J. Microbiol. Biotechnol. (2007), **17**(5), 868–872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Genetic and Antigenic Characterization of Swine H1N2 Influenza Viruses Isolated from Korean Pigs

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Received: December 11, 2006
Accepted: January 29, 2007

Abstract H1N2 influenza viruses are circulating in pigs worldwide and cause considerable economic losses to the pig industry. We genetically analyzed the genes of our isolates from Korean pigs, and compared the antigenicity of our isolates with swine H1N2 viruses isolated from pigs in the U.S.A. In addition, we serologically surveyed the infection rate of swine H1N2 viruses in pigs. We found that H1N2 isolates from Korean pigs are genetically more related to swine H1N2 viruses isolated from pigs in the U.S.A. than those in European countries. When antigenicity was compared, our isolates were weakly reacted to antibodies against swine H1N2 viruses isolated from pigs in the U.S.A. The serological surveillance using sera from pigs in Korea showed that about 46% was positive for H1N2 viruses. Our results suggest that swine H1N2 viruses are widespread in Korean pigs, and the development of a vaccine against H1N2 viruses may help to control their infection in pigs.

Keywords: Influenza virus, swine, H1N2, pigs, serology, antigenicity

Swine influenza viruses are important to the swine industry and humans. Three types of influenza A viruses are currently circulating in pigs worldwide; H1N1, H3N2, and H1N2 [3, 6, 19, 21] . In Europe, H1N1 viruses containing wholly avian genes that are antigenically distinct from the classical swine H1N1 viruses were introduced to pigs in 1979 [20]. The distinct H1N2 swine influenza viruses emerged in the U.K. in the early 1990s and have become the dominant subtype infecting pigs in Europe [2, 4, 18, 26]. These H1N2 viruses contain hemagglutinin (HA), which is related to of the HA from human H1N1, and human-like N2 neuraminidase (NA). The remaining genes are derived from European avian-like H1N1 viruses. In the United States, reassortant influenza A viruses, H1N2 and H3N2, which contain genes from a human, classical swine, and avian origin, have been circulating in pigs since 1998 [11, 27]. In Japan, H1N2 viruses derived from the classical H1N1 and H3N2 swine influenza viruses are circulating in pigs [9, 24]. In Korea, H1N1, H1N2, and H3N2 subtypes of influenza viruses have been reported in pigs [5, 10, 23].

Receptor specificity determines the host range of influenza A viruses. It is known that human influenza viruses preferentially bind to sialic acid bound to galactose by α2,6 linkage [25], whereas avian influenza viruses preferentially bind to sialic acid bound to galactose by α2,3 linkage. The previous studies showed that human tracheal epithelial cells predominantly express α2,6-linked sialic acids, whereas avian intestinal cells predominantly express α2,3-linked sialic acids [14]. However, when the respiratory tracts of pigs were stained, the porcine respiratory epithelial cells expressed both α2,6-linked and α2,3-linked sialic acids. Pigs are regarded as "mixing vessels" for the creation of human pandemic viruses because they can be infected with both avian and human influenza viruses [13, 16, 22].

This study characterized the genetic composition, antigenicity, and serological prevalence of H1N2 subtypes recently isolated from Korean pigs.

Swine influenza viruses were isolated from MDCK cells inoculated with a total of 30 lung samples from pigs suffering from severe respiratory signs on farms in Korea from late 2005 to early 2006. Four isolates were successfully isolated, and the isolates were plaque purified three times in MDCK cells before undergoing genetic analysis.

The ferret hyperimmune sera of the swine H1N2 and H3N2 viruses isolated from pigs in the U.S.A. were kindly provided by Dr. Robert G. Webster (St. Jude Children's Research Hospital, Memphis, TN, U.S.A.). Hemagglutination inhibition (HI) assays were performed. In 96-well plates, 25 μl of the sera treated with the receptor-destroying enzyme (RDE) (Denka Seiken Co., Ltd., Chuo-ku, Tokyo, Japan) (1:10) were added to the well, and a 2-fold dilution

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was performed in 25 μl of PBS (pH 7.4). Twenty-five μl of the H1N2 influenza virus samples was added to each well, and the plates were incubated at room temperature for 15 min before adding 50 μl of 0.5% turkey red blood cells (RBC) in PBS (pH 7.4). The HI titers were recorded 40 min after adding the RBC.

The serological surveillance was performed using porcine sera collected from pigs of 6 provinces using HI assays. The collected porcine sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co., Ltd., Chuo-ku, Tokyo, Japan) (1:10). Twenty-five μl of treated sera was serially 2-fold diluted in 25 μl PBS (pH 7.4) before being reacted with 25 μl of A/Swine/Korea/S5/05 (H1N2) viruses for 15 min. The reacted sera were added to 50 μl of 0.5% turkey red blood cells (RBC) before HI titers were evaluated. The HI titers over 40 were recorded as positive samples.

The viral RNA was extracted directly from infected allantoic fluids using an Rneasy Protect Mini Kit (Qiagen, Valencia, CA, U.S.A.). The RNA was transcribed into the cDNA using an ImProm-II TM Reverse Transcription System (Promega, Madison, WI, U.S.A.) with the Uni12 primer (AGCAAAAGCAGG). The cDNA was amplified with GoTaq DNA Polymerase (Promega, Madison, WI, U.S.A.) and segment-specific primer sets. (The primer sequences are available upon request.) The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.) and sequenced directly by the Cosmo Corporation, Seoul, Korea. The DNA sequences were compiled and edited using the Lasergene sequence analysis software package (DNA Star software version 4.0, Madison, WI, U.S.A.). Phylogenic trees were created using the MegAlign program in the Lasergene sequence analysis software package (DNA Star software version 4.0, Madison, WI, U.S.A.) [7, 15, 17].

The nucleotide sequences of the isolates obtained from this study were deposited in GenBank under the following accession numbers: DQ666925-DQ666947.

Genetic Analysis of Korean Awine H1N2 Influenza Viruses

Four H1N2 influenza viruses were isolated from 30 samples in MDCK cells from late 2005 to early 2006. Genetic information of the isolates was obtained by partial sequencing the genes of the isolates and performing phylogenic analysis. The phylogenic analysis showed that the HA, NA, and M genes of our four isolates belonged to the North American lineage (Figs. 1A–1C). When the HA1 of HA genes were compared, all four H1N2 isolates from the Korean pigs were clustered with those of the H1N2 viruses isolated from pigs in the U.S.A. instead of the H1N2 isolates from European pigs or classical swine H1N1 viruses. However, the Korean isolates formed an independent branch from the H1N2 isolates from North

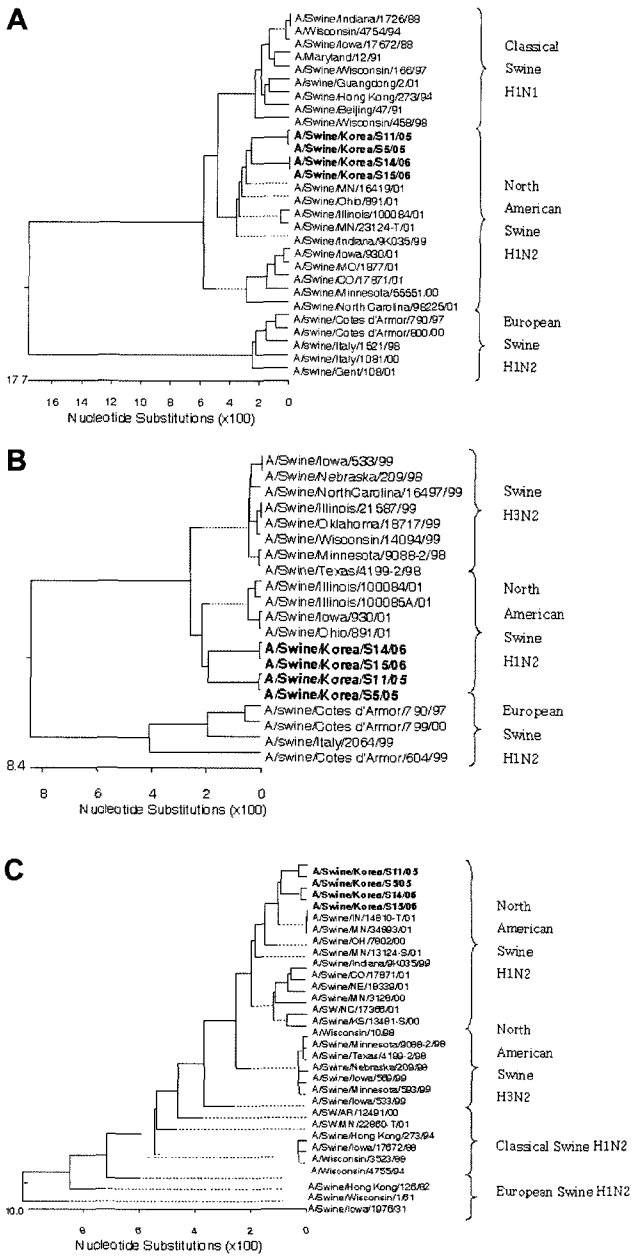

Fig. 1. Phylogenic analysis of HA (**A**), NA (**B**), and M (**C**) of swine H1N2 isolates.

The nucleotide sequences were analyzed with the Lasergene sequence analysis software package (DNA Star software version 4.0, Madison, WI, U.S.A.). The lengths of the horizontal lines are proportional to the minimal number of nucleotide differences required to join the nodes. Vertical lines are used for spacing branches and labels. Viruses sequenced in the present studies are shown in bold and the remaining sequences were found in GenBank. The regions of the analyzed nucleotides are 84-1064 for HA, 81-823 for NA, and 7-945 for M.

America. Phylogenic analysis indicated that the N2 genes of the isolates in this study are clustered with the lineage of those of the swine H1N2 circulating among pigs in North America. One internal gene of these isolates was also

Table 1. Antigenic comparison of swine H1N2 isolates by hemagglutination inhibition assay.

Virus	Subtype	Hyperimmune Ferret Sera	
		A/Sw/NC/2976/02	A/Sw/NC/50270/02
A/Sw/NC/2976/02	H1N2	5,120	<10
A/Sw/NC/50270/02	H3N2	<10	1,280
A/Sw/Kor/S5/05	H1N2	20	<10
A/Sw/Kor/S14/06	H1N2	40	<10

Sw: swine; NC: North Carolina; Kor: Korean.

compared. A comparison of the M sequences of the Korean swine H1N2 viruses with those of the other swine H1N2 and H3N2 viruses showed that the Korean isolates belonged to the lineage of the M genes from the swine H1N2 circulating in U.S. pigs. The M genes of the Korean isolates showed the closest similarity to that of A/Swine/IN/14810-T/01 (H1N2) on the phylogenic trees. When other genes of our isolates were compared, the PB1, PB2, PA, NP, and NS genes were clustered with the swine H1N2 viruses circulating in North American pigs (data not shown), which suggests that the Korean isolates are reassorted viruses created by human, swine, and avian viruses, similar to the swine H1N2 viruses in the U.S.A. [19].

Antigenic Analysis of Korean Swine H1N2 Influenza Virus

Because genetic analysis indicated that the Korean swine H1N2 viruses belong to the lineage of H1N2 influenza viruses circulating among pigs in the United States, the antigenic reactivity of these isolates with ferret hyperimmune sera against U.S.A. swine H1N2 and H3N2 viruses was determined using an HI assay (Table 1). The A/SW/Kor/S5/05 (H1N2) and A/SW/Kor/S14/06 (H1N2) viruses reacted weakly to the antisera against A/SW/NC/297/02 (H1N2) with HI titers of 20 and 40, respectively. No HI titers were detected when the hyperimmune sera of A/SW/NC/50270/02 (H3N2) had been used.

Serological Surveillance

Since swine H1N2 influenza viruses were isolated from pigs in Korea, we are interested in finding out what proportion of pigs in Korea have been infected with this virus. Therefore, we performed the serological surveillance using sera collected from pigs of 6 provinces including Chugnam, Chungpook, and Kyungpook. Out of total 595 sera, 276 were positive for swine H1N2 viruses (46.3%). Among growers, 152 out of 405 sera were positive, whereas among sows, 124 out of 190 sera were positive (65%) (Table 2).

Table 2. Serological surveillance of swine H1N2 influenza viruses in Korean pigs.

	Antigen <A/Sw/Kor/S11/05 (H1N2)>	
	Positive/Total	%
Growers	152/405	37.5
Sows	124/190	65
Growers & Sows	276/595	46.3

Sw: Swine; Kor: Korean.

Pigs can be readily infected with avian and human influenza because the cells in the respiratory tracts of pigs contain receptors for both avian and human influenza viruses [8]. A study of the epidemiology and pathogenesis of swine influenza viruses is very important for identifying and controlling potential pandemic viruses in humans. This study showed that swine H1N2 influenza viruses are circulating continuously in Korean pigs, and the inflammatory responses occur in the tracheas, bronchioles, and lungs of the infected pigs with the induction of the pro-inflammatory cytokines. Genetic analysis showed that the Korean H1N2 influenza viruses isolated from pigs belong to the lineage of swine H1N2 viruses circulating in North America. In addition, the origins of the gene constellations of the Korean swine H1N2 viruses are similar to those of the H1N2 viruses. The HA, NP, M, and NS genes in the Korean H1N2 viruses originated from swine, the NA and PB1 genes originated from humans, and the PA and PB2 genes had an avian origin (data not shown). There might be two reasons for why similar H1N2 viruses circulating among pigs in North America were found in Korean pigs. One is that H1N2 viruses circulating among pigs in North America might have been introduced to Korean pigs by pigs imported from Canada or America to Korea. Another possibility is that Korean H1N2 viruses were reassorted in pigs on Korean farms by the co-infection of classical swine H1N1 and swine H3N2 viruses. In Europe, swine H1N2 viruses containing human-like HA and NA and avian-like PB2, PB1, PA, NP, M, and NS genes are circulating [4]. In Japan, H1N2 viruses contain human-like NA, and swine-like PB2, PB1, PA, HA, NP, M, and NS genes [8]. In Canada, wholly human or human-swine H1N2 viruses have been circulating in pigs since 2003 [12].

The serological surveillance showed that over 40% of pigs in Korea have been infected with swine H1N2 influenza viruses and that sows showed the higher rate of infections by H1N2 viruses than growers. This discrepancy

may be due to the difference of lifespan between growers and sows. Farmers usually raise growers for about 6 months before pigs are sent to markets, while sows stay at farms for about 3–4 years to produce offspring. Since sows stay longer in farms than do growers, sows may have more of a chance to be infected by swine H1N2 influenza viruses.

Our data showed that Korean H1N2 isolates are antigenically different from those of U.S. isolates, even though both strains are genetically closely related. We compared the glycosylation sites of HA proteins of H1N2 isolates. We found that Korean isolates had one additional glycosylation sites on the globular head of HA compared with U.S. isolates (data not shown). More study is needed to understand whether these additional glycosylation sites are responsible for the antigenicity difference between both strains. Previous study showed that human H3N2 viruses containing additional glycosylation sites were antigenically different from those viruses that did not have an additional glycosylation site on the globular head of HA [1].

In summary, swine H1N2 influenza viruses that are antigenically different from those circulating in pigs in the U.S.A. are continuously circulating in pigs in Korea. The continuous surveillance and development of an effective vaccine is needed to control swine H1N2 influenza viruses.

Acknowledgment

This work was supported by Korea Research Foundation Grant KRF-2003-041-E20236.

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