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Genetic Analysis of H7N7 Avian Influenza Virus Isolated From Waterfowl in South Korea in 2016

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Type A influenza virus is circulating in wild birds and can infect wide ranges of hosts such as humans, pigs, domestic birds, and other mammals. Many subtypes of avian influenza viruses are circulating in aquatic birds. Most avian influenza viruses found in aquatic birds are low pathogenic avian influenza viruses. Highly pathogenic avian influenza viruses have been found in waterfowls since 2005. It is known that H5 and H7 subtypes of avian influenza viruses can be mutated into highly pathogenic avian influenza viruses in domestic poultry. In this study, we isolated novel reassortant H7N7 avian influenza virus from the fecal materials of migratory birds in the Western part of South Korea in 2016, and analyzed the sequences of all its eight genes. The genetic analysis of our isolate, A/waterfowl/Korea/S017/2016 (H7N7) indicates that it was reassortant avian influenza virus containing genes of both avian influenza viruses of wild birds and domestic ducks. Phylogenetic analysis showed that our isolate belongs to Eurasian lineage of avian influenza virus. Since avian influenza viruses continue to evolve, and H7-subtype avian influenza virus can mutate into the highly pathogenic avian influenza viruses, which cause the great threat to humans and animals, we closely survey the infections in both wild birds, and domestic poultry, and mammals.

Key words : H7N7, influenza A virus, South Korea, waterfowl

Introduction

Avian influenza viruses belong to the Orthomyxoviridae family. Among four types of influenza viruses, A, B, C and D, influenza A virus can infect a wide range of hosts compared to the other three influenza viruses. Both humans and animals can get infected by influenza A Viruses. The natural reservoirs of influenza A viruses are wild aquatic birds such as shorebirds, ducks and geese [4, 10, 19]. Aquatic birds infected with many subtypes of avian influenza viruses do not show the clear clinical signs, but they play important role in creation of reassortment of influenza A viruses [3, 13].

The genome of avian influenza viruses consists of eight negative-sense segmented genes, PB2 (RNA polymerase basic subunit 2), PB1 (RNA polymerase basic subunit 1), PA (RNA polymerase acidic subunit), HA (haemagglutinin), NP (nucleoprotein), NA (neuraminidase), M (Matrix) and NS (nonstructural). The two proteins on the surface of influenza virus, HA and NA are used to divide influenza A viruses into many subtypes. Currently, there are 18 different hemagglutinin (H1-H18) and 11 different neuraminidase subtypes (N1-N11) in influenza A viruses [4, 19]. Influenza A viruses are also classified into low pathogenic avian influenza virus (LPAI) and highly pathogenic avian influenza virus (HPAI) based on the severity of the disease in the infected animals. H5 and H7 subtypes of influenza A have been associated with HPAI viruses among 18 HA subtypes, and have the polybasic amino acids such as arginine (R) and lysine (K) in the cleavage sites of HA proteins [4, 20].

H7N7-subtype influenza A virus was first detected in chickens in Italy in 1902 [8]. Since then, it has been isolated from domestic poultry and wild birds around the world [2, 18]. The first human case of H7N7 influenza A virus was found in England in 1996 [12]. Three cases of human conjunctivitis in humans infected with H7N7 influenza A virus infection were reported during the 2013 outbreak of H7N7 HPAI virus in Italy [1]. H7N7 influenza A viruses were also isolated from magpie in northwestern part of South Korea [11] and live poultry markets in China [6]. Owing to their continued detection in birds and their ability to cause human

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infection, H7 subtypes of influenza viruses are one of the public health threats [1, 15]. In this study, we isolated H7N7 influenza virus from the fecal samples of wild birds in South Korea in winter, 2016, and genetically characterized its genome.

Materials and Methods

Virus Isolation

About 100 fecal samples of migratory birds were collected in migratory bird site, Chunsu Man which is located in the Western part of South Korea, 2016. This area is known to attract migratory birds during winter season. Isolation media containing glycerol (50%) was used to collect fecal samples. The samples were then inoculated into 10-day-old embryonated specific pathogenic free (SPF) eggs. The inoculated eggs were incubated for 72 hr at 37℃ under humid conditions and then were chilled overnight at 4℃. Presence of influenza A virus in allantoic fluid was checked by hemagglutination assay (HA) with 0.5% turkey red blood cells in phosphate buffered solution (PBS, pH 7.4). We designated the isolate as A/waterfowl/Korea/S017/2016 (H7N7).

RNA extraction and PCR amplification

Viral RNAs from the HA-positive allantoic fluid were extracted using RNeasy protect Mini kit (Qiagen, CA, USA) in accordance with the manufacturer's instructions. Extracted viral RNAs were used to determine genetic information of the isolate. Uni-12 primer (5′-AGCAAAAGCAGG-3′) was used to reverse transcribe viral RNA to complementary DNA (cDNA). Eight viral genes of the isolate were amplified by Polymerase chain (PCR) with GoTaq® DNA polymerase and a segment specific primer set which are available up on request. The primers used for amplifying the genes were; forward (5′TCCGAAGTTGGGGGGGAGCGAAAGCA GGTC-3′) and reverse (5′-GGGCCGCCGGGTTATTAGTAG AAACAA GGTCG-3′) for PB2, forward (5′-TCCGAAGTTGGGGGG GAGCGAAAGCAGGCA-3′) and reverse (5′-GGGCCGCCG GGTTATTAGTAGAAACAAGGCAT3′) for PB1, forward (5′ TCCGAAGTTGGGGGGGAGCGAAAGCAGGTA3′) and reverse (5′GGGCCGCCGGGTTATTAGTAGAAACAAGGTAC3′) for PA, forward (5′TCCGAAGTTGGGGGGGAGCAAAAGC AGGGG3′) and reverse (5′GGGCCGCCGGGTTATTAGTAG AAACAAGGGTG3′) for HA, forward (5′TCCGAAGTTGGG GGGGAGCAAAAGCAGGGT3′) and reverse (5′GGGCCGC CGGGTTATTAGTAGAAACAAGGGTA3′) for NP, forward (5′TCCGAAGTTGGGGGGGAGCAAAAGC AGGAG3′) and reverse (5′GGGCCGCCGGGTTATTAGTAGAAACAAGGA GT3′) for NA, forward (5′TCCGAAGTTGGGGGGGAGCAA AAGCAGGTA3′) and reverse (5′GGGCCGCCGGGTTATTA GTAGAAACAAGGTAG3′) for M, and forward (5′TCCGA AGTTGGGGGGGAGCAAAAGCAGGGT3′) and reverse (5′GG GCCGCCGGGTTATTAGTAGAAACAAG GG TG-3′) for NS. Gel electrophoresis was used to separate amplicons and then the bands were purified using QIAquick Gel Extraction Kit (Qiagen, CA, USA) after they were excised from the gel.

Sequencing and phylogenetic analysis

TA vector (Promega, USA) was used to clone the purified DNA. Transformation was carried out using *Escherichia coli* competent cell, X- gal indicator and Luria Bertani (LB) medium by incubating it overnight at 37℃. The white colonies were selected and grown in LB broth overnight at 37℃ in shaking incubator. Plasmid extractions were conducted and were sent for sequencing. Sequencing of genes was performed by Macrogen (Daejeon, South Korea). Seqman model of DNA star package was used to edit the nucleotide sequences. The nucleotide sequences were deposited into GenBank under accession numbers, MH169353-MH169360**.** Phylogenetic analysis was carried out by using molecular evolutionary genetic analysis software MEGA 7 (MEGA 7.0) with the maximum-likelihood method. Bootstrap analysis using 1,000 replicates was used to evaluate the reliability of the phylogenetic tree. The input nucleotide sequence included both the isolate and published influenza virus sequences from GenBank database.

Results and Discussion

Homology comparison of the isolate, A/Waterfowl/Korea/S017/2016 (H7N7)

The closest related influenza viruses to our isolate, A/waterfowl/Korea/S017/2016(H7N7) were examined using BLAST (basic local alignment search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi,gisaid.org/epi3/frontend#4ead5c) (Table 1). The data in Genbank indicated the NP, M and NS genes were closely related to those of the A/duck/Ganzhou/G25/2015(H4N6), A/duck/Mongolia/543/2015(H4N6) and A/wild duck/Korea/SH5-26/2008(H4N6) with 99% identity respectively. PB1, PA, and HA were closely related to the A/Anseriformes/Anhui/L167/2014(H1N1), A/duck/Mongolia/83/2015(H3N6) and A/duck/Bangladesh/26992/2015

Table 1. Nucleotide homology of A/Waterfowl/Korea/S017/2016(H7N7) with the closest related influenza viruses

Viral genes	Closest related viruses	Nucleotide identity (%)
PB2	A/barnacle goose/Netherlands/2/2014(H3N6),	99
PB1	A/Anseriformes/Anhui/L167/2014(H1N1)	98
PA	A/duck/Mongolia/83/2015(H3N6)	98
HA	A/duck/Bangladesh/26992/2015(H7N9)	98
NP	A/duck/Ganzhou/G25/2015(H4N6)	99
NA	A/duck/Mongolia/742/2015(H10N7)	99
M	A/duck/Mongolia/543/2015(H4N6)	99
NS	A/wild duck/Korea/SH5-26/2008(H4N6)	99

(H7N9) with 98% identity respectively. PB2 and NA were closely related to the A/barnacle goose/Netherlands/2/2014(H3N6) and A/duck/Mongolia/742/2015(H10N7) with 99% identity respectively.

Genetic analysis showed that our isolate, A/Waterfowl/Korea/S017/2016(H7N7) is a reassorted avian influenza virus between the origins of domestic ducks and wild birds. PB2 and NS genes were from the origin of wild birds, while the rest of genes, PB1, PA, HA, NP, NA, and M were from the origin of domestic ducks (Table 1).

Our genetic analysis showed that the isolate was a reassorted avian influenza virus using viral genes of domestic ducks and wild birds. It suggests that migratory birds make frequent contacts with domestic poultry, resulting in the creation of novel avian influenza virus. To prevent the outbreaks of new subtypes of influenza viruses in the domestic poultry and wild birds, it is recommended to set up a barrier to prevent the contact between wild birds and domestic poultry. Since H7-subtypes of influenza viruses can mutate into a highly pathogenic avian influenza virus in poultry [16], the intensive surveillance in poultry is very important to minimize the economic losses in poultry industry.

Analysis of important amino acids involved in binding to human-type influenza receptor, enhancing antiviral drug resistance, and causing pathogenesis in mammal of the isolate, A/Waterfowl/Korea/S017/2016(H7N7)

To know the characteristics of the isolate more, the selected regions of the A/Waterfowl/Korea/S017/2016(H7N7) genome were further analyzed (Table 2). Our isolate had alanine (A) at position 138, glutamine (Q) at position 226, and glycine (G) at position 228 of the HA protein, suggesting the efficient binding to avian influenza receptors [17]. Analysis of the NA gene of this isolate revealed that there was arginine (R) at positions 152 and 292, and histidine (H) at position 274, suggesting the susceptibility to the NA inhibitors, oseltamivir and zanamivir [7]. The M1 mutation T

Table 2. Identification of amino acids of A/Waterfowl/Korea/S017/2016(H7N7) involved in binding to human-type influenza receptor, enhancing antiviral drug resistance, and causing pathogenesis in mammal [6, 7, 9, 14, 17]

Viral protein	Amino acid position	A/Waterfowl/Korea/S017/2016(H7N7)	Comments
HA (H3 numbering)	138	A	A138S: Increased binding to human
	226	Q	Q226L: Increased binding to human
	228	G	G228S: Increased binding to human
NA (N2 numbering)	152	R	R152K: Resistance to oseltamivir and zanamivir
	274	H	H274Y: Resistance to oseltamivir and zanamivir
	292	R	R292K: Resistance to oseltamivir and zanamivir
M1	215	A	T215A: Increased pathogenesis in mice
M2	31	S	S31N: Resistance to amantadine and remantadine
NS1	42	S	P42S: Increased pathogenesis in mice

PB2: RNA polymerase basic subunit 2; PB1: RNA polymerase basic subunit 1;HA, haemagglutinin; NA: neuraminidase; M1: Matrix gene 1; M2: Matrix gene 2; NS1: non-structural gene 1.
A: Alanine; D: Aspartic acid E: Glutamic acid; G: Glycine; H: Histidine; K: Lysine; L: Leucine; N: Asparagine; P: Proline; Q: Glutamine; R: Arginine; S: Serine; T: Threonine, V: Valine; Y: Tyrosine.

(threonine) 215A (alanine), that is known to increase viral pathogenesis in mice, was found [6]. Our isolate had serine (S) at position 31 of M2, suggesting its sensitivity to ion-channel inhibitors, such as amantadine and rimantadine [14]. NS1 had serine (S) at position 42, which is involved in the increased virulence in mice [9]. Other genes involved in causing the pathogenicity and transmission in mammals were not found in our isolate.

The amino acid analysis of our isolate showed that this virus prefers binding to avian species than humans. It suggests that our isolate, H7N7 may not easily infect humans. However, since avian influenza virus continues to mutate, we need to survey domestic pigs and poultry where avian influenza viruses can mutate by the multiple replications in these hosts. Our analysis also showed that NA protein of our isolate does not have the mutation which is responsible for the resistance against the drugs of influenza virus. It suggests that the infected humans, if occurred, can be treated with NA inhibitors, oseltamivir and zanamivir.

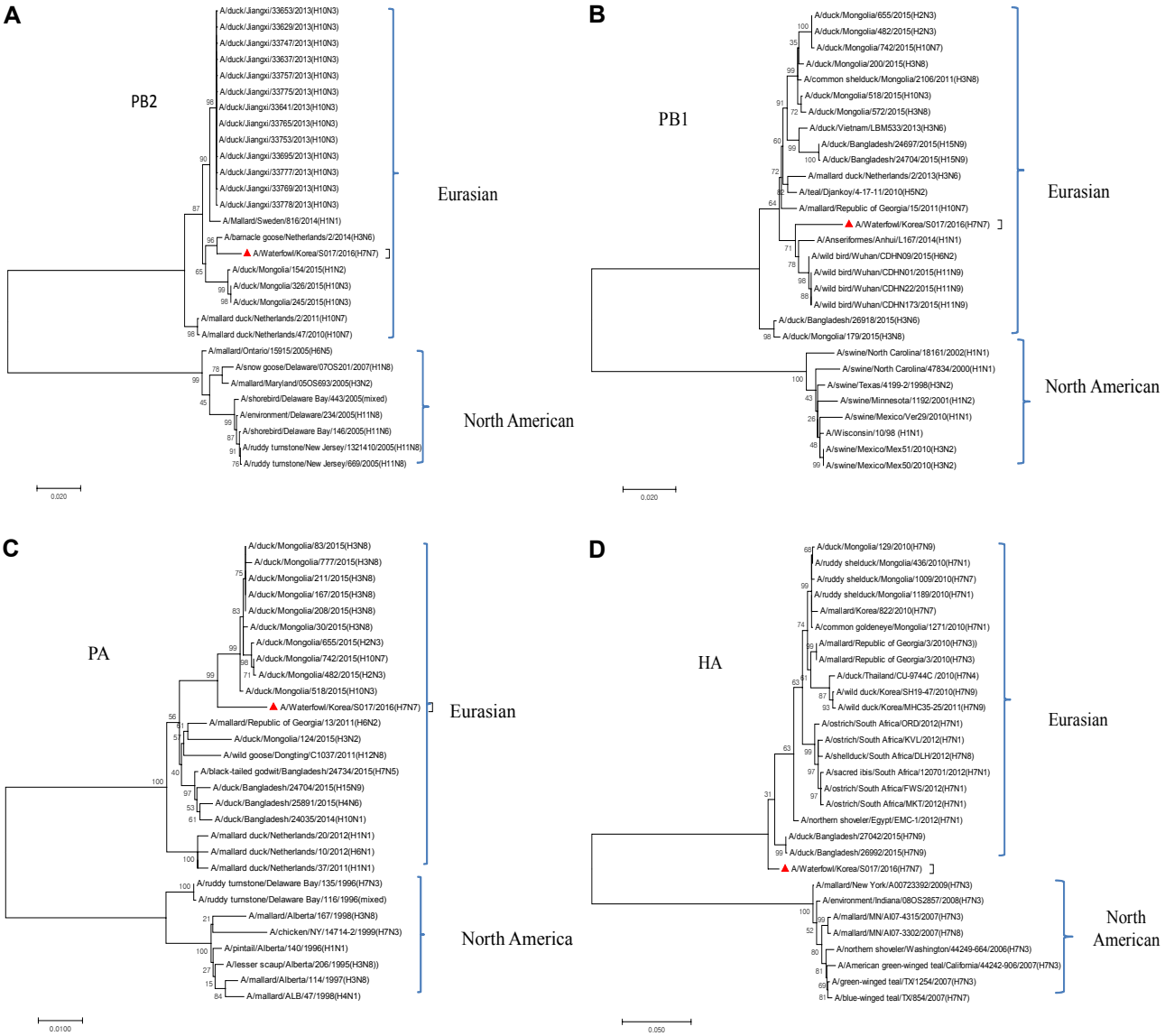

Fig. 1. Phylogenetic analysis of PB2, PB1, PA, HA, NP, NA, M and NS genes of A/Waterfowl /Korea/S017/2016(H7N7). The tree was constructed using Maximum-likelihood method in MEGA7 (www.megasoftware.net) with 1000 bootstrap replicates. Scale bar shows nucleotide substitutions per site. A) PB2; B) PB1; C) PA; D) HA; E) NP; F) NA; G) M; H) NS. Nucleotide region used in the phylogenetic analysis includes; PB2:1-1341, PB1:1-314, PA: 1-2232, HA: 1-1708; NP: 1-1566, NA: 1-1450, M: 1-1017, NS: 1-890.

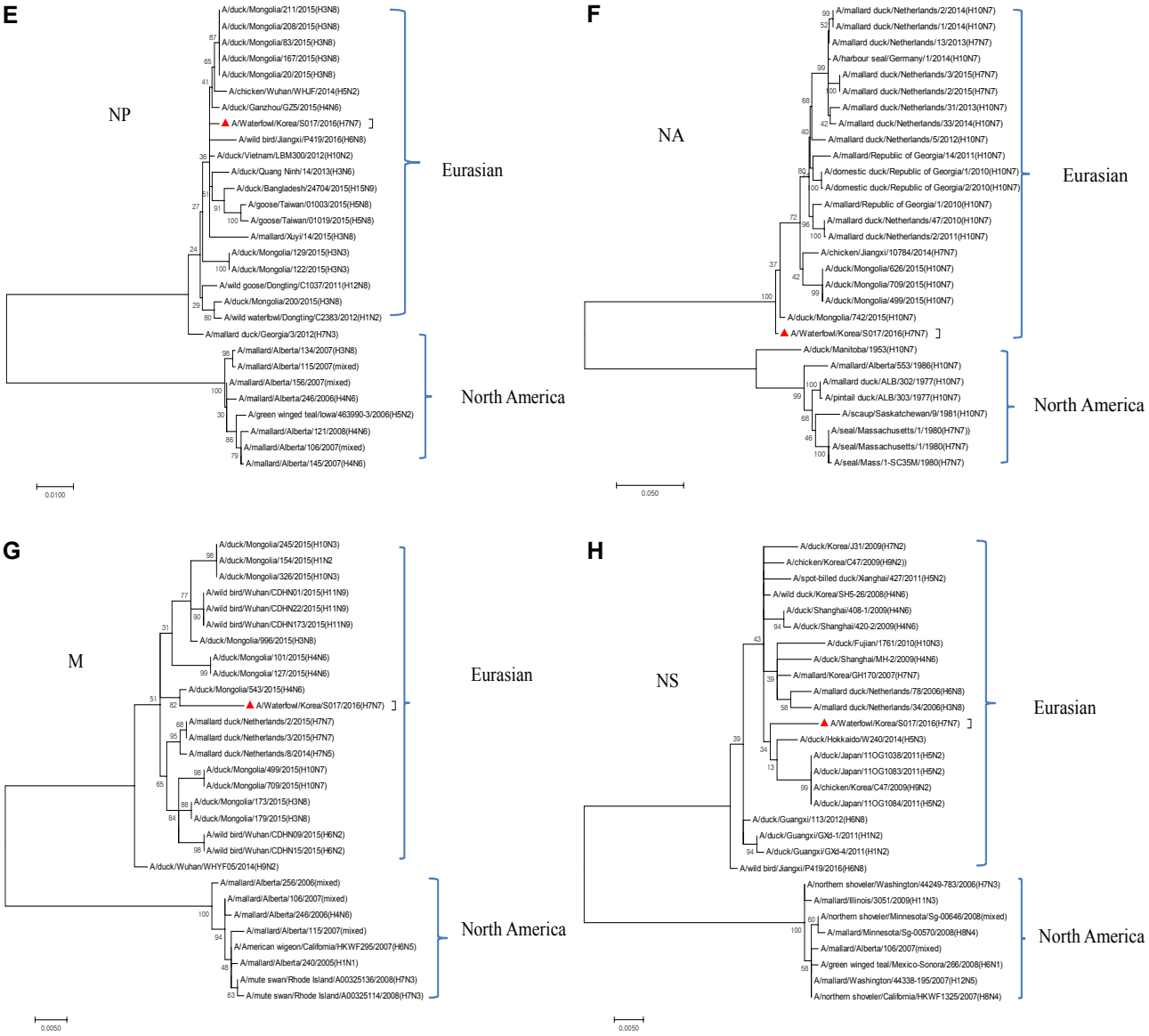

Fig. 1. Continued.

Phylogenetic analysis

The phylogenetic analysis of the isolate showed that all of the eight genes, PB2, PB1, PA, HA, NP, NA, M, and NS belonged to the Eurasian lineage of avian influenza viruses (Fig. 1A-H). We found out that all gene segments of our isolate (A/waterfowl/Korea/S017/2016(H7N7)) had 90-99% nucleotide similarity with A/magpie/Korea/YJD174/2007 (H7N7) reported by Kim et al [11]. The nucleotide similarities between them were; 93% (PB2 gene), 92% (PB1 gene), 96% (PA gene), 90% (HA gene), 96% (NP gene), 94% (NA gene), 97% (M gene), and 99% (NS gene). Furthermore, the Phylogenetic analysis of all H7-subtype avian influenza viruses previously isolated in South Korea were belonged to a Eurasian lineage [11], which correlates with our findings. However, the reassortment statuses of H7N7 viruses isolated in South Korea were not indicated on the previous study. None of our isolate belonged to North American lineages, suggesting that migratory birds migrating in Eurasian regions carried our isolate. No H7N7 HPAI was isolated from the domestic ducks in Korea.

In conclusion, our study suggests that continuous surveillance of wild birds and domestic poultry needs to be carried out to prevent the creation of reassortant avian influenza viruses, which may pose threat to the domestic animals and humans.

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초록 : 2016년 한국 야생조류에서 분리한 H7N7 조류인플루엔자 바이러스 유전자 분석

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A형 인플루엔자바이러스는 야생조류에 존재하며, 사람, 돼지, 가금 및 다른 포유류등 다양한 숙주를 감염한다. 본 연구에서는, 2016년 한국 서쪽의 철새도래지에서 채취한 철새 분변에서 재조합된 새로운 H7N7 조류인플루엔자를 분리하였으며 이 바이러스의 8개 유전자를 분석하였다. 분리된 A/waterfowl/Korea/S017/2016(H7N7) 바이러스의 유전자 분석 결과 이 바이러스는 야생조류 및 가금 오리에서 유래한 조류인플루엔자로 구성된 재조합된 유전자를 가지고 있었다. 계통 분석결과 이 바이러스는 유럽과 아시아계에 속하였다. 조류인플루엔자 바이러스가 계속 진화를 하고 H7 형 조류인플루엔자는 고 병원성 조류인플루엔자로 변하여 사람과 동물에게 커다란 위협이 될 수 있기에 계속 된 역학조사가 필요하다.