


SHORT REPORT

Open Access



Identification of Two novel reassortant avian influenza a (H5N6) viruses in whooper swans in Korea, 2016

Jipseol Jeong^{1*} , Chanjin Woo¹, Hon S. Ip², Injung An¹, Youngsik Kim¹, Kwanghee Lee¹, Seong-Deok Jo¹, Kidong Son¹, Saemi Lee¹, Jae-Ku Oem¹, Seung-Jun Wang¹, Yongkwan Kim¹, Jeonghwa Shin¹, Jonathan Sleeman² and Weonhwa Jheong^{1*}

Abstract

Background: On November 20, 2016 two novel strains of H5N6 highly pathogenic avian influenza virus (HPAIVs) were isolated from three whooper swans (*Cygnus cygnus*) at Gangjin Bay in South Jeolla province, South Korea. Identification of HPAIVs in wild birds is significant as there is a potential risk of transmission of these viruses to poultry and humans.

Results: Phylogenetic analysis revealed that Gangjin H5N6 viruses classified into Asian H5 clade 2.3.4.4 lineage and were distinguishable from H5N8 and H5N1 HPAIVs previously isolated in Korea. With the exception of the polymerase acidic (PA) gene, the viruses were most closely related to A/duck/Guangdong/01.01SZSGXJK005-Y/2016 (H5N6) (98.90 ~ 99.74%). The PA genes of the two novel Gangjin H5N6 viruses were most closely related to AIV isolates previously characterized from Korea, A/hooded crane/Korea/1176/2016 (H1N1) (99.16%) and A/environment/Korea/W133/2006 (H7N7) (98.65%). The lack of more recent viruses to A/environment/Korea/W133/2006 (H7N7) indicates the need for analysis of recent wild bird AIVs isolated in Korea because they might provide further clues as to the origin of these novel reassortant H5N6 viruses.

Conclusions: Although research on the origins and epidemiology of these infections is ongoing, the most likely route of infection for the whooper swans was through direct or indirect contact with reassortant viruses shed by migratory wild birds in Korea. As H5N6 HPAIVs can potentially be transmitted to poultry and humans, continuous monitoring of AIVs among wild birds will help to mitigate this risk.

Keywords: H5N6, Highly pathogenic avian influenza viruses, Whooper swans, Korea

Short report

As a natural reservoir for avian influenza viruses (AIVs), wild birds do not typically exhibit clinical signs of infection [1]. In most instances when AIVs of wild-bird origin are transmitted to poultry, infections are initially mild and represent a low pathogenic phenotype. Highly pathogenic AIVs (HPAIVs) arise following adaptation in domestic poultry, and some strains are known to cause significant illness or death if transmitted back to wild birds. Infection

by HPAI H5N1 led to the deaths of thousands of wild birds at Lake Qinghai, western China in 2005 [2], and infection with HPAI H5N8 killed over a hundred wild birds of multiple species in the Republic of Korea in 2014/2015 [3].

On 16 November, 2016 the government of South Korea reported outbreaks of H5N6 HPAI in poultry farms in South Jeolla province [4]. On 20 November, 2016 one juvenile whooper swan (*Cygnus cygnus*) exhibiting neurological signs including torticollis and ataxia, along with the carcasses of two adults were found at Gangjin Bay in South Jeolla. Samples collected from all three birds were positive for H5 subtype AIV by RT-PCR [5]. To isolate the viruses, oropharyngeal and cloacal

* Correspondence: czar21@korea.kr; purify@korea.kr

¹Environmental Health Research Department, National Institute of Environmental Research, Hwangeong-ro 42, Seo-gu, Incheon, Republic of Korea

Full list of author information is available at the end of the article



swabs, as well as tissue samples (trachea, liver, spleen and kidney) were inoculated into specific pathogen-free chicken eggs. Three isolates were identified as H5N6 AIVs by subtyping RT-PCR [6] and the entire genomes were determined [7]. No other AIV subtypes or Newcastle disease viruses were detected. The isolates from three whooper swans were designated as A/whooper swan/Korea/Gangjin 48/2016 (H5N6) (Gangjin 48, juvenile), A/whooper swan/Korea/Gangjin 49-1/2016 (H5N6) (Gangjin 49-1, adult) and A/whooper swan/Korea/Gangjin 49-2/2016(H5N6) (Gangjin 49-2, adult). The sequences of these isolates were deposited in GenBank with accession numbers < KY402046-KY402077 > .

The viruses contained conservative residues within the receptor-binding pocket of the hemagglutinin protein (HA, including Q226 and G228, H3 numbering), which

is associated with a preference for “avian” type cell surface receptors containing alpha 2,3-sialic acid residues [8]. The viruses did not contain amino acid substitutions conferring resistance to adamantane and neuraminidase (NA) inhibitors [9]. The deduced amino acid sequence of the HA protease cleavage site contains a series of basic amino acid residues (LRERRRKR/GLF) characteristic of highly pathogenic AIVs. Experimental infection of 8-week old chickens by intravenous inoculation of the Gangjin 48 virus resulted in the deaths of all 8 birds within 25 h, conferring a intravenous pathogenicity index of 3.0. [5].

Phylogenetic analysis of HA gene sequences from the three viral isolates classified them within Asian H5 clade 2.3.4.4 lineage (Fig. 1a), which emerged in China during 2010–2011, and unlike earlier clades is known for a novel

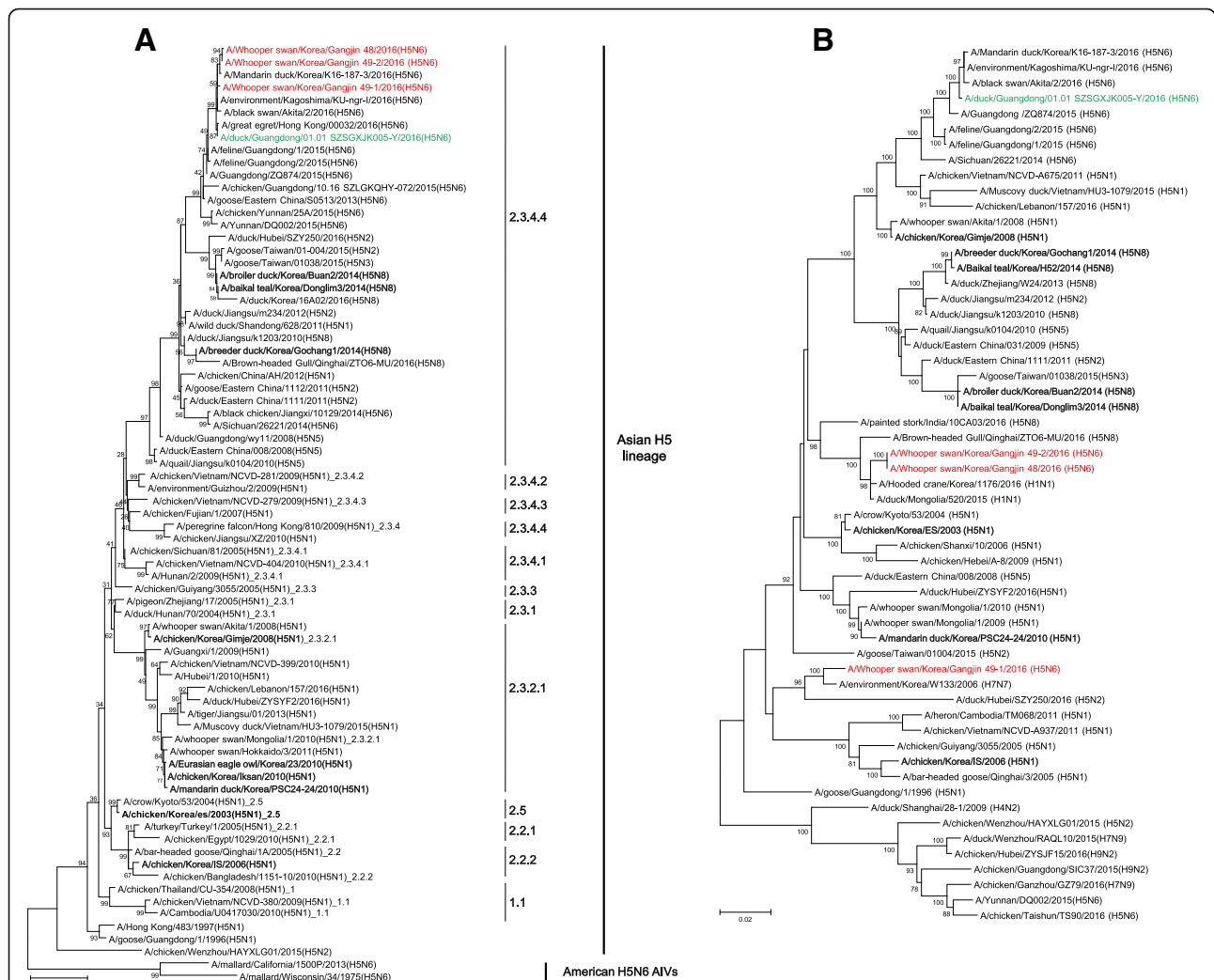


Fig. 1 Phylogenetic tree of HA (a) and PA (b) genes of H5N6 viruses isolated from whooper swans. Clade 2.3.4.4 H5N6 HPAI isolates in Korea and Guangdong, Southern China in 2016 are shown in red and green, respectively. Phylogenetic trees were constructed using the maximum-likelihood algorithm of MEGA 6 with 1,000 bootstrap trials. Other HPAIVs detected in South Korea are indicated in boldface. The numbers at the nodes represent bootstrap values. The scale bar indicates the nucleotides substitutions per site

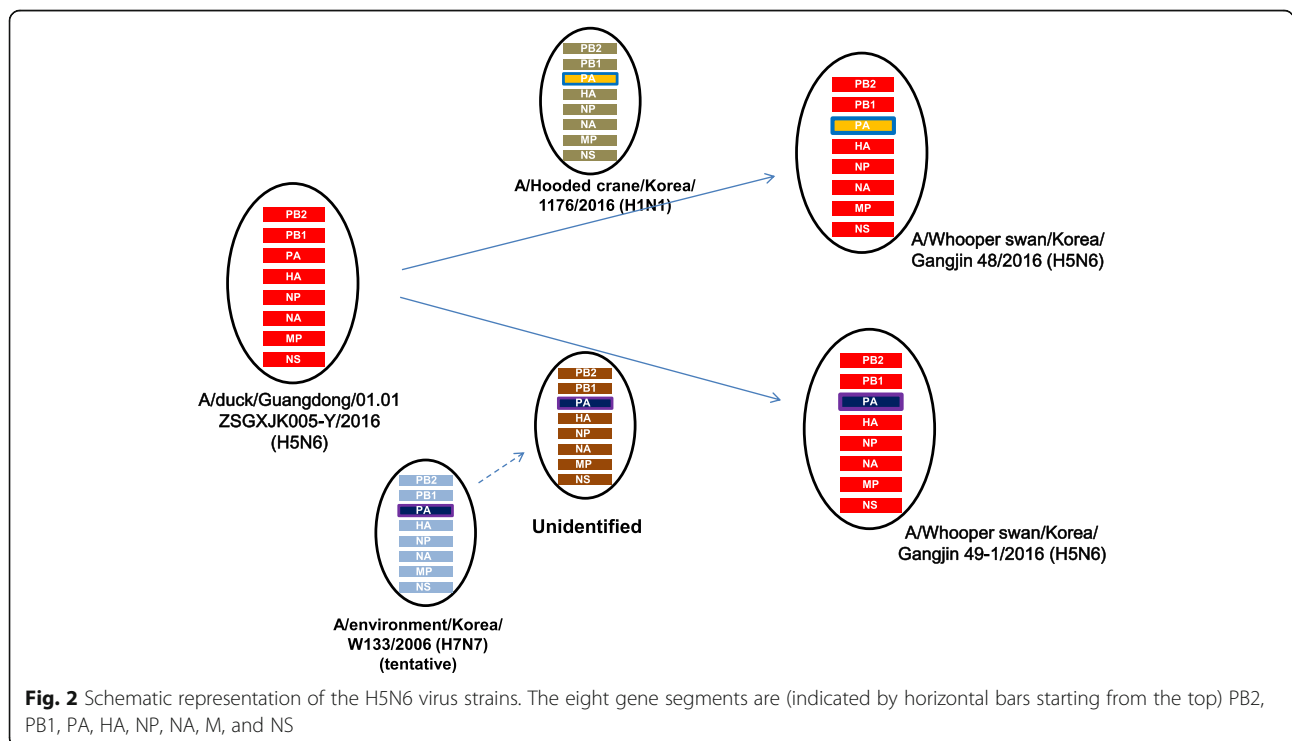
propensity to reassort with NA subtypes other than N1 [10]. The clade 2.3.4.4 H5N6 has been the prevalent lineage in Guangdong, Southern China since 2013 and spread to northeast China in 2014 [11]. Notably, the viruses are distinct from both groups A (A/Baikal teal/Korea/Donglim3/2014) and B (A/breeder duck/Korea/Gochang1/2014) of the 2.3.4.4 H5N8 viruses, and they are clearly distinguishable from H5N1 HPAIVs previously isolated in Korea, including (A/chicken/Korea/ES/2003 (clade 2.5), A/chicken/Korea/IS/2006 (clade 2.2), A/chicken/Korea/Gimje/2008 (clade 2.3.2.1), and A/mallard/Korea/1195/2010 (clade 2.3.2.1)) [3].

Nucleotide identity analysis with BioEdit version 7.2.5 (<http://bioedit.software.informer.com/>) and Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) revealed that all three viruses were homologous with each other, sharing 99.03 ~ 100% nucleotide identity among seven out of eight segments (polymerase basic 2 (PB2), polymerase basic 1 (PB1), HA, nuclear protein (NP), NA, matrix (M), nonstructural (NS)). The PA subunit segments of Gangjin 48 and Gangjin 49–2 were identical to each other but differed (94.05% identity) from Gangjin 49–1, which suggests that at least two kinds of novel H5N6 HPAIV strains have circulated among wild birds in Korea. A BLAST (www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) search and phylogenetic analysis showed that all three viruses likely originated from A/duck/Guangdong/01.01SZ SGXJK005-Y/2016 (H5N6, hereafter Guangdong 2016) (98.90 ~ 99.74% identity) (Fig. 2). The PA genes for Gangjin

48 and Gangjin 49–2 were closely related (99.16% identity) to A/hooded crane/Korea/1176/2016 (H1N1), while the PA gene of Gangjin 49–1 was more closely related (98.65% identity) to A/environment/Korea/W133/2006 (H7N7) (Fig. 1b). The data does not preclude the possibility that a as yet unidentified, but more contemporary, virus might be the actual donor of the PA gene to Gangjin 49–1 (Fig. 1b).

Whooper swans breed in northern Eurasia and winter in Europe and eastern Asia, including South Korea. Previous satellite data describing their southern migration to South Korea indicate the birds follow the Flyway along inner Mongolia, northeast China and the Korean peninsula during October and November [12]. Indeed, on 27 November many migratory bird species including whooper swans (>1,700), mallards (*Anas platyrhynchos*, > 3,500) and spot billed ducks (*Ana poecilorhyncha*, > 1,500) were observed around Gangjin Bay.

It is possible that whooper swans introduced both H5N6 viruses into Korea. However, it is also possible that whooper swans which are highly susceptible to HPAI H5N1 infection [13], may have been exposed locally through direct or indirect contact with other H5N6-infected but asymptomatic migratory species, such as mallards or spot-billed ducks, or perhaps through exposure to infected poultry [14]. Wild bird species differ in their response to HPAIV infection, and it is likely that unidentified asymptotically-infected migratory birds could have introduced an ancestral virus, similar to Guangdong 2016, into Korea. Moreover, these or other species of wild birds



could then play a role in generating novel reassortant viruses when co-infected with other AIVs. Indeed, our research group has isolated more than sixty AIVs from wild birds and waterfowl feces since October 2016 (unpublished data). Genetic analysis of these AIVs might provide clues as to the origin of these viruses; the characterization of which will be further detailed in a subsequent publication.

In conclusion, we have identified the two novel HPAIV H5N6 strains from three whooper swans in Korea. Phylogenetic and genetic analyses have shown that the two strains resulted from reassortment of the clade 2.3.4.4 H5N6 viruses that circulated in Guangdong, and they are distinguishable from HPAIVs that caused previous poultry and wild bird outbreaks in Korea. Increased understanding of clade 2.3.4.4 H5N6 virus transmission and pathogenesis in wild birds would serve to increase knowledge of the risk these viruses present to wildlife, domestic animals, and humans. Furthermore, as the H5N6 HPAIVs are potentially transmissible to poultry and humans, continuous monitoring of AIVs among wild birds is warranted.

Abbreviations

AIVs: Avian Influenza Viruses; HA: Hemagglutinin protein; HPAIVs: Highly Pathogenic Avian Influenza Viruses; M: Matrix protein; NA: Neuraminidase protein; NP: Nuclear protein; NS: Nonstructural protein; PA: Polymerase Acidic protein; PB1: Polymerase Basic 1 protein; PB2: Polymerase Basic 2 protein; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

Acknowledgements

We thank the National Institute of Environmental Research (NIER) and the Ministry of Environment for their efforts in the control of HPAIVs in wild birds. We thank Dr. David Bleher for helpful comments on this manuscript. Mention of products does not indicate endorsement by the US Government.

Availability of data and materials

The dataset supporting the conclusions of this article is available in the Genbank, [https://www.ncbi.nlm.nih.gov/genbank/].

Authors' contributions

JJ conducted virus identification, genome sequencing, phylogenetic analysis and drafted the manuscript. CW, IA, YK, KL, SDJ, KS and SL participated in virus isolation. JKO, SJW, YK, SJ participated in sample collection. JJ, HI, JS and WJ designed the wild bird surveillance, conducted data analysis and provided final approval of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All experiments including chicken infection were approved by the Institutional Animal Care and Use Committees (IACUC) of National Institute of Environmental Research (NIER), Incheon, Korea (IACUC Number: NIER-16-4).

Financial support

This work was supported by National Institute of Environmental Research (NIER), NIER-2016-01-01-033. The funder had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Environmental Health Research Department, National Institute of Environmental Research, Hwangyeong-ro 42, Seo-gu, Incheon, Republic of Korea. ²U.S. Geological Survey, National Wildlife Health Center, Madison, WI, USA.

Received: 4 January 2017 Accepted: 16 March 2017

Published online: 21 March 2017

References

- Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 1992;56(1):152–79.
- Chen H, Smith GJ, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y. Avian flu: H5N1 virus outbreak in migratory waterfowl. *Nature.* 2005; 436(7048):191–2.
- Jeong J, Kang HM, Lee EK, Song BM, Kwon YK, Kim HR, Choi KS, Kim JY, Lee HJ, Moon OK, Jeong W, Choi J, Baek JH, Joo YS, Park YH, Lee HS, Lee YJ. Highly pathogenic avian influenza virus (H5N8) in domestic poultry and its relationship with migratory birds in South Korea during 2014. *Vet Microbiol.* 2014;173(3–4):249–57.
- World Organization for Animal Health: <http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2016/>.
- World Organization for Animal Health: OIE Terrestrial Manual. 2009. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf.
- Fereidouni SR, Starick E, Grund C, Globig A, Mettenleiter TC, Beer M, Harder T. Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. *Vet Microbiol.* 2009;135(3–4):253–60.
- Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol.* 2001; 146(12):2275–89.
- Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science.* 2006;312(5772):404–10.
- Orozovic G, Orozovic K, Lennerstrand J, Olsen B. Detection of resistance mutations to antivirals oseltamivir and zanamivir in avian influenza A viruses isolated from wild birds. *PLoS One.* 2011;6(1):e16028.
- Claes F, Morzaria SP, Donis RO. Emergence and dissemination of clade 2.3.4.4 H5Nx influenza viruses—how is the Asian HPAI H5 lineage maintained. *Curr Opin Virol.* 2016;16:158–63.
- Bi Y, Liu H, Xiong C, Di L, Shi W, Li M, Liu S, Chen J, Chen G, Li Y, Yang G, Lei Y, Xiong Y, Lei F, Wang H, Chen Q, Chen J, Gao GF. Novel avian influenza A (H5N6) viruses isolated in migratory waterfowl before the first human case reported in China, 2014. *Sci Rep.* 2016;6:29888.
- Newman SH, Iverson SA, Takekawa JY, Gilbert M, Prosser DJ, Batbayar N, Natsagdorj T, Douglas DC. Migration of whooper swans and outbreaks of highly pathogenic avian influenza H5N1 virus in eastern Asia. *PLoS One.* 2009;4(5):e5729.
- Brown JD, Stallknecht DE, Swayne DE. Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. *Emerg Infect Dis.* 2008;14(1):136–42.
- Newman SH, Siembieda J, Kock R, McCracken T, Khomenko S, Mundkur T. Update on highly pathogenic avian influenza in animals (type H5 and H7) <http://www.fao.org/avianflu/en/wildlife/index.html>. World Organization for Animal Health 2016.