Virus Genes (2016) 52:142–145
DOI 10.1007/s11262-015-1272-z

Isolation of a novel H3N2 influenza virus containing a gene of H9N2 avian influenza in a dog in South Korea in 2015

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Received: 19 August 2015 / Accepted: 9 December 2015 / Published online: 12 January 2016

Abstract We isolated a serotype H3N2 influenza virus from a dog with severe respiratory distress in an animal clinic in South Korea in 2015 and characterized the sequences of its eight genes. The following seven genes were derived from canine influenza virus: PB2, PB1, HA, NP, NA, M, and NS. However, the PA gene was derived from avian H9N2 influenza virus that is circulating in poultry in Korea. These findings suggest that the continued surveillance of the influenza virus in dogs is warranted because humans have close contact with dogs, which may promote viral transmission.

Keywords Canine · H3N2 · Influenza virus

The influenza virus belongs to the *Orthomyxoviridae* family of viruses and is grouped into three types: A, B, and C [1]. Influenza A has a broader spectrum of hosts than type B and C viruses do, and it can infect both humans and animals, including birds, pigs, and dogs [1]. The species crossover of influenza A virus from animals to humans has caused prior pandemics associated with many human deaths and substantial economic burdens. The outbreak responsible for the 1918 pandemic was caused by a novel H1N1 influenza virus, which caused more than 50 million human deaths [2].

The H3N8 and H3N2 serotypes of influenza A virus are currently circulating in dogs in the USA and Asia, respectively [3–8]. In January 2004, equine-origin H3N8 influenza A virus was isolated from the lung tissues of racing greyhounds in Florida, USA. The infected dogs displayed classic clinical symptoms, including moderate fever, coughing, severe hemorrhagic tracheitis, bronchopneumonia, pleuritis, vasculitis, and death [4]. There have been reported cases of canine H3N8 in 25 US states as of 2008 [8]. Additionally, avian-origin H3N2 influenza virus was isolated from dogs with severe respiratory symptoms in South Korea in 2007 [6]. Avian-origin H3N2 influenza virus was isolated from farmed dogs with similar respiratory symptoms in China in 2011 [7]. In Thailand, avian-origin H3N2 influenza virus was isolated from dogs with flu-like signs of coughing, sneezing, nasal discharge, and fever in 2012. These isolates were found to be genetically similar to the H3N2 influenza virus isolated in South Korea and China [3].

We isolated influenza A virus from a dog in an animal clinic in South Korea on April 24, 2015. The animal presented with respiratory symptoms such as nasal discharge, sneezing, and coughing. The virus was designated A/Canine/Korea/S3001/2015 (H3N2). The virus was isolated in isolation media (PBS, pH 7.4) supplemented with glycerol (50 %) using a nasal swab. The virus was then inoculated into 10-day-old specific pathogen-free (SPF) eggs. The inoculated eggs were incubated for 72 h at 35 °C and then

Edited by William Dundon.

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chilled to 4 °C overnight. The presence of virus in the allantoic fluids was confirmed by a hemagglutination assay (HA) with 0.5 % red blood cells from turkeys.

The viral RNAs were extracted from the HA-positive allantoic fluid using the RNeasy Protect Mini Kit (gen, CA, USA). The RNA was used to determine the genetic information of the isolate. The RNAs were reverse transcribed to cDNAs using the ImProm-II TM Reverse Transcription System (Promega, Madison, USA) and the Uni12 primer (AGCAAAAGCAGG). Eight viral genes were amplified by polymerase chain reaction (PCR) with GoTaq DNA polymerase and a segment-specific primer set [9]. The amplicons were separated by gel electrophoresis. The bands were then excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, CA, USA). The purified DNA was cloned into a TA vector. The sequences were determined by Solgent (Daejeon, Korea). The gene sequences were compiled and edited using the Lasergene sequence analysis software package (DNA Star version 4.0, Madison, USA). We sequenced three clones of each segment, and the sequence information was the same for each clone. The nucleotide sequences were deposited into GenBank under accession numbers KT446472-KT446479.

We conducted phylogenic analyses using neighbor-joining trees and molecular evolutionary genetics analysis software (MEGA4 version 4.0). The input nucleotide sequences included both the isolate and published influenza virus sequences from the GenBank database. The following nucleotide regions were used in the phylogenic analyses: PB2:1-2311, PB1:1-2341, PA:1-2233, HA:1-1765, NP:1-1535, NA:1-1467, M:1-1027, and NS:1-890.

The phylogenic analysis of the isolate revealed that seven genes (PB2, PB1, HA, NP, NA, M, and NS) belonged to canine lineages. However, the PA gene was derived from the avian H9N2 influenza virus that is circulating in Korea (Fig. 1a–h). We also examined the closest related

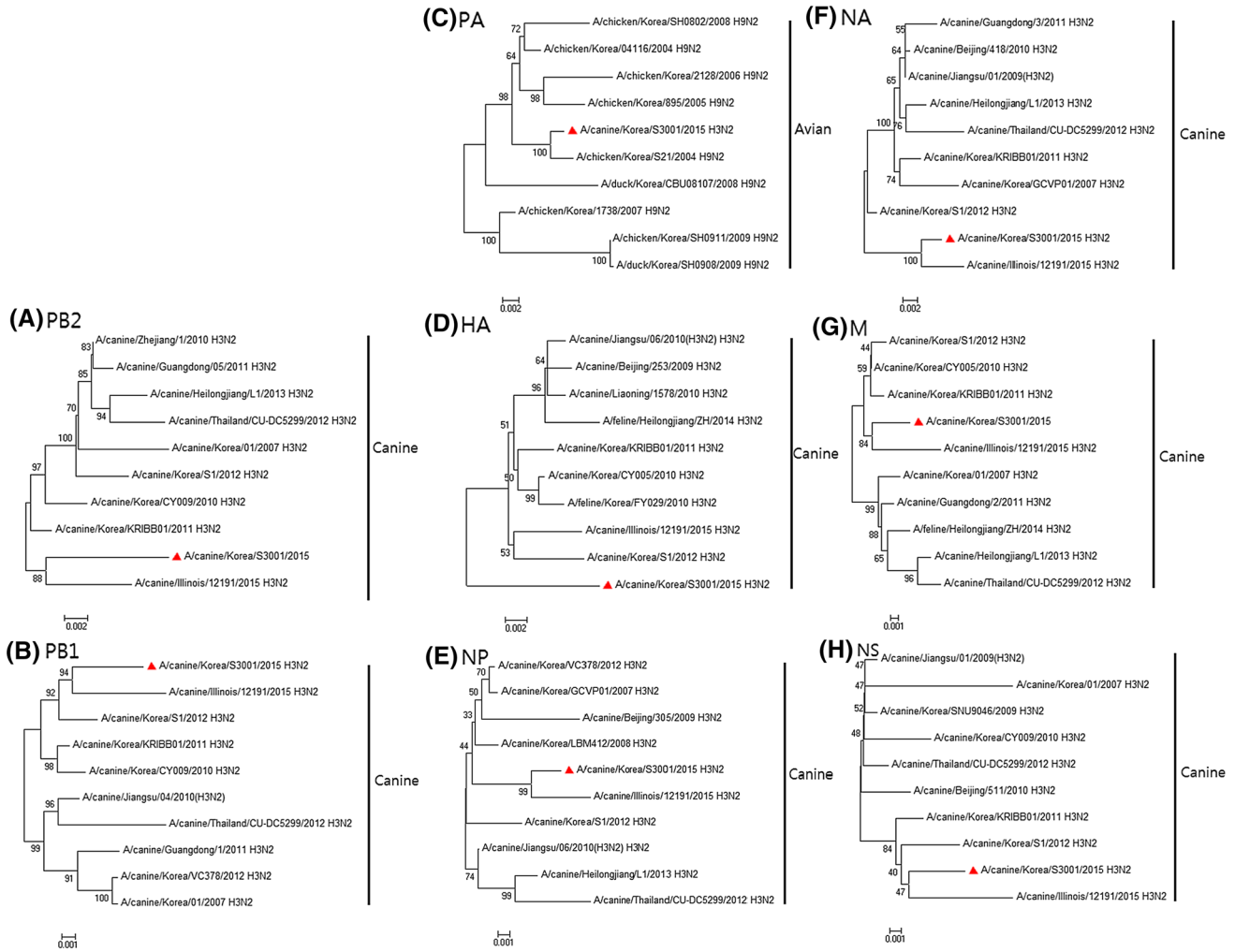

Fig. 1 Phylogenetic analysis of PB2, PB1, PA, HA, NP, NA, M, and NS genes of A/Canine/Korea/S3001/2015 (H3N2). The tree was constructed using the neighbor-joining method in MEGA5 (www.megasoftware.net) with 1000 bootstrap replicates. Scale bar shows nucleotide substitutions per site. **a** PB2; **b** PB1; **c** PA; **d** HA; **e** NP; **f** NA; **g** M; **h** NS

Table 1 Identification of amino acids of A/Canine/Korea/S3001/2015 (H3N2) involved in binding to human-type influenza receptor, enhancing antiviral drug resistance, and causing pathogenesis in mammals

Viral protein	Amino acid position	A/Canine/Korea/S3001/2015 (H3N2)	Comments
HA (H3 numbering)	138	A	A138S:Increased binding to human-type influenza receptor
	226	Q	Q226L:Increased binding to human-type influenza receptor
	228	G	G228S: Increased binding to human-type influenza receptor
NA (N2 numbering)	152	R	R292 K:Resistance to oseltamivir and zanamivir
	274	H	H274Y: Resistance to oseltamivir and zanamivir
	292	R	R292 K: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 mammal

PB2:RNA polymerase basic subunit 2; *PB1:RNA* polymerase basic subunit 1; haemagglutinin; *NA* neuraminidase; *M1* Matrix gene 1; *M2* Matrix gene 2; *NS1* non-structural gene 1

influenza virus to isolate A/Canine/Korea/S3001/2015 (H3N2). The data in GenBank indicated the PB2, PB1, HA, NP, NA, M, and NS genes were closely related to those of A/canine/Korea/KRIBB01/2011(H3N2), A/canine/Korea/S1/2012(H3N2), A/canine/Korea/CY005/2010(H3N2), A/canine/Illinois/12191/2015(H3N2), A/canine/Illinois/12191/2015(H3N2), A/canine/Korea/CY005/2010(H3N2), and A/canine/Korea/KRIBB01/2011(H3N2). The sequence identity was greater than 97 %. However, the PA gene in our isolate was closely related to A/chicken/Korea/S21/2004(H9N2) and had 99 % identity.

We also examined selected regions of the A/Canine/Korea/S3001/2015 (H3N2) genome to better characterize the isolate (Table 1). We found that amino acid 138 in the HA protein sequence was Alanine (A). It was reported that the alanine-to-serine mutation was known to increase binding to the human-type influenza receptor [10]. We assessed the NA gene of the isolate, which is involved in resistance to neuraminidase inhibitors such as oseltamivir and zanamivir. The results indicate that there was an R at positions 152 and 292 and an H at position 274. These mutations suggest sensitivity to drugs [11]. We also identified the M1 mutation T215A. This mutation is known to increase viral pathogenesis in mice [12]. Our isolate has an S at position 31 of M2, which suggests increased sensitivity to ion-channel inhibitors such as amantadine and rimantadine [13]. NS1 has an S at position 42, and this residue is associated with increased virulence in mice [14]. Additional phenotype studies with the isolate are required to confirm the receptor-binding preference, sensitivity to drugs, and pathogenesis in mice.

The genetic analysis of the H3N2 isolate revealed that it is a novel re-assorted virus containing the PA gene from avian H9N2 influenza virus circulating in poultry in Korea. To our knowledge, this is the first report of canine H3N2 influenza virus containing the PA gene of avian H9N2 influenza virus. The gene contributed to the creation of a novel H7N9 influenza virus that has a human mortality rate of approximately 20 % in China. The novel H7N9 influenza virus is a re-assorted virus containing 6 internal genes (PB2, PB1, PA, NP, M, and NS) from avian H9N2 influenza virus [15]. A previous study of A/Canine/Korea/MV1/2012 (H3N2) isolated from dogs in South Korea identified the presence of the M (matrix) gene from the 2009 pandemic H1N1 influenza virus [16].

We examined genes involved in anti-influenza activity. However, we did not identify any mutations that are known to confer resistance to anti-influenza drugs such as neuraminidase inhibitors (oseltamivir and zanamivir) and M2 ion-channel inhibitors (amantadine and rimantadine). Therefore, our results support the prediction that animals infected with canine H3N2 influenza viruses can be successfully treated with these drugs.

Additional surveillance is needed to determine whether the detection of canine H3N2 containing the PA gene from avian H9N2 influenza virus is an isolated case or an outbreak in Korean dogs.

In conclusion, our report suggests that the continuous surveillance of influenza viruses in dogs is necessary for the health of both dogs and humans.

Acknowledgments This work was funded in part by the Basic Science Research Program through the National Research Foundation of Korea (NRF) from the Ministry of Education, Science and Technology (2015R1A2A2A01003943). The manuscript was edited by an editor of American Journal Experts.

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