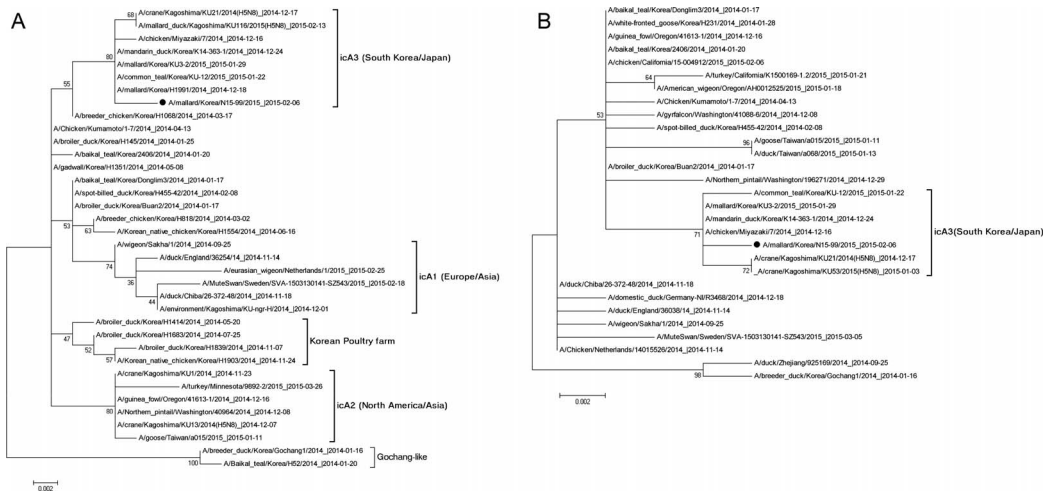
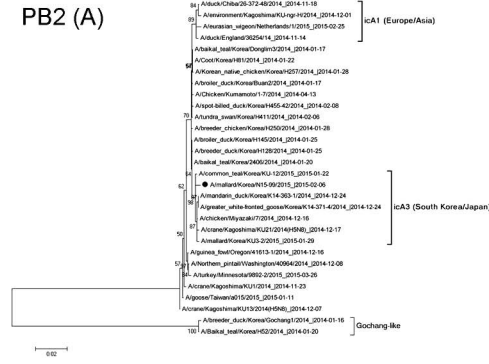
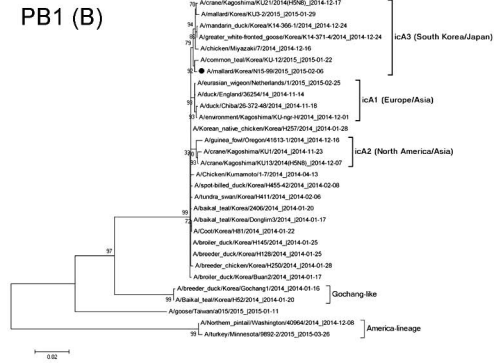


FIGURE 2. Maximum likelihood phylogenetic tree of the (A) hemagglutinin (HA) and (B) neuraminidase (NA) genes of clade 2.3.4.4 highly pathogenic avian influenza viruses. The black circle (•) indicates the HA and NA genes of the Seoul H5N8 isolate (A/mallard/Korea/N15-99/2015) used in this study. The percentages of replicate trees in which the associated taxa formed a cluster among 1,000 total bootstrap replicates are shown next to the branches.

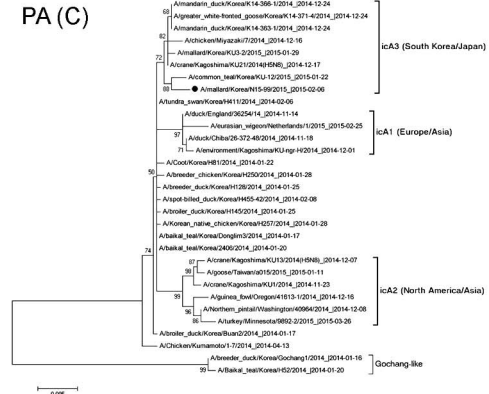
PB2 (A)



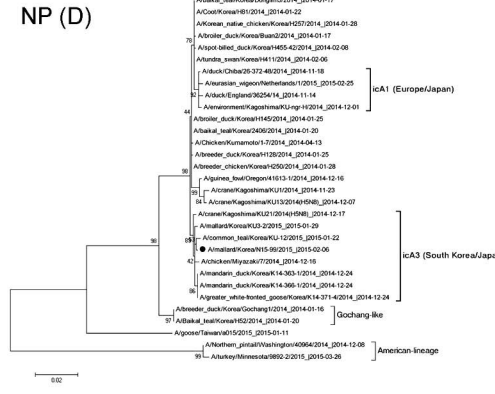
PB1 (B)



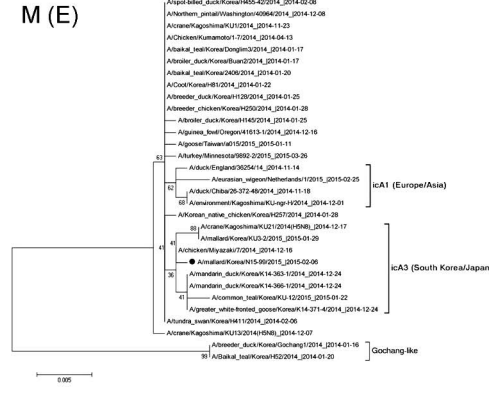
PA (C)



NP (D)



M (E)



NS (F)

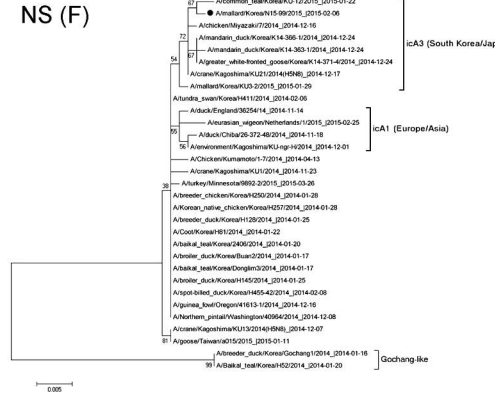


FIGURE 3. Maximum likelihood phylogenetic tree of the (A) PB2=polymerase basic 2, (B) PB1, (C) PA=polymerase acidic, (D) NP=nucleoprotein, (E) M=influenza virus matrix, and (F) NS=nonstructural genes of clade 2.3.4.4 highly pathogenic avian influenza viruses. The black circle (●) indicates the gene of the Seoul H5N8 isolate (A/mallard/Korea/N15-99/2015) from a Mallard (*Anas platyrhynchos*) used in this study. The percentages of replicate trees in which the associated taxa formed a cluster among 1,000 total bootstrap replicates are shown next to the branches.

strap support (PB2 98; PB1 79; PA 72; NP 83; M 36; NS 54) for clustering with icA3 H5N8 strains isolated from wild birds (Fig. 3). These results indicate that this Seoul H5N8 virus did

not originate from poultry farms and likely originated from migratory wild birds. However, it is also possible that domestic poultry species can serve as intermediate hosts of the

icA3 subgroup between wild birds in South Korea.

Previously, H5N1 HPAIVs of clades 2.2 and 2.3.2.1 had disseminated to a wide geographic area via wild birds (Liu et al. 2005; Lee et al. 2011). However, the geographic dissemination of H5N8 HPAIVs of clade 2.3.4.4, via wild birds, was more widespread than that of H5N1 HPAIVs of clades 2.2 and 2.3.2.1. We previously reported that icA3 H5N8 HPAIVs circulated in wild birds for more than 1 yr and were reintroduced into South Korea and Japan from wild bird breeding areas, such as Mongolia and Russia, by migratory wild birds. However, such reintroductions were not detected in previous H5N1 outbreaks (Kwon et al. 2016). Considering these facts and the high viral shedding in the absence of illness in experimentally H5N8-infected waterfowl (Kang et al. 2015), the H5N8 virus has likely adapted to wild waterfowl hosts. These characteristics of H5N8 HPAIVs might increase the opportunity for spread to a wider geographic area, including urbanized cities.

The movement of infected poultry and their products has caused HPAIVs to spread to urbanized cities in Korea since 2008. The first recorded outbreak of an HPAIV in the city of Seoul was H5N1 clade 2.3.2.1 in captive pheasants held in an exhibition for public viewing at a local district office in 2008. These pheasants were introduced to the exhibit 4 d before death at the live bird market in a satellite city of Seoul (Yoon et al. 2010). During the HPAIV outbreak in 2008, H5N1 HPAIV also spread to live bird markets and backyard farms in other urbanized cities of South Korea (World Organization for Animal Health 2008). However, HPAIVs had not previously been isolated from wild birds in Seoul before the four H5N1 HPAI outbreaks in South Korea from 2003 to 2011, despite active surveillance in this region.

In the present study, we report an H5N8 HPAIV strain isolation from a wild bird in Seoul. A phylogenetic analysis indicated that wild birds likely introduced this virus. Wild birds in cities can carry HPAIV and may play a role in its introduction into urbanized cities. Consistent with our findings, a previous

influenza surveillance study in an urbanized city in the Netherlands also indicated that wild birds might play a role in the introduction of low pathogenic influenza viruses to cities (Verhagen et al. 2012). To monitor the spread of HPAIVs, enhanced active surveillance in urbanized cities is required, not only in live bird markets but also in wild bird populations; this could provide a better epidemiologic understanding of HPAIVs and aid in the design of prevention strategies.

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