Archives of Virology (2020) 165:1141–1150
https://doi.org/10.1007/s00705-020-04572-z

ORIGINAL ARTICLE

Higher virulence of swine H1N2 influenza viruses containing avian-origin HA and 2009 pandemic PA and NP in pigs and mice

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Received: 14 November 2019 / Accepted: 31 January 2020 / Published online: 28 March 2020
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Abstract
Pigs are capable of harbouring influenza A viruses of human and avian origin in their respiratory tracts and thus act as an important intermediary host to generate novel influenza viruses with pandemic potential by genetic reassortment between the two viruses. Here, we show that two distinct H1N2 swine influenza viruses contain avian-like or classical swine-like hemagglutinins with polymerase acidic (PA) and nucleoprotein (NP) genes from 2009 pandemic H1N1 influenza viruses that were found to be circulating in Korean pigs in 2018. Swine H1N2 influenza virus containing an avian-like hemagglutinin gene had enhanced pathogenicity, causing severe interstitial pneumonia in infected pigs and mice. The mortality rate of mice infected with swine H1N2 influenza virus containing an avian-like hemagglutinin gene was higher by 100% when compared to that of mice infected with swine H1N2 influenza virus harbouring classical swine-like hemagglutinin. Further, chemokines attracting inflammatory cells were strongly induced in lung tissues of pigs and mice infected by swine H1N2 influenza virus containing an avian-like hemagglutinin gene. In conclusion, it is necessary for the well-being of humans and pigs to closely monitor swine influenza viruses containing avian-like hemagglutinin with PA and NP genes from 2009 pandemic H1N1 influenza viruses.

Introduction

Swine influenza viruses belong to the group of influenza A viruses (IAVs), which contain eight segmented negative-sense RNA as a genome [1]. The IAV genome contains the polymerase basic gene 2 (PB2), polymerase basic gene 1 (PB1), polymerase acidic gene (PA), hemagglutinin gene (HA), nucleoprotein gene (NP), neuraminidase gene (NA), matrix gene (M), and non-structural gene (NS) [1]. IAV infects a variety of hosts, including humans, poultry, pigs, dogs, and horses [2–9]. Aquatic birds are the main reservoirs for influenza A viruses, and 16 distinct HA and 9 NA subtypes of influenza viruses are known to circulate in them [10]. IAV is responsible for human pandemics, which occur at intervals of 10-40 years [11–15]. There are four HA lineages of influenza viruses circulating in pigs: the classical 1A lineage (1A, swine classical lineage), which is derived from the ancestors of the 1918 human influenza pandemic, the 2009 pandemic lineage (1A, cH1-derived), which is derived from 2009 human pandemic influenza viruses, the human seasonal-like 1B lineage (1B lineage), which is derived from human seasonal H1 viruses, and the Eurasian avian-like 1C lineage (1C, EA Eurasian lineage), which is derived from avian H1 viruses [16, 20].

Swine H1N1 influenza A virus (known as classical swine H1N1), first isolated in the 1930s, was found to be derived from the 1918 H1N1 human pandemic influenza virus [17]. These classical swine influenza viruses dominantly circulated in pig populations until the late 1990s, when reassorted H3N2 swine influenza viruses containing genes from swine, human, and avian influenza viruses emerged [18, 19]. This novel swine H3N2 influenza virus contains NP, M, and NS from classical swine H1N1 influenza viruses; PB2 and PA

Handling Editor: Roman Pogranichniy.

Yunyueng Jang and Taehyun Seoequally contributed to this work

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00705-020-04572-z) contains supplementary material, which is available to authorized users.

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from North American avian influenza viruses; and HA, NA, and PB1 from human seasonal influenza viruses [18, 19]. The introduction of reassorted H3N2 influenza viruses accelerated the development of genetic diversity among influenza viruses in pigs. The internal genes of these H3N2 influenza viruses served as a backbone for the creation of novel H1N1 and H1N2 influenza viruses in pigs [20]. The introduction of 2009 pandemic H1N1 influenza viruses (A (H1N1)pdm09) in pigs further diversified the gene constellations of swine influenza viruses [21–24]. The 2009 pandemic H1N1 influenza viruses have been shown to be reassorted viruses containing PB2 and PA genes from avian influenza viruses related to the North American lineage, a PB1 gene from H3N2 seasonal human influenza viruses, HA and NA genes from classical swine influenza viruses related to the North American lineage, and NA and M genes from avian influenza viruses [15, 25, 26]. In South Korea, H1N1, H1N2 and H3N2 influenza viruses have been isolated from pigs [5, 27–31]. A novel reassortant H1N2 virus containing internal genes from 2009 pandemic H1N1 influenza viruses was found in Korean pigs in 2010 [27], and H3N2 influenza viruses with three internal genes from 2009 pandemic H1N1 influenza viruses were found in Korean pigs in 2015 [5].

The receptor binding preference of IAV HA protein is regarded as one of the major determinants of host specificity [2]. Avian influenza viruses preferentially bind alpha 2,3-linked sialic acid (α 2,3-SA), while human influenza viruses preferentially bind alpha 2,6-linked sialic acid (α 2,6-SA), and swine influenza viruses preferentially bind α 2,3-SA or both α 2,3-SA and α 2,6-SA [32–34]. Pigs are regarded as a mixing vessel capable of generating novel IAVs, since cells of the porcine respiratory tract contain both α 2,3-SA and α 2,6-SA, rendering them susceptible to infection by both human and avian influenza viruses [35, 36].

In this study, we isolated two distinct strains of H1N2 influenza virus from Korean pigs in 2018, analysed their genetic composition, and studied their pathogenicity in mice and pigs.

Materials and methods

Sample collection and isolation of swine influenza viruses

Nasal swabs (n = 30) were collected from pigs with respiratory clinical signs such as sneezing and runny nose from two Korean pig farms in Chungnam province in southern South Korea in 2018 and preserved in minimal essential medium (MEM) (pH 7.4) supplemented with Antibiotic Antimycotic Solution (100 units of penicillin, 1 mg of streptomycin and 2.5 µg of amphotericin B) (Sigma) for further use. The collected samples were filtered with syringe filters (0.22 µm) prior to inoculation onto Madin-Darby canine kidney (MDCK) cells in a 6-well cell culture plate. N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (1 µg/ml) (Sigma) was added to MEM. Supernatants of the infected cells were screened for the presence of influenza A virus by reverse transcription polymerase chain reaction (RT-PCR) with primers specific for the influenza A matrix (M) gene [37]. The isolates were subtyped by sequencing the HA and NA genes. The isolates from one farm were designated as A/swine/Korea/s802/2018 (H1N2) and A/swine/Korea/s803/2018 (H1N2), and the viruses from another farm were designated as A/swine/Korea/s804/2018 (H1N2), A/swine/Korea/s805/2018 (H1N2), and A/swine/Korea/s806/2018 (H1N2).

Cloning and sequencing

Viral RNAs from isolated swine influenza viruses were purified using a Viral RNA Mini Kit (QIAGEN). cDNA was synthesized using an ImProm-II™ Reverse Transcription System (Promega, Madison, USA) with the influenza A virus Uni 12 primer (AGCAAAAGCAGG). Eight segments of the isolated swine influenza viruses, PB2, PB1, PA, HA, NP, NA, M and NS, were amplified using GoTaq® Green Master Mix (Promega, Madison, USA) with universal primer sets as described [38]. The amplicons were separated by electrophoresis on an agarose gel, purified using a QIAquick Gel Extraction Kit (QIAGEN), and cloned into a TA vector (Promega, Madison, USA). The sequences of the cloned amplicons were determined at Cosmo Co. (Daejeon, Korea).

Phylogenetic analysis

Viral sequence data were compiled and edited using the sequence analysis software package DNAStar version 4.0 (Madison, USA). The nucleotide sequences of the swine influenza viruses were deposited in the GenBank database under the accession numbers MN094322-MN094329, MN094334-MN094341, MN094342-MN094349, MN094312-MN094319, and MN094350-MN094357.

Neighbour-joining trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA4) version 7.0. Nucleotide sequences from this study and published influenza virus sequences from the GenBank database were used for analysis. The nucleotide regions used in the phylogenic analysis are as follows: 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.

Pathogenicity of swine H1N2 viruses in pigs

Four-week-old Yorkshire pigs (four per group) that were negative for H1N1, H1N2, and H3N2 swine influenza viruses based on a serological assay and M-gene-specific real-time PCR were infected intranasally (i.n.) with 1 ml of a virus suspension containing 10^6 $TCID_{50}$ of A/Swine/Korea/s803/2018 or A/Swine/Korea/s806/2018. Pigs were monitored for any changes in body weight and body temperature until 14 days postinfection (p.i.).

Viral titres and histopathology of lung tissues of pigs infected with swine H1N2 influenza viruses

Four-week-old pigs (three per group) were infected i.n. with 1 ml of 10^6 $TCID_{50}$ of a virus suspension containing A/swine/Korea/s803/2018 or A/swine/Korea/s806/2018, and at 5 days p.i., they were euthanized by intravenous injection of T61 (Intervet) with isoflurane anesthetization. Approximately 1.0 g of lung sample (left cranial lobe) was homogenized in PBS (pH 7.4), and the viral titre was determined in units of $log_{10}TCID_{50}$/ml using MDCK cells. Approximately 1.0 g of lung sample was used for histopathology. Lung tissues were submerged in 10% neutral buffered formalin and were embedded in paraffin. Lung sections (5 μm) were prepared and were stained with haematoxylin solution for 4 minutes and then washed with tap water for 10 minutes. The washed lung sections were stained with eosin solution. The stained sections were observed under a microscope (Olympus, Tokyo, Japan).

Pathogenicity of the isolated swine H1N2 influenza viruses in mice

Six-week-old female BALB/c mice (10 per group) were infected i.n. with a 50-μl suspension containing 10^6 $TCID_{50}$ A/swine/Korea/s803/2018 or A/swine/Korea/s806/2018 per ml. Mice were monitored for any changes in body weight and mortality until 14 days p.i.

Viral titres and histopathology in lung tissues of mice infected with swine H1N2 influenza viruses

Six-week-old female BALB/c mice (three per group) were infected i.n. with a 50-μl suspension containing 10^6 $TCID_{50}$ A/Swine/Korea/S803/2018 or A/Swine/Korea/S806/2018 per ml, and at 5 days p.i., they were euthanized by intravenous injection of T61 (Intervet) with isoflurane anesthetization. Half of the lung tissue (about 0.1 g per lung sample) was homogenized in PBS (pH 7.4), and the viral titre was determined using MDCK cells in units of $log_{10}TCID_{50}$/ml. The remaining half of the lung tissues (about 0.1 g per sample) was used for histopathology. Lung tissues were submerged in 10% neutral buffered formalin and were embedded in paraffin. Lung sections (5 μm) were prepared, stained with haematoxylin solution for 4 minutes, and washed with tap water for 10 minutes. The washed lung sections were stained with eosin solution (1% in ethanol) and viewed under a microscope (Olympus, Tokyo, Japan). The stained tissues were graded for pathology based on the following criteria: a score of 1 for inflammatory change in less than 20% of the examined section, a score of 2 for inflammatory change covering 20 to 50% of the examined section, and a score of 3 for the inflammation change covering over 50% of the examined section.

In order to detect antigens in the lung tissues of pigs and mice, lung sections were stained with a rabbit antibody against influenza A virus nucleoprotein (Sino Biological, China). Deparaffinized sections were treated with antigen retrieval solution in a microwave oven and then were blocked with normal rabbit serum in PBS (pH 7.4). The blocked sections were labeled with a rabbit antibody against influenza A virus nucleoprotein (1:100 dilution) and then with biotin-labeled goat anti-rabbit immunoglobulin (Vector, USA), Vectastain ABC-AP (Vector, USA), and Vector Red alkaline phosphatase substrate (Vector, USA). The stained tissue sections were counterstained with hematoxylin QS (Ve Laboratories, Burlingame, CA) and evaluated under an Olympus DP70 microscope (Olympus Corporation, Tokyo, Japan).

Quantification of genes for inflammatory cytokines and chemokines in the lung tissues of pigs and mice

Total RNA was isolated from pig and mouse lung tissue using an RNeasy Plus Universal Mini Kit (QIAGEN) according to manufacturer's instructions. The mRNA of porcine and mouse inflammatory and chemokine cytokines was quantified by quantitative real-time PCR. To synthesize cDNA, 1 μL of oligo dT primer (0.5 pmol) (Promega, Madison, WI, USA) was added to a total volume of 9 μL of mRNA. The mixture was allowed to react for 5 min at 70 °C prior to incubation for 5 min at 4 °C. Subsequently, 4 μL of 25 mM $MgCl_2$, 4 μL of 5X reverse transcriptase enzyme buffer, 1 μL of RNase inhibitor, 1 μL of reverse transcriptase, and 1 μL of dNTP (10 mM) were added to each of the samples. Each sample was incubated for 5 min at 25 °C, 60 min at 42 °C, and 15 min at 70 °C. SYBR Green–based real-time PCR was performed using a Roto-Gene 6000 apparatus (Corbett, Mortlake, Australia) and SensiMix Plus SYBR (Quantace, London, UK) based on the recommendations of the manufacturer. Samples were tested in duplicate. The data represent the mean of six reactions from three pigs or three mice. The final reaction volume was 20 μL, containing 2 μL of cDNA, 10 μL of SYBR mixture, and inflammatory cytokine- and chemokine-specific

primers (1 μL of forward primer [20 pmol] and 1 μL of reverse primer [20 pmol]). Amplification was carried out for 40 cycles of 5 s at 95 °C, 15 s at 60 °C, and 25 s at 72 °C. Real-time PCR data were quantified using the delta-delta CT method by comparing data from infected animals to those from uninfected animals.

Primer sequences are shown in Supplementary Table 5 and Table 6.

Statistical analysis

Differences between infected and uninfected animals were analysed by Student's *t*-test using IBM SPSS Statistics version 20. *P*-values less than 0.05 were considered statistically significant.

Ethical statement

Chungnam National University (CNU) Internal Animal Use Committee approved the protocol (201906A-CNU-105) for the pathogenicity study for animals, pigs and mice and for collection of clinical samples. All experiments and methods were performed with approval and in accordance with the respective relevant legal guidelines and regulations of Chungnam National University, Republic of Korea.

Results

Genetic analysis of swine H1N2 influenza viruses in South Korea in 2018

We isolated five H1N2 influenza viruses from pigs on farms in South Korea in 2018 where the animals were suffering from respiratory distress. The isolated swine H1N2 influenza viruses were sequenced, and their genomes were analysed. Phylogenetic analysis showed that the PB2, PB1, NA, M, and NS genes of five isolates clustered with swine H1N2, H1N1 and H3N2 influenza viruses (Supplementary Fig. 1a, b, f, g, and h). The HA genes of A/swine/Korea/s802/2018 (H1N2) and A/swine/Korea/s803/2018 (H1N2) were grouped with those of avian-origin swine H1N2 influenza viruses (1C, EA Eurasian lineage), while the HA genes of A/swine/Korea/s804/2018 (H1N2) A/swine/Korea/s805/2018 (H1N2), and A/swine/Korea/s806/2018 (H1N2) were related to those of classical swine H1N2 influenza viruses (1A, swine classical lineage) (Supplementary Fig. 1d). PA and NP genes of the five isolates were related to those of 2009 pandemic H1N1 influenza viruses (Supplementary Fig. 1c and e).

The PB2, PB1, NA, M and NS genes of A/swine/Korea/s803/2018 (H1N2) were most closely related to A/swine/Korea/A18/2011 (H3N2) genes, with 97.46% sequence identity; A/West Virginia/06/2011 (H3N2), with 97.80% identity; A/swine/Korea/CY03-11/2012 (H1N2), with 95.46% identity; A/swine/Korea/CY03-19/2012 (H3N2), with 98.34% identity; and A/swine/Ohio/11TOSU559/2011 (H3N2), with 97.85% identity, respectively. The PB2, PB1, M and NS genes of A/swine/Korea/s803/2018 (H1N2) were related to those of the TRIG-like lineage of H3N2, and the NA gene of A/swine/Korea/s803/2018 (H1N2) was of Korean classical swine H1N2 origin.

The PA and NS genes of A/swine/Korea/s803/2018 (H1N2) were found to be closely related to those of A/Korea/CJ24/2009 (H1N1), with 97.54% sequence identity, and A/Chile/3760/2009 (H1N1), with 97.23% sequence identity, respectively. The HA gene of A/swine/Korea/s803/2018 (H1N2) was closest to that of A/swine/Korea/CY0423-12/2013 (H1N2) (Supplementary Table 1). The PB2, PB1, NA, M, and NS genes of A/swine/Korea/s806/2018 (H1N2) were closest to those of A/swine/Korea/CY03-19/2012 (H3N2), with 98.20% sequence identity; A/swine/Korea/CY03-13/2012 (H3N1), with 98.18% homology, A/swine/Korea/CY03-11/2012 (H1N2), with 95.53% identity; A/swine/Korea/CY03-19/2012 (H3N2), with 98.78% identity; and A/swine/Ohio/11TOSU559/2011 (H3N2), with 98.21% identity, respectively. The PB2, PB1, M, and NS genes of A/swine/Korea/s806/2018 (H1N2) were related to those of the TRIG-like lineage of H3N2, and the NA gene of A/swine/Korea/s806/2018 (H1N2) was of Korean classical swine H1N2 origin.

The PA and NP genes of A/swine/Korea/s806/2018 (H1N2) were most closely related to those of A/Wisconsin/629-D01017/2009 (H1N1), with 97.21% sequence identity, and A/Hong Kong/H090-757-V10/2009 (H1N1), with 97.10% sequence identity, respectively. The HA gene of A/swine/Korea/s806/2018 (H1N2) was closest to that of A/swine/Korea/CY03-11/2012 (H1N2), with 96.30% sequence identity (Supplementary Table 2).

Further, we analysed the major proteins encoded by genes responsible for binding to human-type influenza receptor, increasing antiviral-drug resistance, and enhancing pathogenesis in mammals in two representative isolates, A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) (Supplementary Table 3). Amino acids alanine (A) and aspartic acid (D) were found at amino acid positions 138 and 190 of the HA proteins of A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2), which are responsible for increased binding to human type influenza viruses [39, 40]. The aspartic acid residue (D) at position 225 from A/swine/Korea/s806/2018 (H1N2) is predicted to be involved in binding to human-type influenza viruses [40], and alanine (A) at position 215 in the M1 proteins of A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) is predicted to be involved in pathogenesis in mice [41]. Asparagine (N) was found at position 31 in the M2 proteins of A/swine/Korea/s803/2018 (H1N2)

and A/swine/Korea/s806/2018 (H1N2). This residue confers resistance to M2 ion channel inhibitors such as amantadine and rimantadine [42]. The Serine (S) at position 42 in the NS1 proteins of A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) are predicted to be involved in pathogenesis in mice [43].

We identified potential N-linked glycosylation sites in the HA proteins of A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) because of their possible involvement in pathogenesis in animals (Supplementary Table 4). These isolates had eight potential N-linked glycosylation sites in the HA protein. Five of these, at amino acid positions 27, 28, 40, 498, and 557, were present in both isolates. In addition, A/swine/Korea/s803/2018 (H1N2) had potential N-glycosylation sites at amino acid positions 71, 212, and 291, while A/swine/Korea/s806/2018 (H1N2) had potential N-glycosylation sites at amino acid positions 136, 142, and 304.

Swine H1N2 influenza viruses containing avian-like HA exhibit increased pathogenicity in pigs

We inoculated 4-week-old pigs (four per group) with two representative viruses, A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2), to determine whether swine H1N2 influenza viruses containing different HA genes exhibit differences in their pathogenicity in pigs. Pigs infected with avian-origin-HA-containing H1N2 (A/swine/Korea/s803/2018) had higher body temperatures and more growth retardation than pigs infected with classical-swine-origin HA-containing H1N2 (A/swine/Korea/s806/2018) (Supplementary Fig. 2 a and b). The mean peak body temperature for pigs infected with A/swine/Korea/s803/2018 (H1N2) was 41.0 °C at day 5 p.i., while that for pigs infected with A/swine/Korea/s806/2018 (H1N2) was 39.6 °C at 5 day p.i. ($P < 0.05$) (Supplementary Fig. 2a). At 14 days p.i., the average body weight of the pigs infected with A/swine/Korea/s803/2018 (H1N2) was 134.3% of that before infection, while that of the pigs infected with A/swine/Korea/s806/2018 (H1N2) was 146.8% of that before infection. In PBS-mock-infected control pigs, the average weight at 14 days p.i. was 155.6% of the original weight (Supplementary Fig. 2b). The growth retardation in infected pigs was not significant with either virus. Viral titres in nasal swabs and lung tissues of pigs infected with A/swine/Korea/s803/2018 (H1N2) or A/swine/Korea/s806/2018 (H1N2) were measured (Fig. 1a and b). At 3 days p.i., the mean viral titre in nasal swabs from pigs infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) was 3.5 and 4.0 $TCID_{50}$/ml, respectively (Fig. 1a). At 5 days p.i., the mean viral titre in the lung tissues of pigs infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) was 5.0 ($P < 0.05$) and 4.75 $TCID_{50}$/g, respectively (Fig. 1b). The differences in viral titers in pigs were not significant for the two strains.

We stained pig lung tissues with haematoxylin and eosin (H&E) at day 5 p.i. The lung tissues **??**of pigs infected with A/swine/Korea/S803/2018**??** showed severe interstitial pneumonia with high infiltration of inflammatory cells (Fig. 2b), while those of pigs infected with A/swine/Korea/s806/2018 (H1N2) showed much milder pneumonia (Fig. 2c). The lung tissue of PBS-mock infected pigs did not

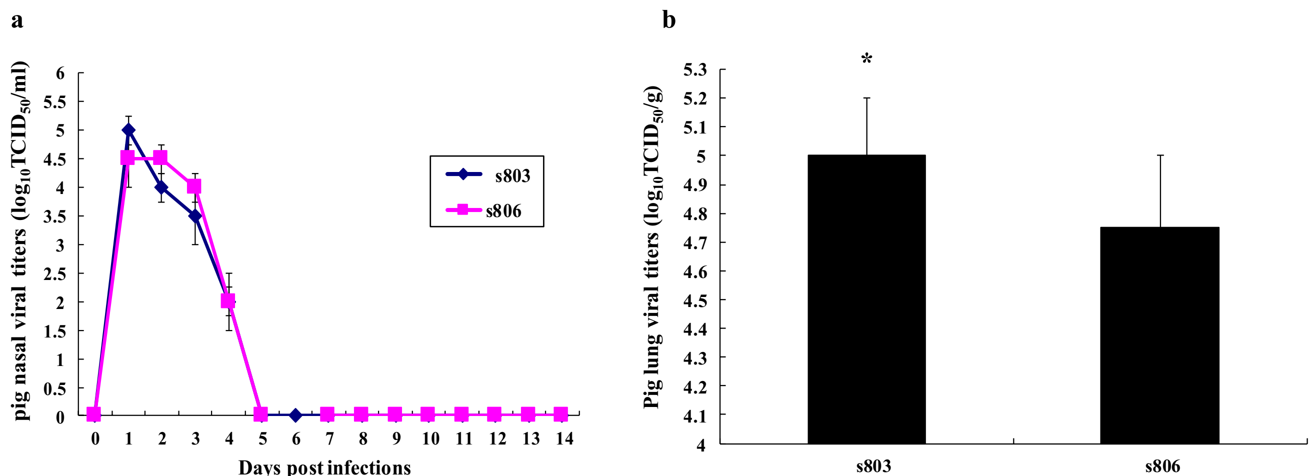

Fig. 1 Viral titers in nasal swabs and lung tissues of infected pigs. Nasal swabs were collected daily from infected pigs (Supplementary Fig. 2) and placed in PBS (pH 7.4), and their viral titers were measured in MDCK cells in units of $log_{10}TCID_{50}$/ml (a). To measure viral titers in lung tissues (b), four-week-old pigs (three per group) were infected i.n. with 1 ml of a suspension containing 10^6 $TCID_{50}$ of A/swine/Korea/s803/2018 or A/swine/Korea/s806/2018, and at 5 days p.i., the pigs were euthanized for the collection of lung tissues. Tissues were homogenized, and their viral titers were measured in MDCK cells in units of $log_{10}TCID_{50}$/g. The data represent the mean ± standard deviations. s803, A/swine/Korea/s803/2018 (H1N2); S806, A/swine/Korea/s806/2018 (H1N2). *, $p < 0.05$

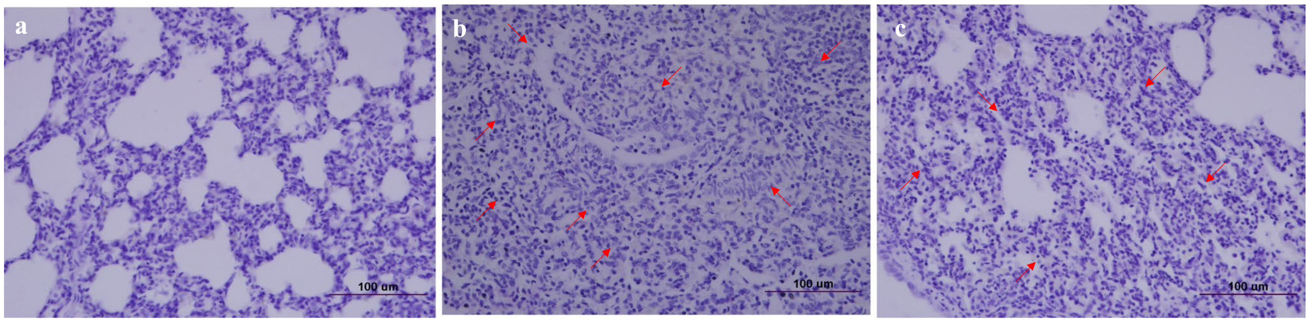

Fig. 2 Histopathology of lung tissues of infected pigs. The parts of lung tissues used to determine viral titers in Fig. ??1b?? were fixed with neutral buffered formalin and were stained with H&E. (a) Lung tissue from a PBS-mock-infected pig. (b) Lung tissue from a pig infected with A/swine/Korea/s803/2018 (H1N2). (c) Lung tissue from a pig infected with A/swine/Korea/s806/2018 (H1N2). Arrow: interstitial pneumonia and infiltration of inflammatory cells

show any sign of pneumonia (Fig. 2a). The infected porcine lung tissues were positively stained with influenza A NP antibody (Supplementary Fig. 3). A/swine/Korea/s803/2018 (H1N2) dominantly infected alveolar cells (Supplementary Fig. 3b), while A/swine/Korea/s806/2018 (H1N2) mainly infected bronchial alveolar cells (Fig. 5c). The pathology scores of porcine lung tissues infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) were 1.3 and 1.0, respectively (Supplementary Fig. 4).

Inflammatory cytokines and chemokines in the lung tissues of pigs infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) were quantified on day 5 p.i., since inflammatory responses contribute to the pathogenesis of influenza viruses in animals [44] (Fig. 3). CCL1 and CXCL9 were found to be upregulated in the lung tissues of pigs infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2). Compared to the lung tissues of PBS-mock-infected pigs, the lungs of pigs infected with A/swine/Korea/s803/2018 (H1N2), exhibited a 65.7-fold increase in CCL1, which attracts monocytes, natural killer (NK) cells, immature B cells, and dendritic cells ($P < 0.001$), those of pigs infected with A/swine/Korea/s806/2018 (H1N2) shared a 48.2-fold increase [45]. The increase in CXCL9, which attracts cytotoxic lymphocytes (CTLs), natural killer (NK) cells, natural killer T (NKT) cells, and macrophages, in the lung tissue of pigs infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) was 29.85- ($P < 0.05$) and 20.3-fold, respectively [46].

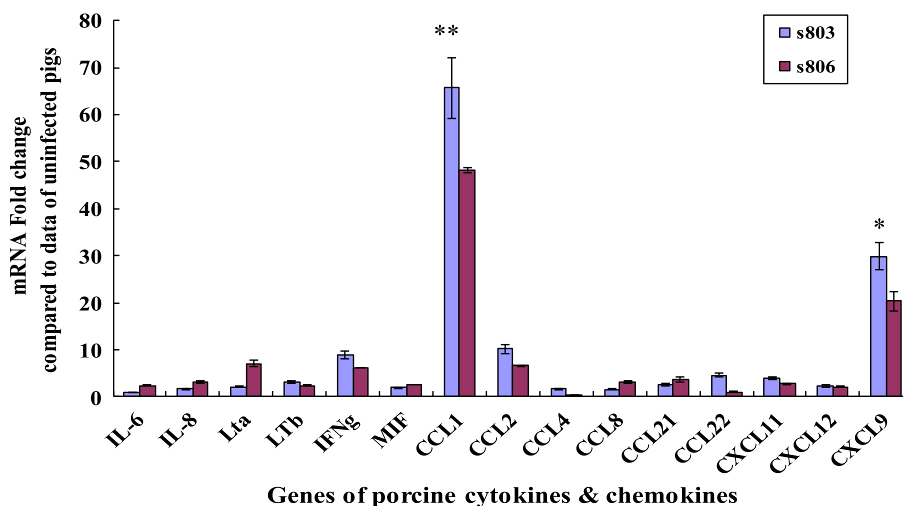

Fig. 3 Quantification of expression of genes for inflammatory cytokines and chemokines in the lung tissues of infected pigs. Total RNA was isolated from the parts of lung tissues used to determine viral titers in Fig. ??1b??. Genes of porcine inflammatory cytokines and chemokines were quantified by SYBR Green–based real-time PCR. The fold change was calculated using the delta-delta CT method by comparing data from infected pigs to those from uninfected pigs. s803, A/swine/Korea/s803/2018 (H1N2); S806, A/swine/Korea/s806/2018 (H1N2). **, $P < 0.001$; *, $p < 0.05$

Swine H1N2 influenza viruses containing avian-like HA show increased pathogenicity in infected mice

In order to study pathogenesis in another mammal, we infected mice (n = 10) with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2). Infected mice were monitored for survival and changes in body weight up to 14 days p.i. (Fig. 4). The survival rate for mice infected with A/swine/Korea/s803/2018 (H1N2) was 0%, while that for mice infected with A/swine/Korea/s806/2018 (H1N2) was 100% (Fig. 4a). The loss in body weight in mice infected with A/swine/Korea/s803/2018 (H1N2) was similar to that in mice infected with A/swine/Korea/s806/2018 (H1N2) (Fig. 4b).

On day 5 p.i., the H&E-stained lung tissue of mice that had been infected with A/swine/Korea/s803/2018 (H1N2) showed severe interstitial pneumonia with a large number of inflammatory cells (Supplementary Fig. 5b), while that of mice infected with A/swine/Korea/s806/2018 (H1N2) had milder pneumonia (Supplementary Fig. 5c). The lung tissue of a PBS-mock-infected mouse did not show any signs of pneumonia (Supplementary Fig. 5a). Mouse lung tissues were positively stained with influenza A NP antibody (Supplementary Fig. 5). The pathological scores of mouse lung tissues infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) were 2.7 and 1.6, respectively (Supplementary Fig. 4)

Viral titres in lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) were similar, at 4.5 $TCID_{50}$/g and 4.0 $TCID_{50}$/g, respectively (Supplementary Fig. 6a).

We also quantified mRNA for inflammatory cytokines and chemokines in the lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2). Among the cytokines and chemokines, CCL4, CCL22, CXCL9, and CXCL10 were dominantly induced in the lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2). The levels of CCL4, CXCL19, and CXCL10 were higher in the lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) than in those infected with A/swine/Korea/s806/2018 (H1N2) (Supplementary Fig. 6b). The levels of CCL4, CXCL19, and CXCL10 in the lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) were 298.1- ($P < 0.05$), 608.8- ($P < 0.001$), and 501.4- ($P < 0.05$) fold higher than in PBS-mock-infected mice, while those in mice infected with A/swine/Korea/s806/2018 (H1N2) were 160.8-, 302.3-, and 317.3-fold higher, respectively. The levels of CCL22 in the lung tissues of mice infected with A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) were 160.89- and 213.7-fold higher than the control, respectively.

Discussion

Swine influenza viruses cause severe respiratory disease in pigs and have potential to infect humans. Pigs act as a mixing vessel to create novel influenza viruses, since they are easily infected with human and avian influenza viruses. We isolated five of H1N2 influenza viruses from Korean pigs in 2018. These isolates are divided into two groups: avian-origin-H1- and classical swine-origin-H1-containing

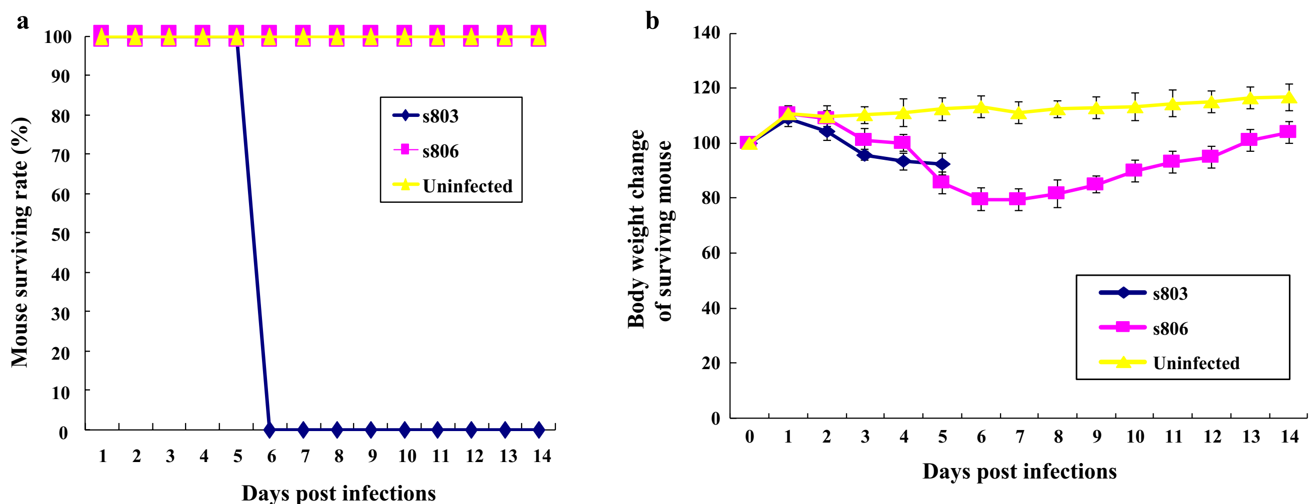

Fig. 4 Survival rate and body weight of infected mice. Six-week-old female BALB/c mice (10 per group) were infected i.n. with 50 µl of a suspension containing10^6 $TCID_{50}$ of A/Swine/Korea/s803/2018 or A/Swine/Korea/s806/2018. The survival rate and the body weight of each mouse were daily recorded until 14 days p.i. The data represent the mean ± standard deviation. s803, A/swine/Korea/s803/2018 (H1N2); S806, A/swine/Korea/s806/2018 (H1N2). (a) Survival rate. (b), Body weight

Table 1 Amino acid difference in eight genes between A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2)

Gene segment	Amino acid difference: A/swine/Korea/s803/2018 (H1N2) (position) A/swine/Korea/s806/2018 (H1N2)
PB2	R126K, E249R, M292T, V295I, S328F, V344M, R368K, S465P, K482R, V511I, S569T, R664G, R702K, A717T
PB1	L166F, A182T, I200V, K211R, D213N, E229K, G361R, A461V, L604F, G614E, V646M, L683I, Q687L, N694D,
PA	E27D, T85I, K113N, R174G, P259T, L336R, T357A, T441M, L543P, P601S, E603K, E610G
HA	E2K, K4V, L5I, F6V, F9L, C10Y, A11T, L15T, K16D, I20L, V22I, I36V, T52D, S53R, N55D, K57R, S60K, N62R, K64I, N65A, Q68H, N71K, T73N, V74I, I78L, D85E, L88N, S90A, N91S, I97V, K103N, N104I, A106T, E111D, A113I, D114N, T124S, K136N, T138S, D144T, T146D, T149V, V151A, S154P, S156A, A158T, N159S, R163Q, L166I, I168L, D173N, Y174S, P176Q, L178I, V193L, V196I, P200S, T201N, N202S, S203N, Q206R, T207V, H211N, N212E, H213D, T214A, S217F, K222R, Y224S, R226K, V232A, V233N, E239D, A241E, L251V, D252E, Q253P, T256K, I266V, W269R, K276R, S277N, S279G, M283I, M284I, D286E, Q288S, N291D, T293D, K295T, Y300K, L303I, K304N, S305T, T306S, V312I, E319K, Q328K, I338T, I362V, Q382L, K383T, I387N, D390N, S393T, V399I, K402R, I405T, S409A, N415S, N416H, M421I, V435I, F454Y, L457S, S461N, K465R, K467R, R471K, N490D, E491T, N502D, S510A, N513T, E516K, M525T, G526R, H528Y, L544V
NP	R109K, T111I, H141Y, I192V, I228M, T379I, T402N, N439S, T479N
NA	I8M, C42Y, Y44S, I51V, E54K, T86N, D127N, R249K, I312T, A313V, G346N, I401S, Q432L
M1	V51I, M65T
M2	P24L, F25L, I32M, A38V, F90L
NS1	S31P, N53G, K78N, T79M, T87S, T123I, I124M, I129V, V182I
NS2	D12G, N45S, K49R, A74E, F101L

viruses. Avian-origin-H1-containing H1N2 viruses cause more-severe disease in mice and pigs than classical swine-origin-H1-containing H1N2 influenza viruses.

The H1N2 influenza viruses isolated from Korean pigs in 2018 were divided into two groups, avian-origin H1 (A/swine/Korea/s803/2018 (H1N2) and classical swine-origin A/swine/Korea/s806/2018 (H1N2), based on similar constellations of the PB2, PB1, PA, NP, NA, N, and NS genes. We found that the PA and NP genes were derived from the 2009 pandemic H1N1 influenza virus. Previously isolated swine H1N2 influenza viruses in Korean pigs in 2013 contained HA from a Eurasian avian-like H1 swine influenza virus and NA genes from swine H1N2 influenza viruses circulating in Korean pigs [30, 31]. The internal genes of these isolates were derived from circulating triple-reassorted H3N2 swine influenza viruses containing an M gene from the 2009 pandemic H1N1 influenza virus, which were found to be circulating in pigs in North America [30, 31]. H1N2 swine influenza virus A/swine/Korea/VDS1/2010, which was isolated from Korean pigs in 2010, contained HA and NA genes from circulating swine influenza viruses, whereas the rest of the genes were from the 2009 pandemic H1N1 influenza virus [27]. H1N1 influenza virus A/swine/Korea/61/2016, which was isolated from Korean pigs in 2016, had the HA gene from the 2009 pandemic H1N1 influenza virus, while the rest of the genes were from circulating swine influenza viruses [27]. To summarize, we think that the swine H1N2 influenza viruses in Korean pigs are continuously evolving by recombination with genes from 2009 pandemic H1N1 influenza viruses.

Our results of experimental infections of mice and pigs with two representative viruses, A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2), showed that the avian-origin-HA-containing A/swine/Korea/s803/2018 (H1N2) caused more-severe disease in these animals. Mice infected with A/swine/Korea/s803/2018 (H1N2) showed 100% mortality, while A/swine/Korea/s806/2018 (H1N2) did not cause fatal infections in mice. In contrast, the Korean 2013 isolate A/swine/Korea/CY0423/2013, which contains an avian-like HA, a Korean swine H1N2-like neuraminidase, and H3N1pM-like internal genes did not cause fatal infections in mice [31].

A previously reported pathogenicity study of variant H1N1 and H1N2 influenza viruses isolated from humans who had been infected by contact with diseased pigs showed that most of these viruses did not cause fatal infections in mice [47]. All mice infected with A/Minnesota/45/2016 (H1N2v), A/Iowa/39/2015 (H1N1v), or A/Minnesota/19/2011 (H1N2v) survived, while mice infected with A/Ohio/09/2015 (H1N1v) showed 80% mortality [47]. A study of viruses arising by reassortment between Eurasian avian-like swine H1N1 influenza virus and the 2009 pandemic H1N1 influenza virus showed that most reassorted viruses containing an avian-like H1 and the rest of genes from 2009 pandemic H1N1 influenza virus were lethal for mice [48]. A/swine/Korea/s803/2018 (H1N2) virus had an

avian-origin H1 with PA and NP genes from the 2009 pandemic H1N1 influenza virus, and this virus exhibited higher pathogenicity in mice and pigs.

Our results show that infection with A/swine/Korea/s803/2018 (H1N2) induces the expression of chemokine genes that are involved in recruiting inflammatory cells in lung tissues of mice and pigs more strongly than A/swine/Korea/s806/2018 (H1N2). In lung tissues, the chemokines CCL1 and CXCR9 were induced more strongly in pigs infected with A/swine/Korea/s803/2018 (H1N2) than in pigs infected with A/swine/Korea/s806/2018 (H1N2). In lung tissues, the chemokines CCL4, CXCR9, and CXCL10 were more strongly induced in mice infected with A/swine/Korea/s803/2018 (H1N2) than in mice infected with A/swine/Korea/s806/2018 (H1N2). It seems that differences in chemokine induction affected the pathogenicity of our swine H1N2 isolates in both pigs and mice.

When we compared the amino acid sequences of eight genes between A/swine/Korea/s803/2018 (H1N2) and A/swine/Korea/s806/2018 (H1N2) (Table 1 and Supplementary Fig. 7), none of the amino acid differences in A/swine/Korea/s803/2018 (H1N2) were found to be the same as those reported previously to be involved in increased pathogenicity in mice [49–51]. Amino acid mutations in PB2 (L134H, I164L, D701N), HA (G228S), and M1 (D221N) have been shown to be involved in enhanced virulence in mice [49]. Mouse-adapted H1N2 avian influenza virus showed increased virulence in mice when they contained the amino acid mutations M147L and E627K in PB2 [50]. Eurasian avian-like H1N1 swine influenza viruses with the mutation D701N in PB2 showed increased pathogenicity in mice [51].

In summary, we can confidently state that two distinct groups of swine H1N2 influenza viruses were circulating in Korean pigs in 2018. Swine H1N2 virus containing avian-origin HA was lethal for mice. In the future, there is a need to monitor swine influenza viruses, given their zoonotic potential, in order to prevent any possible infections in humans and pigs.

Acknowledgements We thank the local veterinarians for providing pig swab samples. This work was in part supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (no. 2019002168).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval Chungnam National University (CNU) Internal Animal Use Committee approved the protocol (201906A-CNU-105) for the pathogenicity study in pigs and mice and collection of clinical samples.

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