FULL PAPER *Virology*

Genomic Reassortants of Pandemic A (H1N1) 2009 Virus and Endemic Porcine H1 and H3 Viruses in Swine in Japan

Rikio KIRISAWA[1)]*, Yoshitaka OGASAWARA[1)], Hayato YOSHITAKE[1)], Asuka KODA[1)] and Tokujiro FURUYA[2)]

[1)]*Laboratory of Veterinary Virology, Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyoudai Midori-machi, Ebetsu, Hokkaido 069–8501, Japan*
[2)]*Zoetis Japan Co., Ltd., 3–22–7 Yoyogi, Shibuya-ku, Tokyo 151–0053, Japan*

(Received 15 April 2014/Accepted 4 July 2014/Published online in J-STAGE 24 July 2014)

ABSTRACT. From 2010 to 2013 in Japan, we isolated 11 swine influenza viruses (SIVs) from pigs showing respiratory symptoms. Sequence and phylogenetic analyses showed that 6 H1N1 viruses originated from the pandemic (H1N1) 2009 (pdm 09) virus and the other 5 viruses were reassortants between SIVs and pdm 09 viruses, representing 4 genotypes. Two H1N2 viruses contained H1 and N2 genes originated from Japanese H1N2 SIV together with internal genes of pdm 09 viruses. Additionally, 1 H1N2 virus contained a further NP gene originating from Japanese H1N2 SIV. One H1N1 virus contained only the H1 gene originating from Japanese H1 SIV in a pdm 09 virus background. One H3N2 virus contained H3 and N2 genes originating from Japanese H3N2 SIV together with internal genes of pdm 09 virus. The results indicate that pdm 09 viruses are distributed widely in the Japanese swine population and that several reassortments with Japanese SIVs have occurred.

KEY WORDS: Japan, pandemic 09, reassortant, swine influenza

doi: 10.1292/jvms.14-0194; *J. Vet. Med. Sci*. 76(11): 1457–1470, 2014

Influenza A virus of the *Orthomyxoviridae* family is an enveloped RNA virus with eight segmented negative-stranded RNAs. In April 2009, a new swine-origin virus emerged in humans and caused a worldwide pandemic. This new virus was named pandemic (H1N1) 2009 virus (pdm 09). It is composed of genes of the North American triple reassortant swine influenza virus (SIV) and European avian-like H1N1 SIV [9, 49]. Soon after emergence of pdm 09, human-to-swine transmission was observed in many countries [15, 17, 34, 43, 44, 50, 51, 60]. Susceptibility of pigs to pdm 09 was confirmed by experiments showing that the virus induced disease similar to that induced by endemic SIV and transmitted efficiently in pigs [3, 28, 59]. Reassortants of pdm 09 and circulating SIVs were later detected in many countries [1, 6, 7, 12, 13, 16, 27, 29, 35, 37, 52, 57, 58, 64]. SIV causes respiratory disease in pigs with symptoms of high fever, depression, anorexia and labored abdominal breathing. Such symptoms result in weight loss of fattening pigs and generate an economic loss in the pig industry.

In Japan, classical H1N1 SIVs are thought to have entered the Japanese pig population in the late 1970s [40, 62]. There have been few reports of its isolation after 1980s. H3N2 virus of human origin was isolated from a pig in 1969 [39]. Serological and virological examinations revealed that H3N2 viruses were maintained in pigs in the 1990s [23, 24]. However, isolation of H3N2 viruses from pigs has only occurred sporadically in Japan [46]. A reassortant H1N2 SIV was first isolated in 1978 [53]. The virus was a reassortant of classical H1N1 SIV and human H3N2 virus. This virus has become the predominant type of SIV in Japan [22, 41, 42, 45, 48, 63]. In 2009, pdm09 viruses were isolated in 2 prefectures in Japan, and in 2011 and 2012, reassortant H1N2 viruses containing H1 and N2 genes from Japanese H1N2 SIVs together with internal genes of pdm 09 viruses were isolated in other 2 prefectures [31]. Another type of H1N2 reassortant containing the N2 gene from Japanese H1N2 SIV in a pdm 09 background was isolated [26]. However, there is little information on the circulating SIV phenotype in Japan after the spread of pdm 09 in humans in Japan. In this study, we investigated the distribution of influenza viruses in the Japanese pig population with clinical symptoms, such as pneumonitis.

MATERIALS AND METHODS

Virus isolation from clinical specimens: Eleven clinical specimens listed in Table 1 were positive for influenza A virus as determined by using a rapid diagnosis kit for detection of influenza nucleoprotein antigen (FUJIREBIO Inc., Tokyo, Japan). Nasal swabs or lung homogenates were inoculated on monolayers of Madin-Darby canine kidney (MDCK) cells and into the allantoic cavities of 10-day-old embryonated chicken eggs. The supernatants and allantoic fluids were harvested and tested by the hemagglutination (HA) test with 0.5% chicken red blood cells or by using the rapid diagnosis kit. Subtypes of the HA and NA genes were determined by reverse transcription polymerase chain reaction (RT-PCR) with primers for HA and NA genes [36].

Sequence and phylogenetic analysis: Viral RNA was ex-

*CORRESPONDENCE TO: KIRISAWA, R., Laboratory of Veterinary Virology, Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyoudai, Midori-machi, Ebetsu, Hokkaido 069–8501, Japan. e-mail: r-kirisa@rakuno.ac.jp

Table 1. Influenza viruses isolated in this study

Virus	Subtype	Sampling period	Sample	Clinical signs	Farm (SIV vaccine status in sows)	Segment sequenced	GenBank Accession No.
A/sw/Ibaraki/46/2010	H1N1	Sep. 2010	Lung from a fattening pig (50 days old)	Pneumonitis	Domestic farm in Ibaraki Prefecture (No)	Full genome	AB911590-AB911597
A/sw/Kagoshima/60/2010	H1N1	Oct. 2010	Lung from a fattening pig (5 months old)	Pneumonitis	Domestic farm in Kagoshima Prefecture (No)	Full genome	AB911606-AB911613
A/sw/Hiroshima/8/2011	H1N1	Jan. 2011	Lung from a fattening pig (50 days old)	Pneumonitis	Domestic farm in Hiroshima Prefecture (No)	Full genome	AB909395-AB909402
A/sw/Hiroshima/52/2011	H1N1	Nov. 2011	Lung from a fattening pig (60 days old)	Pneumonitis	The same farm as above in Hiroshima Prefecture (No)	Full genome	AB911598-AB911605
A/sw/Chiba/14/2012	H1N2	Jan. 2012	Nasal swab from a suckling piglet (16 days old)	Pneumonitis	Domestic farm in Chiba Prefecture (No)	Full genome	AB914481-AB914488
A/sw/Kagoshima/23/2012	H1N1	Mar. 2012	Lung from a fattening pig (70 days old)	Pneumonitis	Domestic farm in Kagoshima Prefecture (No)	Full genome	AB910568-AB910575
A/sw/Chiba/30/2012	H1N1	Apr. 2012	Lung from a suckling piglet (29 days old)	Pneumonitis	Domestic farm in Chiba Prefecture (Yes)	Full genome	AB910576-AB910583
A/sw/Chiba/107/2012	H1N2	Aug. 2012	Nasal swab from a fattening pig (35 days old)	Pneumonitis	Domestic farm in Chiba Prefecture (No)	Full genome	AB914489-AB914496
A/sw/Chiba/119/2012	H1N2	Oct. 2012	Nasal swab from a fattening pig (80 days old)	Pneumonitis	Domestic farm in Chiba Prefecture (No)	Full genome	AB914497-AB914504
A/sw/Kagoshima/65/2012	H1N1	Nov. 2012	Lung from a fattening pig (60 days old)	Pneumonitis	Domestic farm in Kagoshima Prefecture (No)	Full genome	AB911614-AB911621
A/sw/Tochigi/14/2013	H3N2	Feb. 2013	Nasal swab from a sow (2 years old)	Pneumonia	Domestic farm in Tochigi Prefecture (No)	Full genome	AB914505-AB914512

Abbreviation: sw, swine.

tracted from supernatants and allantoic fluids described above using the QIAamp Viral RNA Mini kit (Qiagen K.K., Tokyo, Japan). The extracted RNA was reverse-transcribed to cDNA by Superscript III (Life Technologies Co., Ltd., Tokyo, Japan) with a universal primer for influenza A viruses [14]. Each gene segment was amplified by PCR with KOD-Plus-Neo (Toyobo Co., Ltd., Osaka, Japan) and segment-specific primers. The PCR products were purified with the High Pure PCR Product Purification kit (Roche Diagnostics K.K., Tokyo, Japan) or the QIAquick Gel Extraction kit (Qiagen K.K.), if extrabands were observed. Sequencing was conducted in Hokkaido System Science Co., Ltd. (Sapporo, Japan). Phylogenetic analysis of the nucleotide sequences was conducted by using MEGA5 software with 1,000 bootstrap replicates of the neighbor-joining method [47, 56]. Evolutionary distances were estimated according to the Kimura 2-parameter method [25]. GenBank accession numbers assigned to the gene sequences of the analyzed isolates are AB909395-AB909402, AB910568-AB910583, AB9111590-AB911621 and AB914481- AB914542 (Table 1).

Sera: Immune sera to isolates of influenza A viruses were prepared in rabbits. Briefly, allantoic fluid containing viruses inactivated with 0.05% formalin was concentrated by ultracentrifugation. Concentrated viruses were purified by discontinuous sucrose gradient (20 to 60%) ultracentrifugation. The purified viruses with protein content of 50 μg mixed with TiterMax Gold adjuvant (TiterMax U.S.A., Inc., Norcross, GA, U.S.A.) were subcutaneously injected 4 times at intervals of 1 month. Swine sera were collected from variously aged fattening pigs, gilts and sows of a consistent management pig farm located in Hiroshima Prefecture, where SIVs were isolated 2 times at an interval of 10 months at 6 months after the second SIV isolation.

Hemagglutination inhibition (HI) test: The antigenic reactivity of H1 viruses and antibody titers to H1 viruses in pig sera were examined by the hemagglutination inhibition (HI) test. Briefly, immune sera and pig sera were treated with a receptor-destroying enzyme (RDE II) (Denka Seiken Co., Ltd., Tokyo, Japan) overnight at 37°C to remove nonspecific inhibitors of HA. Then, the sera were heated at 56°C for 30 min and absorbed with an equal volume of 10% chicken red blood cells at room temperature for 60 min. Antigens

Table 2. Genetic origins of swine influenza viruses isolated in this study

Virus	Subtype	Gene origin from							
		HA	NA	PB2	PB1	PA	NP	M	NS
A/sw/Ibaraki/46/2010	H1N1	pdm [a)]	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Kagoshima/60/2010	H1N1	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Hiroshima/8/2011	H1N1	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Hiroshima/52/2011	H1N1	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Chiba/14/2012	H1N2	Cl-Sw[b)]	Cl-Sw	pdm	pdm	pdm	Cl-Sw	pdm	pdm
A/sw/Kagoshima/23/2012	H1N1	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Chiba/30/2012	H1N1	Cl-Sw	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Chiba/107/2012	H1N2	Cl-Sw	Cl-Sw	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Chiba/119/2012	H1N2	Cl-Sw	Cl-Sw	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Kagoshima/65/2012	H1N1	pdm	pdm	pdm	pdm	pdm	pdm	pdm	pdm
A/sw/Tochigi/14/2013	H3N2	Hu-Sw[c)]	Hu-Sw	pdm	pdm	pdm	pdm	pdm	pdm

a) Originated from a Pandemic (H1N1) 2009 virus. b) Originated from a classical swine influenza. c) Originated from a human-swine influenza. Abbreviation: sw, swine.

were adjusted to 8 hemagglutinating units. The sera were titrated by 2-fold dilution and mixed with an equal volume of the antigen (25 μl). The mixtures were incubated for 60 min at room temperature. Fifty μl of 0.5% chicken red blood cells was added to each mixture, and the mixture was left to stand for 60 min at room temperature. HI titer of 20 or greater was regarded as positive [55].

RESULTS

Virus isolation: We isolated eleven hemagglutinating agents from all 11 specimens in MDCK cells and embryonated eggs after the first inoculation of the specimens. A commercial rapid diagnosis kit for human influenza revealed that all of the agents were influenza A viruses. Partial sequencing of the HA and NA genes showed that 7 isolates were H1N1 viruses, 3 isolates were H1N2 viruses, and 1 isolate was H3N2 virus. Designations of the isolates are listed in Table 1.

Genetic analysis of the isolates: Sequences of the full-lengths of the 11 isolates were determined. BLAST analysis and construction of phylogenetic trees revealed genetic origins of genes of the isolates as shown in Table 2. Phylogenetic trees based on H1 and N1 genes are shown in Figs. 1 and 2. Among the 7 H1N1 isolates, H1 and N1 genes of 6 isolates (A/swine/Ibaraki/46/2010, A/swine/Kagoshima/60/2010, A/swine/Hiroshima/8/2011, A/swine/Hiroshima/52/2011, A/swine/Kagoshima/65/23/2012 and A/swine/Kagoshima/65/2012) were found to be those of pdm 09 viruses. The other six genes of the isolates were also found to be those of pdm 09 viruses based on a phylogenetic tree of the 6 genes (data not shown, except for NP gene in Fig. 4). A/swine/Hiroshima/8/2011 and A/swine/Hiroshima/52/2011 were isolated from the same pig farm in Hiroshima Prefecture at intervals of 10 months. The last H1N1 isolate (A/swine/Chiba/30/2012) had the H1 gene of Japanese H1 SIV and N1 gene of pdm 09 virus, and the other 6 internal genes were found to be those of pdm 09 viruses.

In the 3 H1N2 isolates, H1 genes were located within a cluster of reassortants of pdm 09 and Japanese SIVs (A/swine/Tochigi/2/2011 and A/swine/Mie/R02/2012) [31]. The H1 gene of the H1N1 isolate (A/swine/Chiba/30/2012) was located in a different cluster from the 3 H1N2 isolates, despite the fact that the isolates were from the same prefecture, Chiba. Among the 3 H1N2 isolates, N2 genes of A/swine/Chiba/107/2012 and A/swine/Chiba/119/2012 were located in the same cluster (Fig. 3). A/swine/Gunma/1/2012, A/swine/Tochigi/2/2011 and A/swine/Mie/R02/2012, which were reported to be reassortants of pdm09 and classical SIV [26, 31], were located in the cluster. The N2 gene of A/swine/Chiba/14/2012, however, was located in a different cluster. The other six genes of the 3 H1N2 isolates were from pdm09, except for the NP gene of A/swine/Chiba/14/2012, which was located within a cluster of Japanese SIVs (Fig. 4). The evolutionary distance of the NP gene between A/swine/Chiba/14/2012 and other Japanese SIVs was relatively large.

The HA and NA genes of 1 H3N2 isolate (A/swine/Tochigi/14/2013) were derived from a human-swine lineage, and the other 6 genes were from pdm 09 (Figs. 3 and 5). The evolutionary distance of H3 and N2 genes between A/swine/Tochigi/14/2013 and the other Japanese H3N2 SIVs was relatively large. The N2 gene of A/swine/Tochigi/14/2013 was located in a cluster of human-swine lineage that was a different cluster in which the 3 H1N2 isolates in this study were located.

Antigenic analysis of H1 influenza viruses by the HI test: To determine the antigenic relationships among the 10 H1 isolates, the HI test was performed using immune rabbit sera against A/swine/Ibaraki/46/2010 (H1N1) that had the HA gene of pdm 09 and A/swine/Chiba/14/2012 (H1N2) that had the HA gene of Japanese SIV (Table 3). An 8-fold reduction in the titer with the serum against A/swine/Ibaraki/46/2010 (H1N1) was observed for A/swine/Chiba/30/2012 (H1N1) and A/swine/Chiba/14/2012 (H1N2) compared to the HI titer of a homologous virus. The antiserum to A/swine/Chiba/14/2012 (H1N2) showed an 8-fold reduction in the HI titer in A/swine/Kagoshima/60/2010 (H1N1) compared to the titer of a homologous virus and did not react with A/swine/Chiba/30/2012 (H1N1).

Analysis of amino acid sequences in HA of H1 viruses: To

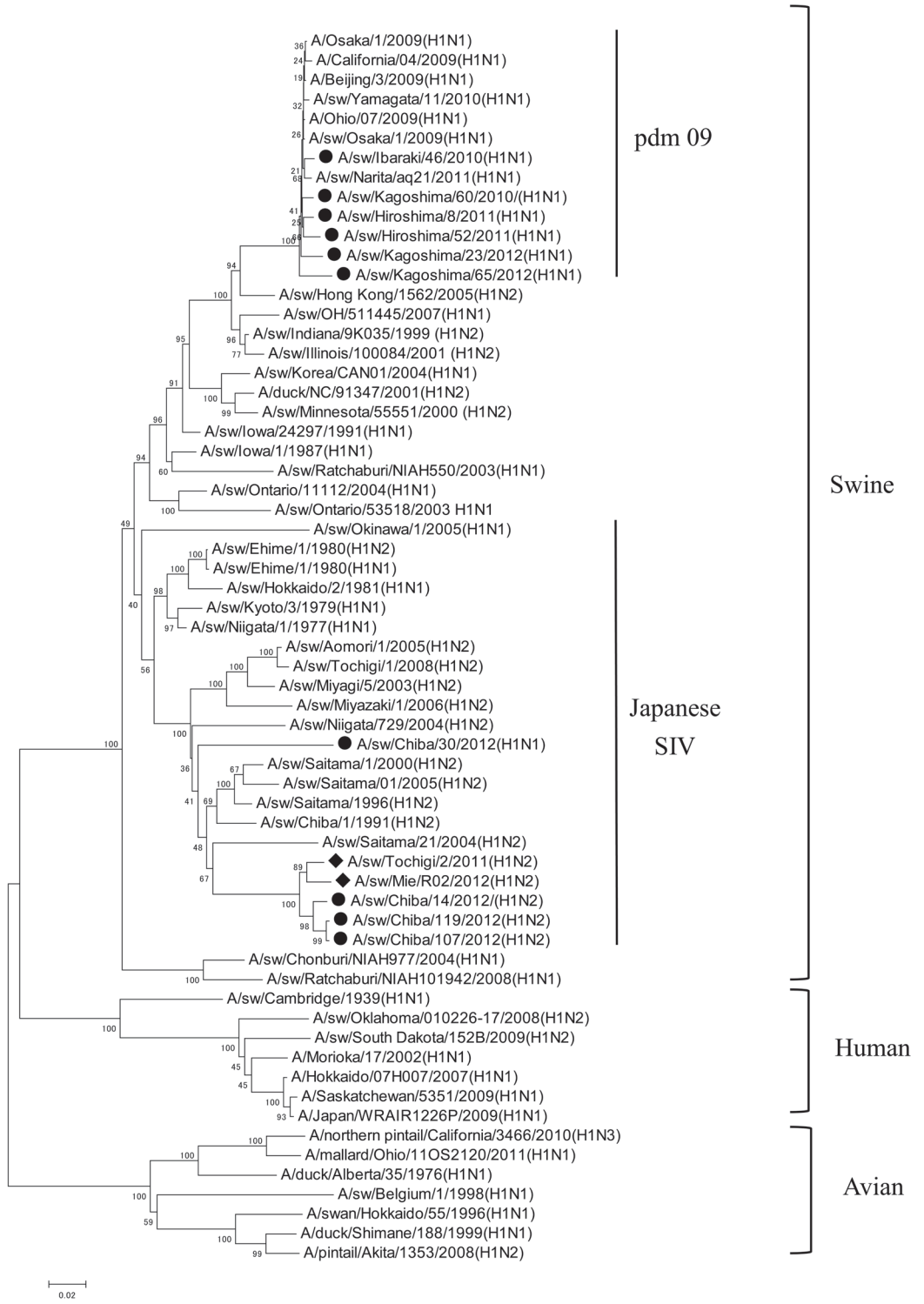

Fig. 1. Phylogenetic tree of H1 HA genes of H1 isolates in this study and others available from GenBank. H1 isolates in this study are indicated by closed circles. Reassortant H1N2 SIVs reported previously in Japan [31] are indicated by closed diamonds. Japanese SIVs and viruses of pdm 09 lineage are indicated by the bar on the right of the tree. Scale bar indicates substitution per site. sw: swine.

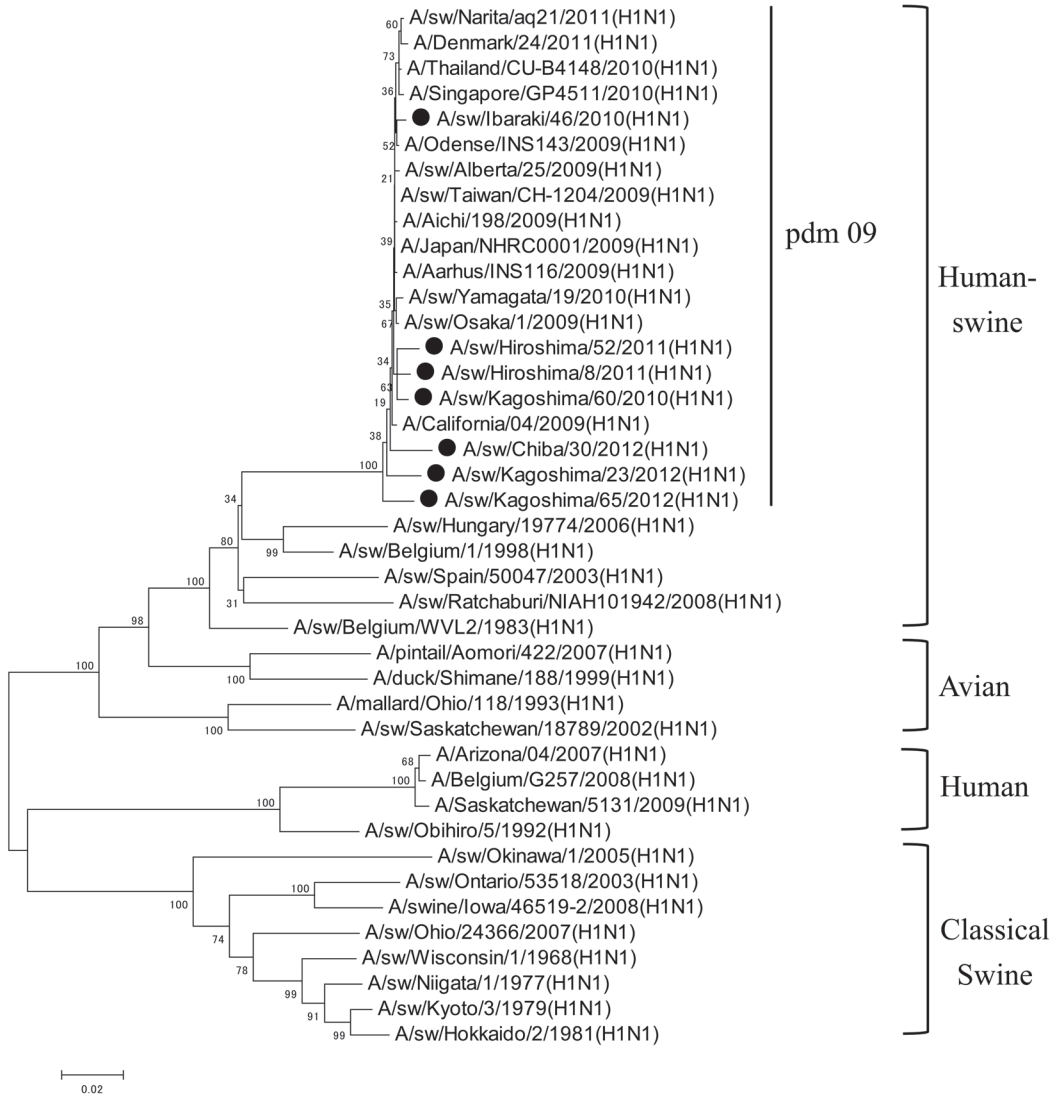

Fig. 2. Phylogenetic tree of N1 NA genes of H1 isolates in this study and others available from GenBank. H1 isolates in this study are indicated by closed circles. Viruses of pdm 09 lineage are indicated by the bar on the right of the tree. Scale bar indicates substitution per site. sw: swine.

investigate the difference in HI activities of H1 viruses, we compared amino acid sequences in 5 antigenic sites, Sa, Sb, Ca1, Ca2 and Cb [4, 5, 10, 19, 30], in the H1 molecule of HA (Tables 4 and 5). Among the 6 isolates of pdm 09 lineage, 1 or 2 amino acid differences were observed at site Sa in 2 isolates from Kagoshima Prefecture, and 1 amino acid difference was observed at site Sb in A/swine/Hiroshima/8/2011 (H1N1) and A/swine/Kagoshima/65/2012 (H1N1). At site Ca1, 1 amino acid difference was observed in A/swine/Kagoshima/23/2012 (H1N1). At site Ca2, 1 and 2 amino acid differences were observed in A/swine/Kagoshima/60/2010 (H1N1) and A/swine/Kagoshima/65/2012 (H1N1), respectively. At site Cb, 1 amino acid difference was observed in A/swine/Kagoshima/65/2012 (H1N1). These amino acid differences did not affect the addition of an *N*-glycosylation site (N-X-S/T). Among the 4 H1 reassortants originating from HA of Japanese SIVs, 1 and 4 amino acid differences at site Sa were observed in A/swine/Chiba/14/2012 (H1N2) and A/swine/Chiba/30/2012 (H1N1), respectively, compared to those of A/swine/Chiba/107/2012 (H1N2) and A/swine/Chiba/119/2012 (H1N2). At site Sb, 4 amino acid differences were observed in A/swine/Chiba/30/2012 (H1N1) compared to those of the other 3 isolates. At sites Ca1, Ca2 and Cb, 2 or 4 amino acid differences were observed in A/swine/Chiba/30/2012 (H1N1) compared to those of the other 3 isolates. Among these amino acid differences, A/swine/Chiba/30/2012 (H1N1) did not have *N*-glycosylation sites at 179 and 212 compared to the other 3 viruses.

Resistance markers of anti-virus drugs in H1 and H3 viruses: In the amino acid sequences of NA protein of all 11

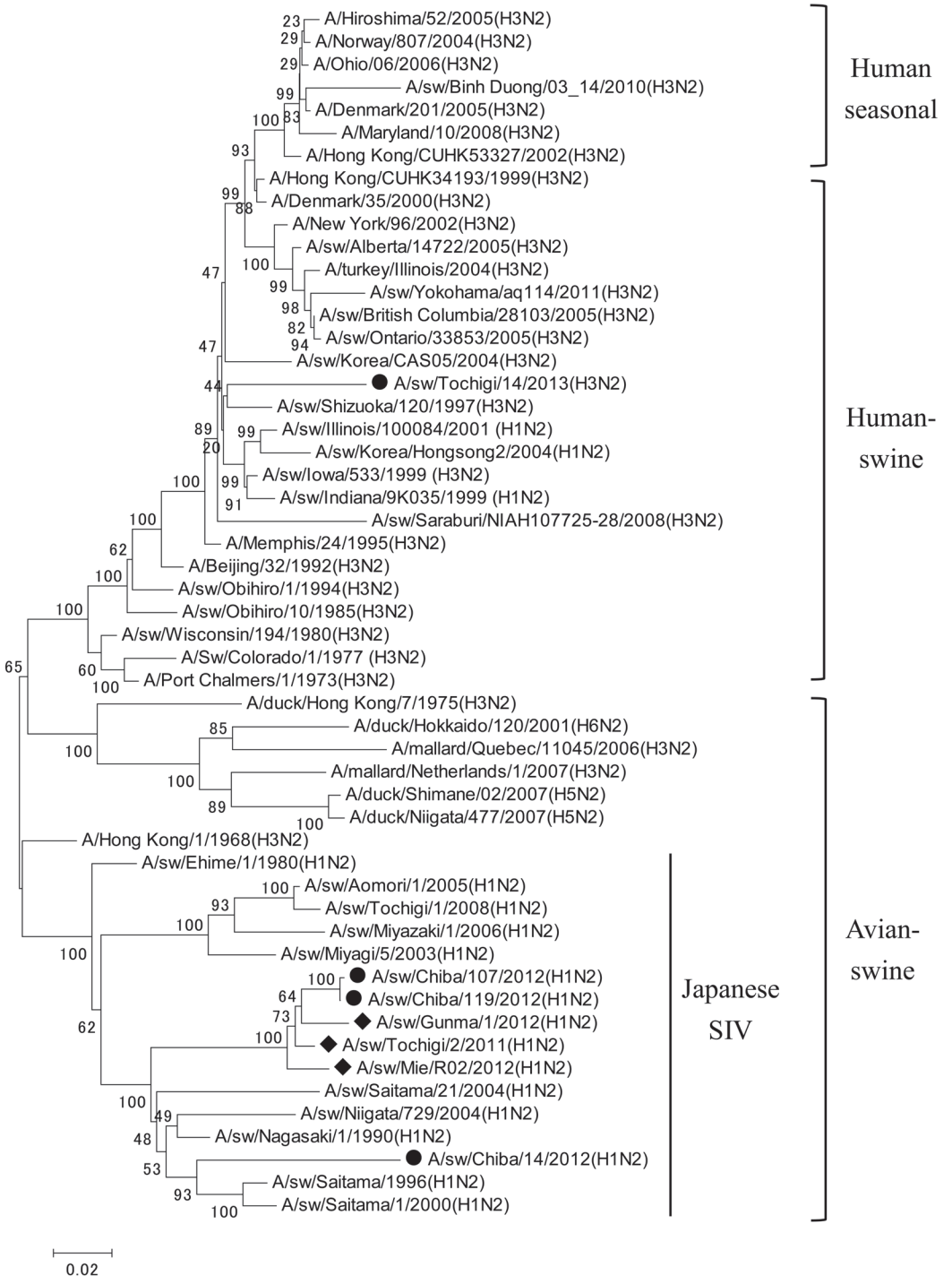

Fig. 3. Phylogenetic tree of N2 NA genes of N2 isolates in this study and others available from GenBank. N2 isolates in this study are indicated by closed circles. Reassortant H1N2 SIVs reported previously in Japan [26, 31] are indicated by closed diamonds. Japanese SIVs are indicated by the bar on the right of the tree. Scale bar indicates substitution per site. sw: swine.

isolates in this study, no resistance markers for oseltamivir (H275Y for N1 protein and R292K for N2 protein) [2, 11] or zanamivir (Q136K) [32] were observed. In analysis of M2 protein of the 11 isolates, the amantadine resistance marker S31N [54] was observed in all of the isolates (data not shown).

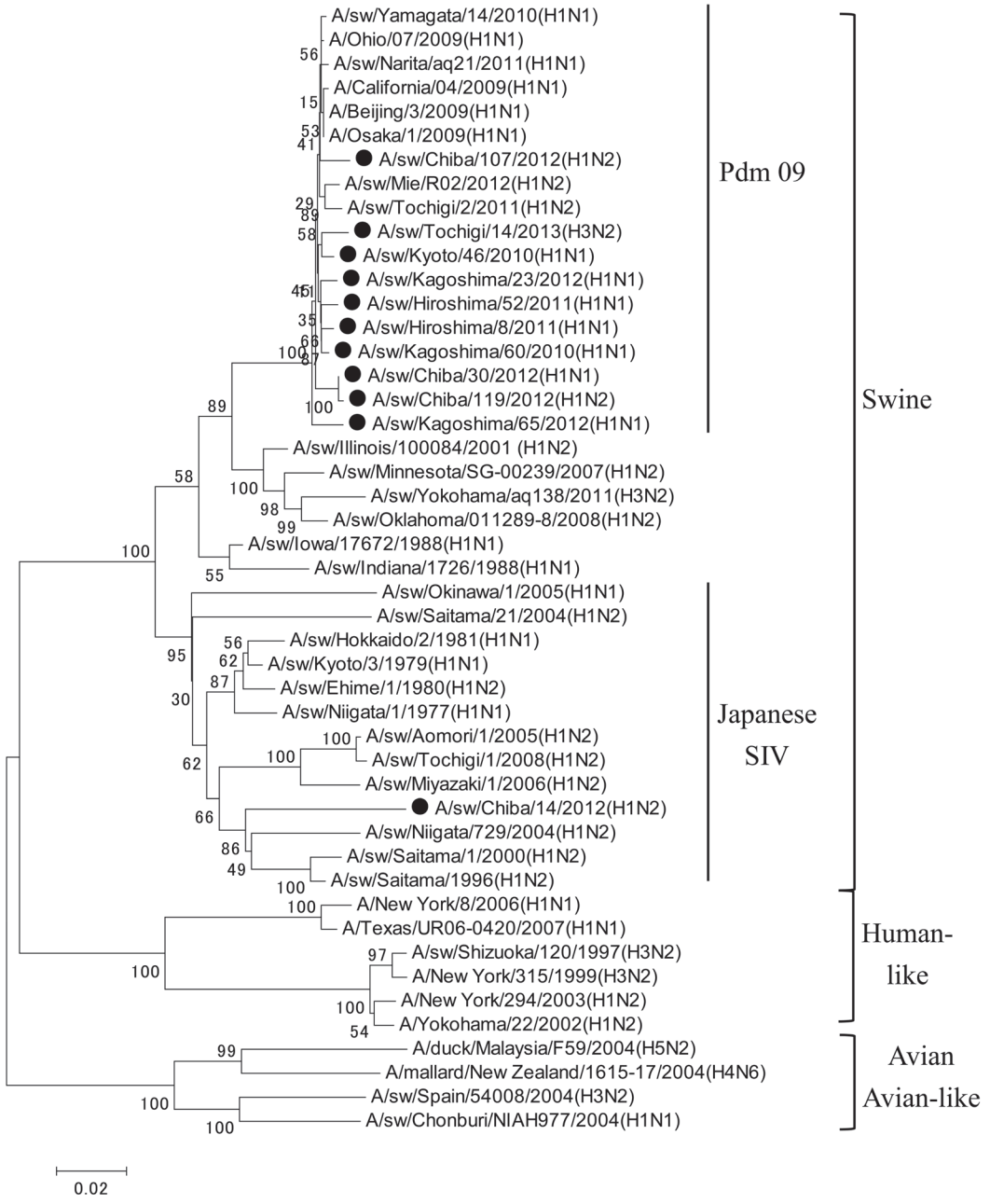

Fig. 4. Phylogenetic tree of NP genes of all eleven isolates in this study and others available from GenBank. Isolates in this study are indicated by closed circles. Japanese SIVs and viruses of pdm 09 lineage are indicated by the bar on the right of the tree. Scale bar indicates substitution per site. sw: swine.

Sero-epidemiology of the pig farm where SIVs were isolated 2 times at an interval of 10 months: A/swine/Hiroshima/8/2011 (H1N1) was isolated from the lung of a 50-day-old pig with pneumonitis in a consistent management farm in Hiroshima in January 2011. Ten months later, A/swine/Hiroshima/52/2011 (H1N1) was isolated from the lung of a 60-day-old pig with pneumonitis in the same farm in November 2011. SIV vaccine was not used in that farm. In order to determine whether SIV had been circulating in the farm, we examined the prevalence of SIV HI antibody against both isolates using sera from pigs of various ages collected 6 months after the second SIV isolation (Table 6). Almost half of the fattening pigs and gilts had low levels of the antibody, and the others did not have the antibody. All sows had relatively high levels of the HI antibody against the first isolate, A/swine/Hiroshima/8/2011 (H1N1), but half

Table 3. Antigenic relationship among pdm 09 lineage viruses and H1 reassortants isolated in this study by HI assay

Virus	subtype	Antiserum	
		A/sw/Ibaraki/46/2010 (H1N1)	A/sw/Chiba/14/2012 (H1N2)
A/sw/Ibaraki/46/2010	H1N1	160	160
A/sw/Kagoshima/60/2010	H1N1	80	40
A/sw/Hiroshima/8/2011	H1N1	80	160
A/sw/Hiroshima/52/2011	H1N1	80	80
A/sw/Kagoshima/23/2012	H1N1	40	160
A/sw/Kagoshima/65/2012	H1N1	160	320
A/sw/Chiba/30/2012	H1N1	20	<20
A/sw/Chiba/14/2012	H1N2	20	320
A/sw/Chiba/107/2012	H1N2	80	320
A/sw/Chiba/119/2012	H1N2	160	320

Abbreviation: sw, swine.

of the sows did not have the antibody against the second isolate. Overall, most of the positive sera against the first isolate tended to show slightly low HI titers against the second isolate. These results indicate that most of the sows were infected with the first isolate but might not have been infected with the second isolate. The HI titers in the fattening pigs and gilts suggested that SIV might not have been circulating in the farm. Antigenicity of the 2 isolates might be slightly different. Furthermore, among the 5 antigenic sites (Tables 4 and 5), 1 amino acid difference was observed at site Sb. To investigate whether the second isolate was a direct descendant strain of the first isolate, we compared all nucleotide sequences of 8 RNA segments of the second isolate, A/swine/Hiroshima/52/2011 (H1N1), with those of the first isolate, A/swine/Hiroshima/8/2011 (H1N1), and 4 isolates of pdm 09 lineage in this study (Table 7). A/swine/Hiroshima/52/2011 (H1N1) was most similar to A/swine/Kagoshima/60/2010 (H1N1) and next to A/swine/Hiroshima/8/2011 (H1N1). These results and the phylogenic tree suggested that A/swine/Hiroshima/52/2011 (H1N1) was not a direct descendant strain of A/swine/Hiroshima/8/2011 (H1N1).

DISCUSSION

In this study, we isolated 6 H1NI viruses of pdm 09 lineage, 4 H1 reassortants between pdm 09 viruses and Japanese H1 SIVs and 1 H3N2 reassortant of pdm 09 virus and H3N2 SIV from several prefectures in Japan. We did not isolate any Japanese SIVs. These results indicated that pdm 09 viruses are distributed widely in Japan and have reassorted with circulating Japanese SIVs. These viruses were isolated from pigs with clinical symptoms, such as pneumonitis. Therefore, pdm 09 viruses and the reassortant viruses might be relatively pathogenic to pigs compared to circulating Japanese SIVs.

Among the 5 reassortants examined in this study, 3 genotypes were detected first in Japan: A/swine/Chiba/14/2012 (H1N2) contained H1, N2 and NP genes of Japanese SIV origin, A/swine/Chiba/30/2012 (H1N1) contained only the H1 gene of Japanese SIVs in a pdm 09 virus background, and A/swine/Tochigi/14/2013 (H3N2) contained H3 and N2 genes of Japanese H3N2 SIV origin together with internal genes of pdm 09 virus. Four of the 5 reassortants replace HA and NA genes simultaneously, suggesting that some genome constellations might be evolutionarily more stable [37]. Recent evidence that the functional balance of activities of HA binding and NA cleavage is found in human viruses including pdm 09 viruses but not in swine influenza viruses might explain the greater restrictions on reassortment between the HA and NA genes in humans [37, 61]. In the swine population, reassortant viruses observed in this study might have spread more efficiently than pdm 09 viruses.

The results of an HI test using antiserum to the reassortant A/swine/Chiba/14/2012 (H1N2) revealed that antigenicity of reassortant A/swine/Chiba/30/2012 (H1N1) was greatly different from that of the 3 H1N2 reassortants. The virus was isolated from a suckling patient piglet that had not been vaccinated with SIV vaccine, but sows in the same farm had been vaccinated with an imported SIV vaccine as described below. The piglet might have possessed the maternal antibody against SIV. Therefore, it was thought that the isolated virus could replicate in the piglet due to its antigenic difference, despite the existence of the possible maternal antibody. It is known that the H1 HA molecule has 4 distinct antigenic sites: Sa, Sb, Ca and Cb. Ca is further divided into Ca1 and Ca2. Through the 5 sites, A/swine/Chiba/30/2012 (H1N1) has 2 to 4 amino acid differences compared to the 3 H1N2 reassortants. The Sa and Sb sites, which contained many amino acids involved in neutralizing epitopes near the receptor binding pockets [4, 5], were different in A/swine/Chiba/30/2012 (H1N1) and the 3 H1 reassortants, suggesting that these might cause the antigenic difference between them. Furthermore, it is well-known that antigenic changes of HA occasionally result in the addition or deletion of carbohydrate side chains on the HA molecule [5, 8, 18]. A/swine/Chiba/30/2012 (H1N1) does not have any *N*-glycosylation sites (N-X -S/T) in the 5 antigenic sites, but the 3 H1N2 reassortants have a total of 2 *N*-glycosylation sites in Sa and Sb. Although A/swine/Chiba/14/2012 (H1N2), A/swine/Chi-

Table 4. Comparison of amino acid residues at antigenic sites Sa and Sb on the HA1 molecule of H1 viruses used in this study with those of A/California/04/2009

Strain	Amino acid position in HA1																						
	Sa													Sb									
	141	142	170	171	172	173	174	176	177	178	179	180	181	201	202	203	204	206	207	208	210	211	212
A/California/04/2009 (H1N1)	P	N	K	K	G	N	S	P	K	L	S	K	S	T	S	A	D	Q	S	L	Q	N	A
A/sw/Ibaraki/46/2010 (H1N1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/60/2010 (H1N1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Hiroshima/8/2011 (H1N1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–	–	–	–
A/sw/Hiroshima/52/2011(H1N1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/23/2012 (H1N1)	A	–	–	–	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/65/2012 (H1N1)	–	Y	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–
A/sw/Chiba/30/2012 (H1N1)	–	–	–	–	–	S	–	–	–	I	–	–	–	–	–	D	–	R	R	–	–	–	E
A/sw/Chiba/14/2012 (H1N2)	–	L	–	–	–	–	–	–	–	I	N[a)]	–	–	–	–	T	–	–	–	–	–	–	N[a)]
A/sw/Chiba/107/2012 (H1N2)	–	L	–	–	–	–	–	–	–	–	N[a)]	–	–	–	–	T	–	–	–	–	–	–	N[a)]
A/sw/Chiba/119/2012(H1N2)	–	L	–	–	–	–	–	–	–	–	N[a)]	–	–	–	–	T	–	–	–	–	–	–	N[a)]

a) Putative *N*-glycosylation site. Abbreviation: sw, swine.

Table 5. Comparison of amino acid residues at antigenic sites Ca1, Ca2 and Cb on the HA1 molecule of H1 viruses used in this study with those of A/California/04/2009

Virus	Amino acid position in HA1																								
	Ca1											Ca2								Cb					
	183	184	185	186	187	220	221	222	252	253	254	154	155	156	157	158	159	238	239	87	88	89	90	91	92
A/California/04/2009 (H1N1)	I	N	D	K	G	S	S	R	E	P	G	P	H	A	G	A	K	R	D	L	S	T	A	S	S
A/sw/Ibaraki/46/2010 (H1N1)	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/60/2010 (H1N1)	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–
A/sw/Hiroshima/8/2011 (H1N1)	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Hiroshima/52/2011 (H1N1)	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/23/2012 (H1N1)	–	–	–	–	–	T	–	–	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/sw/Kagoshima/65/2012 (H1N1)	–	–	–	–	–	T	–	–	–	–	–	–	R	–	–	–	–	–	G	–	Y	–	–	–	–
A/sw/Chiba/30/2012 (H1N1)	V	–	N	–	R	T	–	T	–	–	–	S	Q	G	–	–	S	–	–	–	–	K	V	N	–
A/sw/Chiba/14/2012 (H1N2)	V	–	N	–	E	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–	–
A/sw/Chiba/107/2012 (H1N2)	V	–	N	–	E	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–	–
A/sw/Chiba/119/2012 (H1N2)	V	–	N	–	E	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–	–

Abbreviation: sw, swine.

ba/107/2012 (H1N2) and A/swine/Chiba/119/2012 (H1N2) possessed very similar HA (Fig. 1), the HI titers using antiserum to A/swine/Ibaraki/46/2010 (H1N1) were different between A/swine/Chiba/14/2012 (H1N2) and the other 2 H1N2 isolates. A/swine/Chiba/14/2012 (H1N2) has 1 amino acid difference at the 178th position in the Sa site (isoleucine in A/swine/Chiba/14/2012 (H1N2) and leucine in the other 2 H1N2 isolates). A/swine/Chiba/30/2012 (H1N1) also showed a weak response to the antiserum to A/swine/Ibaraki/46/2010 (H1N1), and the amino acid at the 178th position in the Sa site was isoleucine. In A/swine/Ibaraki/46/2010 (H1N1) and the other 5 isolates of pdm 09 lineage, the amino acid at the 178th position in the Sa site was conserved as leucine. Migunowa *et al.* [33] reported that a single mutation in an antigenic site C (change from isoleucine to leucine at position 51 of the HA1 gene) of influenza virus (H3N2) affected the antigenicity. The amino acid difference at the 178th position (isoleucine and leucine) in the Sa site might affect the antigenicity by an HI test using antiserum to A/swine/Ibaraki/46/2010 (H1N1). Among the 6 isolates of pdm 09 lineage, 4 viruses have almost the same amino acids in the 5 antigenic sites. The other 2 viruses, A/swine/Ka-

Table 6. Prevalence of SIV HI antibody in variously aged pigs in the pig farm located in Hiroshima Prefecture where SIV was isolated 2 times at an interval of 10 months

Pig[a)]	A/sw/Hiroshima/8/2011 (H1N1)	A/sw/Hiroshima/52/2011 (H1N1)	Pig[a)]	A/sw/Hiroshima/8/2011 (H1N1)	A/sw/Hiroshima/52/2011 (H1N1)
Fattening			Fattening		
30 days old			150 days old		
No.1	40	80	N0.21	40	20
No.2	20	20	No.22	<20	<20
No.3	40	20	No.23	20	<20
No.4	20	<20	No.24	<20	<20
No.5	20	20	No.25	20	<20
60 days old			Gilt		
No.6	20	<20	No.26	<20	<20
No.7	20	20	No.27	<20	<20
No.8	20	<20	No.28	<20	<20
No.9	<20	<20	Sow, parity No.:1		
No.10	<20	<20	No.29	40	<20
90 days old			No.30	160	40
No.11	20	<20	No.31	80	40
No.12	<20	<20	Sow, parity No.:3		
No.13	<20	<20	No.32	80	80
No.14	320	320	No.33	160	80
No.15	<20	<20	No.34	40	<20
120 days old			Sow, parity No.:5		
No.16	<20	<20	No.35	40	<20
No.17	<20	<20	No.36	40	<20
No.18	320	320	No.37	40	<20
No.19	20	<20	Sow, parity No.:7		
No.20	<20	<20	No.38	160	80
			No.39	160	40

a) Pig sera were collected at 6 months after the second SIV isolation (A/swine/Hiroshima/52/2011 (H1N1)). Abbreviation: sw, swine.

Table 7. Identity of nucleotide sequences of 8 RNA segments of A/swine/Hiroshima/52/2011 (H1N1) with those of corresponding segments of 5 pdm 09 lineage isolates in this study

Virus	Identity (%)							
	PB2	PB1	PA	HA	NP	NA	M	NS
A/sw/Ibaraki/46/2010 (H1N1)	**99.19[a)]**	98.68	98.61	98.45	98.91	98.74	98.05	99.1
A/sw/Kagoshima/60/2010 (H1N1)	99.10	98.80	**99.10**	98.51	**99.36**	**98.95**	**98.34**	**99.21**
A/sw/Hiroshima/8/2011 (H1N1)	**99.19**	**98.85**	99.06	**98.62**	99.23	98.88	97.96	98.88
A/sw/Kagoshima/23/2012 (H1N1)	98.72	98.33	98.52	97.99	99.04	98.04	97.47	98.20
A/sw/Kagoshima/65/2012 (H1N1)	98.55	97.65	98.57	97.13	98.47	97.82	97.96	98.54

a) Highest identity (%) is indicated in bold. Abbreviation: sw, swine.

goshima/23/2012 (H1N1) and A/swine/Kagoshima/65/2012 (H1N1), showed several amino acid differences, but these differences did not affect the antigenicity by an HI test using antiserum to A/swine/Ibaraki/46/2010 (H1N1) in this study. However, 1 isolate, A/swine/Kagoshima/60/2010 (H1N1), showed a relatively weak response to the antiserum to the reassortant A/swine/Chiba/14/2012 (H1N2), suggesting that lysine at the 238th position in the Cb site affected the antigenicity, because the amino acid of this site was conserved as arginine in the other nine isolates.

A phylogenetic tree of the H3 gene (Fig. 5) shows that the H3 gene of A/swine/Tochigi/14/2013 (H3N2) has a large branch length from Japanese isolates in 2000, 2002, 2007 and 2008. This might be because several genetically evolved H3N2 SIVs were maintained on their own terms in the swine population underground before being detected [38]. Concerning the antigenicity of H3N2 SIVs, Takemae *et al.* [55] reported that Japanese H3N2 SIV isolates in 2000, 2002, 2007 and 2008 showed almost the same antigenicity by the HI test. Therefore, our isolate might also have the same antigenicity as that of those isolates. We would like to compare the antigenicity of our isolate to that of those H3N2 isolates by the HI test in the near future.

SIVs of pdm 09 lineage were isolated twice in the same

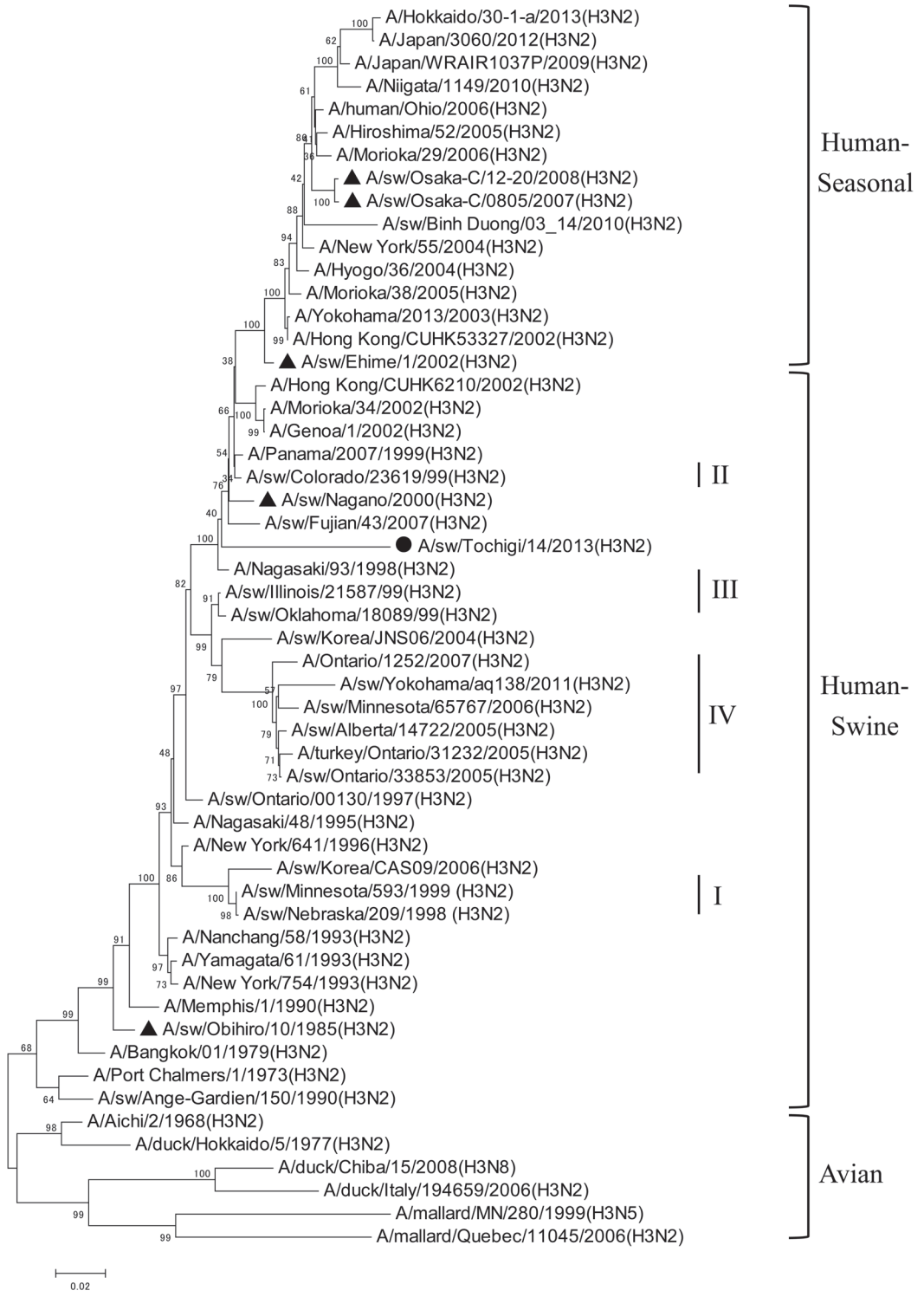

Fig. 5. Phylogenetic tree of H3 HA genes of an H3 isolate in this study and others available from GenBank. The H3 isolate in this study is indicated by a closed circle. Past H3 isolates in Japan are indicated by closed triangles. I, II, III and IV for the clusters of the triple reassortant H3N2 SIVs are indicated by the bar on the right of the tree. Scale bar indicates substitution per site. sw: swine.

pig farm in Hiroshima Prefecture at an interval of 10 months. Since the 2 isolated viruses showed slight genetic and antigenic differences, the second isolate might not be the direct descendant strain of the first isolate, suggesting that the first strain might not have circulated continuously in the pig farm and that another SIV might have re-entered the pig farm. Sero-epidemiological results also support this possibility. However, the possibility of sporadic SIV infection in the farm could not be ruled out, because a few fattening pigs had high levels of SIV antibodies.

Two commercial vaccines are currently available in Japan. One is a domestic vaccine consisting of A/swine/Kyoto/3/79 (H1N1) and A/swine/Wadayama/5/1969 (H3N2), and the other is an imported vaccine containing A/swine/Iowa/08/00 (H1N1) and A/swine/Iowa/06/00 (H3N2). All SIV isolates in this study were from pigs that were not vaccinated with any SIV vaccines. Investigation of the reactivity of the viruses to antisera against the 4 vaccine strains is needed. If the viruses are efficiently inactivated by those antisera, these vaccines might be useful for preventing viral infections. We plan to carry out the serological tests in the near future.

Analysis of the amino acid sequences of all isolates revealed that all isolates might be susceptible to oseltamivir and zanamivir and resistant to amantadine. If the pdm lineage virus is transmitted from pigs to humans and causes clinical symptoms, antiviral drug therapy might be useful.

In conclusion, 6 pdm 09 viruses and 5 reassortants of Japanese SIVs and pdm 09 viruses were isolated in the period from 2010 to 2013 in Japan. Among the reassortants, 3 were the same type of H1N2 reassortant. This type of reassortant had been isolated in 2011 and 2012 in other prefectures. Thus, this type of reassortant SIV could form another lineage in addition to Japanese H1N2 SIVs as reported in Germany [27]. We obtained indirect evidence of the presence of H1N2 and H3N2 SIVs in the Japanese pig population due to the presence of segments (HA and NA) derived from these viruses in the reassortant SIVs examined in this study. The emergence of several types of reassortant SIVs raises further concerns about whether the viruses will undergo further genetic reassortment and gain virulence. In humans in Japan, pdm09 viruses had been displaced with seasonal H3 viruses by the 2011–2012 seasons [20]. However, the pdm09 virus extensively reemerged in the 2013–2014 season in Japan [21]. Although the reason for its reemergence is not clear, one possibility is that swine serve as reservoirs of pdm 09 viruses that can be transmitted to humans. Systematic influenza virus surveillance in pigs in Japan should be considered for swine hygiene and human public health.

REFERENCES

1. Ali, A., Khatri, M., Wang, L., Saif, Y. M. and Lee, C. W. 2012. Identification of swine H1N2/pandemic H1N1 reassortant influenza virus in pigs, United States. *Vet. Microbiol.* **158**: 60–68. [Medline] [CrossRef]
2. Aoki, F. Y., Boivin, G. and Roberts, N. 2007. Influenza virus susceptibility and resistance to oseltamivir. *Antivir. Ther.* **12**: 603–616. [Medline]
3. Brookes, S. M., Núñez, A., Choudhury, B., Matrosovich, M., Essen, S. C., Clifford, D., Slomka, M. J., Kuntz-Simon, G., Garcon, F., Nash, B., Hanna, A., Heegaard, P. M., Quéguiner, S., Chiapponi, C., Bublot, M., Garcia, J. M., Gardner, R., Foni, E., Loeffen, W., Larsen, L., Van Reeth, K., Banks, J., Irvine, R. M. and Brown, I. H. 2010. Replication, pathogenesis and transmission of pandemic (H1N1) 2009 virus in non-immune pigs. *PLoS ONE* **5**: e9068. [Medline] [CrossRef]
4. Brownlee, G. G. and Fodor, E. 2001. The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**: 1871–1876. [Medline] [CrossRef]
5. Caton, A. J., Brownlee, G. G., Yewdell, J. W. and Gerhard, W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**: 417–427. [Medline] [CrossRef]
6. Ducatez, M. F., Hause, B., Stigger-Rosser, E., Darnell, D., Corzo, C., Juleen, K., Simonson, R., Brockwell-Staats, C., Rubrum, A., Wang, D., Webb, A., Crumpton, J. C., Lowe, J., Gramer, M. and Webby, R. J. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. *Emerg. Infect. Dis.* **17**: 1624–1629. [Medline] [CrossRef]
7. Fan, X., Zhu, H., Zhou, B., Smith, D. K., Chen, X., Lam, T. T., Poon, L. L., Peiris, M. and Guan, Y. 2012. Emergence and dissemination of a swine H3N2 reassortant influenza virus with 2009 pandemic H1N1 genes in pigs in China. *J. Virol.* **86**: 2375–2378. [Medline] [CrossRef]
8. Gallagher, P., Henneberry, J., Wilson, I., Sambrook, J. and Gething, M. J. 1988. Addition of carbohydrate side chains at novel sites on influenza virus hemagglutinin can modulate the folding, transport, and activity of the molecule. *J. Cell Biol.* **107**: 2059–2073. [Medline] [CrossRef]
9. Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C. B., Emery, S. L., Hillman, M. J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D. F., Fouchier, R. A., Pappas, C., Alpuche-Aranda, C. M., López-Gatell, H., Olivera, H., López, I., Myers, C. A., Faix, D., Blair, P. J., Yu, C., Keene, K. M., Dotson, P. D. Jr., Boxrud, D., Sambol, A. R., Abid, S. H., St George, K., Bannerman, T., Moore, A. L., Stringer, D. J., Blevins, P., Demmler-Harrison, G. J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H. F., Belongia, E. A., Clark, P. A., Beatrice, S. T., Donis, R., Katz, J., Finelli, L., Bridges, C. B., Shaw, M., Jernigan, D. B., Uyeki, T. M., Smith, D. J., Klimov, A. I. and Cox, N. J. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**: 197–201. [Medline] [CrossRef]
10. Gerhard, W., Yewdell, J., Frankel, M. E. and Webster, R. 1981. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature* **290**: 713–717. [Medline] [CrossRef]
11. Gubareva, L. V. 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. *Virus Res.* **103**: 199–203. [Medline] [CrossRef]
12. Han, J. Y., Park, S. J., Kim, H. K., Rho, S., Nguyen, G. V., Song, D., Kang, B. K., Moon, H. J., Yeom, M. J. and Park, B. K. 2012. Identification of reassortant pandemic H1N1 influenza virus in Korean pigs. *J. Microbiol. Biotechnol.* **22**: 699–707. [Medline] [CrossRef]
13. Hiromoto, Y., Parchariyanon, S., Ketusing, N., Netrabukkana, P., Hayashi, T., Kobayashi, T., Takemae, N. and Saito, T. 2012.

Isolation of the pandemic (H1N1) 2009 virus and its reassortant with an H3N2 swine influenza virus from healthy weaning pigs in Thailand in 2011. *Virus Res.* **169**: 175–181. [Medline] [CrossRef]
14. Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. and Perez, D. R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.* **146**: 2275–2289. [Medline] [CrossRef]
15. Holyoake, P. K., Kirkland, P. D., Davis, R. J., Arzey, K. E., Watson, J., Lunt, R. A., Wang, J., Wong, F., Moloney, B. J. and Dunn, S. E. 2011. The first identified case of pandemic H1N1 influenza in pigs in Australia. *Aust. Vet. J.* **89**: 427–431. [Medline] [CrossRef]
16. Howard, W. A., Essen, S. C., Strugnell, B. W., Russell, C., Barass, L., Reid, S. M. and Brown, I. H. 2011. Reassortant Pandemic (H1N1) 2009 virus in pigs, United Kingdom. *Emerg. Infect. Dis.* **17**: 1049–1052. [Medline] [CrossRef]
17. Howden, K. J., Brockhoff, E. J., Caya, F. D., McLeod, L. J., Lavoie, M., Ing, J. D., Bystrom, J. M., Alexandersen, S., Pasick, J. M., Berhane, Y., Morrison, M. E., Keenliside, J. M., Laurendeau, S. and Rohonczy, E. B. 2009. An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. *Can. Vet. J.* **50**: 1153–1161. [Medline]
18. Igarashi, M., Ito, K., Kida, H. and Takada, A. 2008. Genetically destined potentials for N-linked glycosylation of influenza virus hemagglutinin. *Virology* **376**: 323–329. [Medline] [CrossRef]
19. Igarashi, M., Ito, K., Yoshida, R., Tomabechi, D., Kida, H. and Takada, A. 2010. Predicting the antigenic structure of the pandemic (H1N1) 2009 influenza virus hemagglutinin. *PLoS ONE* **5**: e8553. [Medline] [CrossRef]
20. Infectious Disease Surveillance Center of National Institute of Infectious Diseases. 2012. Isolation/detection of influenza virus in Japan, week 36/2011-week 25/2012 (as of July 19, 2012). http://www.nih.go.jp/niid/en/influenza-e/2099-idsc/iasr-flu-e/2459-iasr-inf-e20120719.html.
21. Infectious Disease Surveillance Center of National Institute of Infectious Diseases. 2012. Isolation/detection of influenza virus in Japan, week 18/2013-week 9/2014 (as of March 6, 2014). http://www.nih.go.jp/niid/en/flu-m/flutoppage/2099-idsc/iasr-flu-e/4473-iasr-inf-e20140306.html.
22. Ito, T., Kawaoka, Y., Vines, A., Ishikawa, H., Asai, T. and Kida, H. 1998. Continued circulation of reassortant H1N2 influenza viruses in pigs in Japan. *Arch. Virol.* **143**: 1773–1782. [Medline] [CrossRef]
23. Katsuda, K., Sato, S., Imai, M., Shirahata, T. and Goto, H. 1995. Prevalence of antibodies to type A influenza viruses in swine sera 1990–1994. *J. Vet. Med. Sci.* **57**: 773–775. [Medline] [CrossRef]
24. Katsuda, K., Shirahata, T., Kida, H. and Goto, H. 1995. Antigenic and genetic analyses of the hemagglutinin of influenza viruses isolated from pigs in 1993. *J. Vet. Med. Sci.* **57**: 1023–1027. [Medline] [CrossRef]
25. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120. [Medline] [CrossRef]
26. Kobayashi, M., Takayama, I., Kageyama, T., Tsukagoshi, H., Saitoh, M., Ishioka, T., Yokota, Y., Kimura, H., Tashiro, M. and Kozawa, K. 2013. Novel reassortant influenza A(H1N2) virus derived from A(H1N1)pdm09 virus isolated from swine, Japan, 2012. *Emerg. Infect. Dis.* **19**: 1972–1974. [Medline] [CrossRef]
27. Lange, J., Groth, M., Schlegel, M., Krumbholz, A., Wieczorek, K., Ulrich, R., Köppen, S., Schulz, K., Appl, D., Selbitz, H. J., Sauerbrei, A., Platzer, M., Zell, R. and Dürrwald, R. 2013. Reassortants of the pandemic (H1N1) 2009 virus and establishment of a novel porcine H1N2 influenza virus, lineage in Germany. *Vet. Microbiol.* **167**: 345–356. [Medline] [CrossRef]
28. Lange, E., Kalthoff, D., Blohm, U., Teifke, J. P., Breithaupt, A., Maresch, C., Starick, E., Fereidouni, S., Hoffmann, B., Mettenleiter, T. C., Beer, M. and Vahlenkamp, T. W. 2009. Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. *J. Gen. Virol.* **90**: 2119–2123. [Medline] [CrossRef]
29. Liu, Q., Ma, J., Liu, H., Qi, W., Anderson, J., Henry, S. C., Hesse, R. A., Richt, J. A. and Ma, W. 2012. Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States. *Arch. Virol.* **157**: 555–562. [Medline] [CrossRef]
30. Luoh, S. M., McGregor, M. W. and Hinshaw, V. S. 1992. Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses. *J. Virol.* **66**: 1066–1073. [Medline]
31. Matsuu, A., Uchida, Y., Takemae, N., Mawatari, T., Yoneyama, S. K., Kasai, T., Nakamura, R., Eto, M. and Saito, T. 2012. Genetic characterization of swine influenza viruses isolated in Japan between 2009 and 2012. *Microbiol. Immunol.* **56**: 792–803. [Medline] [CrossRef]
32. McKimm-Breschkin, J. L. 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir. Viruses* **7** Suppl. 1: 25–36. [Medline] [CrossRef]
33. Migunova, B. V., Plusnin, A. Z., Petrov, N. A., Rivkin, M. I., Grinbaum, E. B., Obuchova, L. V. and Kuznetzov, O. K. 1990. Primary structure of the gene coding for the haemagglutinin of influenza virus A/Leningrad/385/80(H3N2): detection of a point mutation responsible for the antigenic drift. *Acta Virol.* **34**: 209–219. [Medline]
34. Moreno, A., Di Trani, L., Alborali, L., Vaccari, G., Barbieri, I., Falcone, E., Sozzi, E., Puzelli, S., Ferri, G. and Cordioli, P. 2010. First pandemic H1N1 outbreak from a pig farm in Italy. *Open Virol. J.* **4**: 52–56. [Medline] [CrossRef]
35. Moreno, A., Di Trani, L., Faccini, S., Vaccari, G., Nigrelli, D., Boniotti, M. B., Falcone, E., Boni, A., Chiapponi, C., Sozzi, E. and Cordioli, P. 2011. Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. *Vet. Microbiol.* **149**: 472–477. [Medline] [CrossRef]
36. Nagarajan, M. M., Simard, G., Longtin, D. and Simard, C. 2010. Single-step multiplex conventional and real-time reverse transcription polymerase chain reaction assays for simultaneous detection and subtype differentiation of influenza A virus in swine. *J. Vet. Diagn. Invest.* **22**: 402–408. [Medline] [CrossRef]
37. Nelson, M. I., Detmer, S. E., Wentworth, D. E., Tan, Y., Schwartzbard, A., Halpin, R. A., Stockwell, T. B., Lin, X., Vincent, A. L., Gramer, M. R. and Holmes, E. C. 2012. Genomic reassortment of influenza A virus in North American swine, 1998–2011. *J. Gen. Virol.* **93**: 2584–2589. [Medline] [CrossRef]
38. Nelson, M. I., Gramer, M. R., Vincent, A. L. and Holmes, E. C. 2012. Global transmission of influenza viruses from humans to swine. *J. Gen. Virol.* **93**: 2195–2203. [Medline] [CrossRef]
39. Nerome, K., Ishida, M., Nakayama, M., Oya, A., Kanai, C. and Suwicha, K. 1981. Antigenic and genetic analysis of A/Hong Kong (H3N2) influenza viruses isolated from swine and man. *J. Gen. Virol.* **56**: 441–445. [Medline] [CrossRef]
40. Nerome, K., Ishida, M., Oya, A. and Oda, K. 1982. The possible origin H1N1 (Hsw1N1) virus in the swine population of Japan and antigenic analysis of the isolates. *J. Gen. Virol.* **62**: 171–175. [Medline] [CrossRef]
41. Nerome, K., Sakamoto, S., Yano, N., Yamamoto, T., Kobayashi,

S., Webster, R. G. and Oya, A. 1983. Antigenic characteristics and genome composition of a naturally occurring recombinant influenza virus isolated from a pig in Japan. *J. Gen. Virol.* **64**: 2611–2620. [Medline] [CrossRef]
42. Ouchi, A., Nerome, K., Kanegae, Y., Ishida, M., Nerome, R., Hayashi, K., Hashimoto, T., Kaji, M., Kaji, Y. and Inaba, Y. 1996. Large outbreak of swine influenza in southern Japan caused by reassortant (H1N2) influenza viruses: its epizootic background and characterization of the causative viruses. *J. Gen. Virol.* **77**: 1751–1759. [Medline] [CrossRef]
43. Pasma, T. and Joseph, T. 2010. Pandemic (H1N1) 2009 infection in swine herds, Manitoba, Canada. *Emerg. Infect. Dis.* **16**: 706–708. [Medline] [CrossRef]
44. Pereda, A., Cappuccio, J., Quiroga, M. A., Baumeister, E., Insarralde, L., Ibar, M., Sanguinetti, R., Cannilla, M. L., Franzese, D., Escobar Cabrera, O. E., Craig, M. I., Rimondi, A., Machuca, M., Debenedetti, R. T., Zenobi, C., Barral, L., Balzano, R., Capalbo, S., Risso, A. and Perfumo, C. J. 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. *Emerg. Infect. Dis.* **16**: 304–307. [Medline] [CrossRef]
45. Saito, T., Suzuki, H., Maeda, K., Inai, K., Takemae, N., Uchida, Y. and Tsunemitsu, H. 2008. Molecular characterization of an H1N2 swine influenza virus isolated in Miyazaki, Japan, in 2006. *J. Vet. Med. Sci.* **70**: 423–427. [Medline] [CrossRef]
46. Saito, T, Takemae, N., Uchida, Y., Hiromoto, Y. and Hayashi, T. 2010. Evolutional analysis of swine influenza in Japan. pp. 20–25. *In*: Paper Presented at the XIV International Conference on Negative Strand Viruses, Brugge, Belgium.
47. Saitou, N. and Nei, M. 1987. The Neighbor-Joining Method—a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425. [Medline]
48. Shimada, S., Ohtsuka, T., Tanaka, M., Mimura, M., Shinohara, M., Uchida, K., Segawa, Y. and Kimura, K. 2004. Existence of reassortant A (H1N2) swine influenza viruses in Saitama Prefecture, Japan. *Int. Congr. Ser.* **1263**: 749–753. [CrossRef]
49. Smith, G. J., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghwani, J., Bhatt, S., Peiris, J. S. Y. and Rambaut, A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* **459**: 1122–1125. [Medline] [CrossRef]
50. Song, M. S., Lee, J. H., Pascua, P. N., Baek, Y. H., Kwon, H. I., Park, K. J., Choi, H. W., Shin, Y. K., Song, J. Y., Kim, C. J. and Choi, Y. K. 2010. Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea. *J. Clin. Microbiol.* **48**: 3204–3211. [Medline] [CrossRef]
51. Starick, E., Lange, E., Fereidouni, S., Bunzenthal, C., Höveler, R., Kuczka, A., Grosse Beilage, E., Hamann, H. P., Klingelhöfer, I., Steinhauer, D., Vahlenkamp, T., Beer, M. and Harder, T. 2011. Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. *J. Gen. Virol.* **92**: 1184–1188. [Medline] [CrossRef]
52. Starick, E., Lange, E., Grund, C., Grosse Beilage, E., Döhring, S., Maas, A., Noé, T., Beer, M. and Harder, T. C. 2012. Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. *J. Gen. Virol.* **93**: 1658–1663. [Medline] [CrossRef]
53. Sugimura, T., Yonemochi, H., Ogawa, T., Tanaka, Y. and Kumagai, T. 1980. Isolation of a recombinant influenza virus (Hsw 1 N2) from swine in Japan. *Arch. Virol.* **66**: 271–274. [Medline] [CrossRef]
54. Suzuki, H., Saito, R., Masuda, H., Oshitani, H., Sato, M. and Sato, I. 2003. Emergence of amantadine-resistant influenza A viruses: epidemiological study. *J. Infect. Chemother.* **9**: 195–200. [Medline] [CrossRef]
55. Takemae, N., Nguyen, T., Ngo, L. T., Hiromoto, Y., Uchida, Y., Pham, V. P., Kageyama, T., Kasuo, S., Shimada, S., Yamashita, Y., Goto, K., Kubo, H., Le, V. T., Van Vo, H., Do, H. T., Nguyen, D. H., Hayashi, T., Matsuu, A. and Saito, T. 2013. Antigenic variation of H1N1, H1N2 and H3N2 swine influenza viruses in Japan and Vietnam. *Arch. Virol.* **158**: 859–876. [Medline] [CrossRef]
56. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739. [Medline] [CrossRef]
57. Tremblay, D., Allard, V., Doyon, J. F., Bellehumeur, C., Spearman, J. G., Harel, J. and Gagnon, C. A. 2011. Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. *J. Clin. Microbiol.* **49**: 4386–4390. [Medline] [CrossRef]
58. Vijaykrishna, D., Poon, L. L., Zhu, H. C., Ma, S. K., Li, O. T., Cheung, C. L., Smith, G. J., Peiris, J. S. and Guan, Y. 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. *Science* **328**: 1529. [Medline] [CrossRef]
59. Vincent, A. L., Lager, K. M., Faaberg, K. S., Harland, M., Zanella, E. L., Ciacci-Zanella, J. R., Kehrl, M. E. Jr., Janke, B. H. and Klimov, A. 2010. Experimental inoculation of pigs with pandemic H1N1 2009 virus and HI cross-reactivity with contemporary swine influenza virus antisera. *Influenza Other Respir. Viruses* **4**: 53–60. [Medline] [CrossRef]
60. Welsh, M. D., Baird, P. M., Guelbenzu-Gonzalo, M. P., Hanna, A., Reid, S. M., Essen, S., Russell, C., Thomas, S., Barrass, L., McNeilly, F., McKillen, J., Todd, D., Harkin, V., McDowell, S., Choudhurym, B., Irvine, R. M., Borobia, J., Grant, J. and Brown, I. H. 2010. Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs. *Vet. Rec.* **166**: 642–645. [Medline] [CrossRef]
61. Xu, R., Zhu, X., McBride, R., Nycholat, C. M., Yu, W., Paulson, J. C. and Wilson, I. A. 2012. Functional balance of the hemagglutinin and neuraminidase activities accompanies the emergence of the 2009 H1N1 influenza pandemic. *J. Virol.* **86**: 9221–9232. [Medline] [CrossRef]
62. Yamane, N., Arikawa, J., Odagiri, T. and Ishida, N. 1979. Annual examination of influenza virus infection among pigs in Miyagi prefecture, Japan: the appearance of Hsw1N1 virus. *Acta Virol.* **23**: 240–248. [Medline]
63. Yoneyama, S., Hayashi, T., Kojima, H., Usami, Y., Kubo, M., Takemae, N., Uchida, Y. and Saito, T. 2010. Occurrence of a pig respiratory disease associated with swine influenza A (H1N2) virus in Tochigi Prefecture, Japan. *J. Vet. Med. Sci.* **72**: 481–488. [Medline] [CrossRef]
64. Zhu, H., Zhou, B., Fan, X., Lam, T. T., Wang, J., Chen, A., Chen, X., Chen, H., Webster, R. G., Webby, R., Peiris, J. S., Smith, D. K. and Guan, Y. 2011. Novel reassortment of Eurasian avian-like and pandemic/2009 influenza viruses in swine: infectious potential for humans. *J. Virol.* **85**: 10432–10439. [Medline] [CrossRef]