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Genetic Analysis of a Swine H3N2 Influenza Virus Strain Isolated in Korea in 2017

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Abstract

Pigs are regarded as a "mixing vessel", in which novel influenza viruses may emerge. We isolated a novel swine H3N2 influenza virus from a pig that suffered from severe respiratory symptoms on a farm in South Korea in June, 2017. Genetic analysis showed that this virus was a reassortant strain, containing genes from swine H3N2 and H1N2 influenza viruses. Six genes (PB2, PB1, HA, NP, NA, NS) derived from swine H3N2 influenza viruses and two genes (PA and M) came from swine H1N2 influenza viruses. Our results suggest that reassortment indeed occurs in pigs, indicating that continuous monitoring of influenza virus isolates in pigs is needed for animal and human health because influenza viruses infect both humans and animals.

Key Words: Swine influenza virus, H3N2, epidemiology

The genome of 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[1)].

Influenza A virus is an important respiratory pathogen in pigs. The first clinical influenza-like illness in pigs has been reported during the 1918 H1N1 pandemic[10)]. This classical H1N1 swine influenza virus has been circulating for 80 years in pigs around the world. At present, three subtypes of influenza virus, H1N1, H1N2, and H3N2, are mainly circulating in pigs in the majority of countries, including Canada, China, Japan, and USA[1,2,3,5,19,20,21)].

In South Korea, classical H1N1 and H1N2 influenza viruses were reported in pigs in 2007[8,15)] and 2009 pandemic H1N1 influenza virus was introduced into pigs in 2009[13)]. Swine H3N2 and H3N1 influenza viruses containing the M gene of the 2009 pandemic H1N1 influenza virus were circulating in Korean pigs in 2012[14)]. In addition, swine H3N2 influenza viruses

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containing PA, NP, and M genes of the 2009 pandemic H1N1 influenza viruses also circulated in Korean pigs[11].

In this study, we genetically characterized the genomes of H3N2 influenza virus isolated from pigs suffering from the severe respiratory stress on the farm in South Korea in 2017.

On June 8, 2017, one 3-week-old female pig (Duroc) with severe respiratory distress and high fever over 40°C was euthanized for autopsy on a pig farm in Chungnam province in the southern region of South Korea. Pig's lung has multiple petechiae, which are small red spots (1–2 mm), on the left and right cranial lobes. Its lung tissues were homogenized in PBS (pH 7.4). The prepared samples were inoculated into the wells of a 6-well plate with fully grown Madin-Darby canine kidney (MDCK) cells. Inoculated cells showed cytopathic effects on 3 days after inoculation. Influenza A virus detection in cell culture supernatants was confirmed by reverse transcription polymerase chain reaction with influenza A matrix (M) gene primers, which are available upon request. We designated the isolate as A/Swine/Korea/S45/2017 (H3N2). No other important pathogens which cause respiratory diseases in pigs were detected from its lung tissues. We tried to detect Porcine Reproductive and Respiratory Syndrome virus (PRRSV), Porcine circovirus, *Mycoplasma hyopneumoniae*, *Actynobacillus pluropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, and *Pasteurella multocida* using their specific primers, but we could not detect them in our lung tissues.

Chungnam National University (CNU) Internal Animal Use Committee approved the protocol for pig euthanasia and collection of clinical samples for autopsy. Collection of pig lung tissues were performed by obtaining the consent from the pig farm.

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 reaction (PCR) with GoTaq DNA polymerase and a segment specific primer set. 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.

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°C. The white colonies were selected and grown in LB broth overnight at 37°C in shaking incubator. Plasmid extractions were conducted and were sent for sequencing. Sequencing of genes was performed by Microgen (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, MF951077-MF951084. These sequence data and swine viruses used for the study are readily available upon request to corresponding author. 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. The nucleotides of open reading frame (ORF) of genes, PB2 (1-2280), PB1 (1-2274), PA (1-2151), HA (1-1701), NP (1-1515), NA (1-1410), M (1-982), and NS (1-660), were used for phylogenetic analysis.

We fully sequenced this isolate, A/Swine/Korea/S45/2017 (H3N2). A BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search was carried out to find out previously reported influenza viruses with sequences closest to the sequence of our isolate (Table 1). The RNA polymerase basic

Table 1. Nucleotide homology of A/Swine/Korea/S45/2017 (H3N2) to the closest related influenza viruses

Viral genes	Closest related viruses	Nucleotide identity (%)
PB2	A/swine/Korea/A18/2011 (H3N2)	98
PB1	A/swine/Indiana/A00968386/2012 (H3N2)	98
PA	A/swine/Iowa/A01049723/2011 (H1N2)	98
HA	A/swine/Korea/A18/2011 (H3N2)	97
NP	A/swine/Korea/CY02-09/2012 (H3N2)	98
NA	A/swine/Korea/A18/2011 (H3N2)	97
M	A/swine/North Carolina/A01732036/2016 (H1N2)	98
NS	A/swine/Pennsylvania/A01380412/2013 (H3N2)	98

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 gene; NS: non-structural gene.

subunit 2 (PB2), RNA polymerase basic subunit 1 (PB1), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), and non-structural (NS) genes were closely related to those of the swine H3N2 influenza viruses, A/swine/Korea/A18/2011 (H3N2), A/swine/Indiana/A00968386/2012 (H3N2), A/swine/Korea/A18/2011 (H3N2), A/swine/Korea/CY02-09/2012 (H3N2), A/swine/Korea/A18/2011 *(H3N2),* and A/swine/Pennsylvania/A01380412/2013 (H3N2), respectively, with over 97% identity. The genes encoding RNA polymerase acidic subunit (PA), and M were closely related to those of the swine H1N2 influenza viruses, A/swine/Iowa/A01049723/2011 (H1N2), and A/swine/North Carolina/A01732036/2016 (H1N2), respectively, with over 98% identity.

Phylogenetic analysis suggested that all eight genes (PB2, PB1, PA, HA, NP, NA, M, and NS) of our isolate clustered with the swine lineage isolated from Korea and America (Fig. 1, A–H). When we directly sequenced the genes of RNA isolated from lung tissue of a pig, viral gene constellation is the same as that of the isolate in cell culture.

Next, we analyzed amino acid sequences of the isolated swine H3N2 influenza virus (Table 2). Positions 138 and 228 of the HA protein (H3 numbering) had alanine (A) and serine (S) residues, respectively, which were previously shown to underlie enhanced binding to human influenza virus receptor[18]. Positions 152, 274, and 292 of the NA protein had arginine (R), histidine (H), and arginine (R) instead of lysine (K), tyrosine (Y), and lysine (K), respectively. The isolate was predicted to be susceptible to NA inhibitors according to the amino acid sequence analysis[16]. There was Alanine (A) in M1 at position 215, and this variant has been associated with increased virulence in mice[4]. NS1 contained a serine (S) at position 42, and this mutation has been linked with a more severe pathogenesis of influenza infection in mice[7]. Asparagine (N) was found at position 31 of M2, and this substitution has been shown to underlie the resistance to the anti-influenza ion channel blocking drugs amantadine and rimantadine[6].

Our findings suggested that our isolate, A/Swine/Korea/S45/2017 (H3N2), was a reassortant virus, containing genes from swine H3N2 and H1N2 influenza viruses that circulate in Korean pigs. This result indicates that novel influenza virus strains may arise in pigs infected with influenza viruses of different subtypes. In this regard, it should be mentioned that pigs can act as “mixing vessels”, in which novel influenza viruses may emerge, able to infect humans[9]. For example, the 2009 pandemic H1N1 influenza viruses that spread around the world harbored six genes (PB2, PB1, PA, HA, NP, NS) characteristic of swine influenza viruses from North America and two genes (NA and M) derived from swine influenza viruses from Europe and Asia[17].

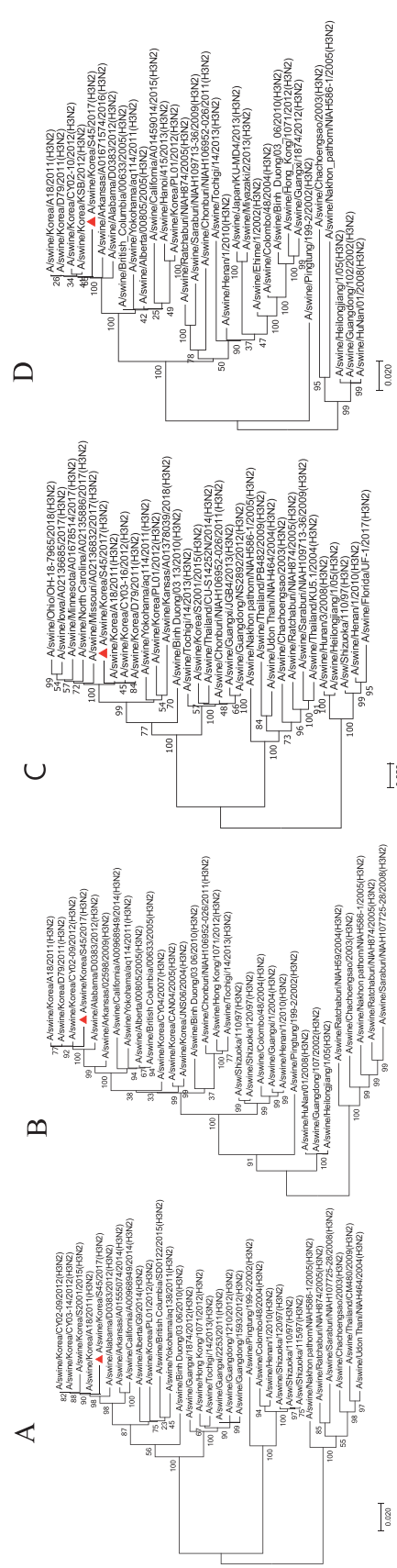

Fig. 1. Phylogenetic analysis of PB2, PB1, PA, HA, NP, NA, M and NS genes of A/Swine/Korea/S45/2017 (H3N2). The tree was constructed by using the neighbor-joining method in MEGA 7.0 (www.megasoftware.net) with 1,000 bootstrap replicates. Scale bar shows nucleotide substitutions per site. **A**, PB2; **B**, PB1; **C**, PA; **D**, HA; **E**, NP; **F**, NA; **G**, M; **H**, NS.

Table 2. Identification of amino acids of A/Swine/Korea/S45/2017 (H3N2) involved in binding to human-type influenza receptor, enhancing antiviral drug resistance, and causing pathogenesis in mammals

Viral protein	Amino acid position	A/Swine/Korea/ S2001/2015 (H3N2)	Comments
PB2	627	E	E627K: Mammalian host adaptation
HA (H3 numbering)	138	A	S138A: Increased binding to human-type influenza receptor
	190	E	E190D: Increased binding to human-type influenza receptor
	226	V	Q226L: Increased binding to human-type influenza receptor
	228	S	G228S: Increased binding to human-type influenza receptor
NA (N2 numbering)	152	R	R292K: Resistance to oseltamivir and zanamivir
	274	H	H274Y: Resistance to oseltamivir and zanamivir
	292	R	R292K: Resistance to oseltamivir and zanamivir
M1	30	S	N30D: Increased pathogenesis in mice
	215	A	T215A: Increased pathogenesis in mice
M2	31	N	S31N: Resistance to amantadine and remantadine
NS1	42	S	P42S: Increased pathogenesis in mammal

Information on the role of amino acids of swine influenza virus in causing pathogenesis in pigs is limited, so further study to find out particular amino acids in each segment of our H3N2 swine influenza virus, which are responsible for causing pathogenesis in pigs is warranted. Interestingly, Manzoor *et al* reported that D256G or E627K mutation in PB2 gene from H5N1 avian influenza virus enhanced viral polymerase activity and replication in pigs[12].

In conclusion, our results suggest that intensive surveillance of influenza virus isolates in pigs may be necessary to prevent cross-over infection of humans with such hybrid and virulent swine influenza virus strains.

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