



Two different genotypes of H1N2 swine influenza virus isolated in northern China and their pathogenicity in animals[☆]



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ABSTRACT

During 2006 and 2007, two swine-origin triple-reassortant influenza A (H1N2) viruses were isolated from pigs in northern China, and the antigenic characteristics of the hemagglutinin protein of the viruses were examined. Genotyping and phylogenetic analyses demonstrated different emergence patterns for the two H1N2 viruses, Sw/Hebei/10/06 and Sw/Tianjin/1/07. Sequences for the other genes encoding the internal proteins were compared with the existing data to determine their origins and establish the likely mechanisms of genetic reassortment. Sw/Hebei/10/06 is an Sw/Indiana/9K035/99-like virus, whereas Sw/Tianjin/1/07 represents a new H1N2 genotype with surface genes of classic swine and human origin and internal genes originating from the Eurasian avian-like swine H1N1 virus. Six-week-old female BALB/c mice infected with the Sw/HeB/10/06 and Sw/TJ/1/07 viruses showed an average weight loss of 12.8% and 8.1%, respectively. Healthy six-week-old pigs were inoculated intranasally with either the Sw/HeB/10/06 or Sw/TJ/1/07 virus. No considerable changes in the clinical presentation were observed post-inoculation in any of the virus-inoculated groups, and the viruses effectively replicated in the nasal cavity and lung tissue. Based on the results, it is possible that the new genotype of the swine H1N2 virus that emerged in China may become widespread in the swine population and pose a potential threat to public health.

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1. Introduction

Swine influenza (SI) is a respiratory disease affecting pigs and is caused by the influenza A virus, which is responsible for regular outbreaks of influenza in these animals. Influenza viruses of the H1N1, H3N2, and H1N2

subtypes have circulated in pig populations all over the world since the classic swine (CS) H1N1 influenza virus was first isolated from pigs in 1930 (Brown, 2000; Webby et al., 2000). Pigs can be easily infected by avian and human influenza viruses in addition to SI viruses (SIVs) because the cells in their respiratory tracts contain receptors for both avian and human influenza viruses (Ito et al., 1998a). Furthermore, if influenza viruses of different subtypes were to simultaneously co-infect a pig, the genes of the two viruses might reassort, resulting in a virus that is readily transmissible between pigs.

The first detection of the H1N2 influenza A virus in pigs was in Japan in 1978. The reassortant virus was a classical swine H1N1 virus that had gained the neuraminidase (NA)

[☆] Nucleotide GenBank accession numbers: The nucleotide sequences obtained in this study have been submitted to GenBank.

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gene from human H3N2 viruses (Nerome et al., 1985), and this virus was re-isolated in subsequent studies in 1989–1992, 2000–2001, and 2006 (Ito et al., 1998b; Ouchi et al., 1996). Since 1987, H1N2 influenza A viruses have caused widespread outbreaks of respiratory disease among pigs in major swine-producing regions in Europe (Brown et al., 1998; Gourreau et al., 1994; Maldonado et al., 2006; Schrader and Suss, 2003; Trebbien et al., 2013), North America (Ali et al., 2012; Karasin et al., 2006, 2000), and Asia (Chutinimitkul et al., 2008; Jo et al., 2007; Jung and Chae, 2004; Takemae et al., 2013). In China, an H1N2 outbreak in pigs was reported in 2006 (Qi and Lu, 2006). The outbreak was caused by a double-reassortant virus with the NA gene most closely related to the corresponding gene from the human H3N2 virus circulating in 1995. The remaining seven genes were most closely related to those from CS H1N1 viruses. H1N2 viruses were also reported in China in 2009 and 2011 (Kong et al., 2011; Xu et al., 2009). Efficient surveillance of SIVs is urgently needed to understand their evolution and epidemiology and to address the challenges of monitoring, understanding, and controlling influenza in pigs after the emergence of a novel reassortant pandemic H1N1 (pH1N1/2009) virus in 2009 (Vincent et al., 2014).

During SIV surveillance conducted between 2006 and 2007 in China, two H1N2 viruses were isolated from pigs in northern China. In this study, we examined the genetic composition, antigenicity, and pathogenicity of the H1N2 subtypes isolated from Chinese pigs in 2006 and 2007. We examined the antigenic characteristics of the hemagglutinin (HA) protein of the H1N2 viruses. To investigate these relationships in detail, the sequences of genes were compared with the existing data to determine their origins and establish the likely mechanisms for genetic reassortment. Furthermore, we evaluated the replication and pathogenicity of these viruses in mice and pigs.

2. Materials and methods

2.1. Virus isolation and identification

In October 2006, 200 samples were collected from apparently healthy pigs in a slaughterhouse in the Hebei province in China as part of a routine virological surveillance project. In November 2007, approximately 3-month-old pigs and sows at a swine farm in Tianjin were reported to have the typical signs and symptoms of a respiratory disease. In total, 60 swab samples were collected into the transport medium containing antibiotics (Yang et al., 2012) and kept at a temperature of 4 °C until they were transported to the laboratory. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's minimal essential media (DMEM) supplemented with fetal bovine serum (5%). Viral samples were inoculated into the MDCK cells. Prior to inoculation, the cell monolayer was washed at least once with a tissue culture medium containing trypsin (2 µg/ml) without serum. The cultures were maintained with a serum-free medium containing 2 µg/ml of trypsin and examined daily for evidence of cytopathic effects. Two swine H1N2 viruses were obtained. Virus isolates were passaged and identified by hemagglutination- and NA-inhibition assays

using a panel of reference sera (National Reference Laboratory for Avian Influenza, Harbin Veterinary Research Institute, CAAS, China). The isolates were plaque-purified three times on primary MDCK cells and then grown in MDCK cells, resulting in a virus stock solution that was then stored at –70 °C.

2.2. Genetic and phylogenetic analyses of the H1N2 isolates

Viral RNA was extracted and reverse-transcribed under standard conditions using the Uni12 (AGCAAAGCAGG) primer. Polymerase-chain reaction (PCR) amplification was performed using segment-specific primers (primer sequences designed in-house and available on request). The PCR products were purified and sequenced using a CEQ DTCS quick-start kit on a CEQ 8800 DNA sequencer (Beckman Coulter). The nucleotide sequences were edited by using the Seqman module of the DNASTar package. To characterize the gene segments of the isolated viruses, we constructed phylogenetic trees with reference viruses that comprised H1N1, H1N2, and H3N2 viruses isolated from humans and swine. Representative sequences of the different SIVs were selected as reported previously (Chen et al., 2013; Karasin et al., 2006; Pascua et al., 2012; Vijaykrishna et al., 2011), including those of human H1N1, EA H1N1, CS H1N1 and triple reassortant H1N2 strains. The selected representative sequences are all available in GenBank. Multiple sequence alignment with default settings and evolutionary analyses were conducted by using MEGA 5.10. Phylogenetic trees were constructed by using the neighbor-joining method with a bootstrap value of 1000. Antigenic sites are regions of molecules involved in antibody binding, and four sites (Sa, Sb, Ca, and Cb) of H1N1 influenza virus have been defined (Xu et al., 2010). The deduced amino acid sequences of HA1 of the two H1N2 isolates were compared with those of the following SIV subtype reference strains: CS H1N1, A/swine/Guangdong/6/2010; North American triple-reassortant internal gene (TRIG) H1N2, A/swine/Minnesota/5763/2003; double reassortant H1N2, A/swine/Zhejiang/1/2004; Eurasian avian-like (EA) swine H1N1, A/swine/Hong kong/1780/2008; and pandemic 2009 H1N1 (pH1N1/2009), A/swine/Heilongjiang/44/2009. Potential N-linked glycosylation sites (NXT/NXS, X may be any amino acid except proline) on the HA1 peptide chain were also predicted.

2.3. Antigenic analysis of the two swine H1N2 influenza viruses

For the antigenic characterization of the isolates, we used five influenza A viruses representing CS H1N1 strain A/swine/Guangxi/12/2005 (H1N1), EA H1N1 strain A/swine/Henan/11/2005 (H1N1), pH1N1/2009 strain A/swine/Heilongjiang/44/2009 (H1N1), and A/swine/Tianjin/1/2004 (H1N2, double-reassortant virus containing CS H1 HA gene). 8-Week-old white leghorn SPF chickens housed in isolator cages were intranasally inoculated with 0.1 ml containing 10^{6.0} EID₅₀ of virus. Sera were collected on day 21 post infection (p.i.) for seroconversion confirmation and antigenic analysis. Sera were heat inactivated at 56 °C for 30 min, and hemagglutination inhibition assay

(HI) tests were performed according to the World Organization for Animal Health (OIE) recommendation. Briefly, 25 μ l of serial two-fold dilutions of the treated serum samples were mixed with 4 HA units of virus in V-shaped microtiter plates and incubated at room temperature for 30 min. Then, 25 μ l of 1% chicken RBCs was added to each well and incubated at room temperature for 40 min. The HI titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of 4 HA units of the virus. All HI assays were performed in duplicate.

2.4. Replication and pathogenicity of the viruses in mice

We used a mouse model to evaluate the pathogenicity of the two H1N2 influenza viruses. Six-week-old female BALB/c mice weighing 16–18 g were purchased from Vital River Laboratories, Beijing, China. Mice in each group ($n=8$) were lightly anesthetized with CO₂ and inoculated intranasally with 10⁶TCID₅₀ of the H1N2 virus at a volume of 50 μ l. Mock-infected control mice were inoculated intranasally with phosphate-buffered saline (50 μ l). Three mice were humanely euthanized 3 days postinfection, and the spleen, kidney, brain, nasal turbinate, and lung were removed from the individual mice and placed and homogenized (10%, w/v) in cold DMEM under sterile conditions. Then the solid debris was pelleted by centrifugation at 5000 $\times$ g for 10 min at 4 °C, and the homogenates were used for virus titration in the MDCK cells. Virus titers were reported as log₁₀TCID₅₀ per ml. The remaining mice were monitored daily for clinical signs, body weight, and mortality over a 14-day follow-up.

2.5. Experimental inoculation in swine

Healthy six-week-old pigs ($n=18$) were randomly divided into three groups. Prior to challenge, the animals were confirmed to be seronegative for swine influenza virus infection by both an enzyme-linked immunosorbent assay (IDEXX Laboratories, product code 99-12119) and HI assay with the Sw/HeB/10/06 and Sw/TJ/1/07 virus as antigen. All serum samples were pretreated with receptor-destroying enzyme (RDE; cholera filtrate; Sigma–Aldrich, MO) overnight at 37 °C to remove nonspecific inhibitors. The animals in each subgroup were anesthetized and then challenged by intranasal inoculation of 2 ml of 10⁷TCID₅₀ of the Sw/HeB/10/06 or Sw/TJ/1/07 virus, respectively.

All pigs were monitored daily by clinical examination, and rectal temperatures were recorded. Nasal swab samples were obtained for viral isolation in eggs every day for two weeks after challenge. Three days later, three pigs from each subgroup were euthanized, and bronchoalveolar lavage fluid (BALF) and brain, liver, lung, kidney, and spleen tissue were collected for analysis by viral titration as previously described (Weingartl et al., 2010). The three remaining animals in each group were observed and euthanized 14 days after challenge.

All animal experiments were performed in biosafety level 2+ facilities at the Harbin Veterinary Research Institute (HVRI), CAAS. All animal studies were approved by the HVRI Animal Care and Use Committee and were conducted according to applicable laws and guidelines.

3. Results

3.1. Virus isolation and identification

MDCK cell cultures demonstrated cytopathic effects and HA positivity, and the HA agent was characterized using hemagglutination- and NA-inhibition assays. Based on the results, the isolates were identified as type A SIV belonging to the H1N2 subtype. The isolates were designated as A/swine/Hebei/10/2006 (H1N2) and A/swine/Tianjin/1/2007 (H1N2), respectively.

3.2. Antigenic analysis of swine influenza isolates

The HI assay, performed with the antigen and antiserum, was used to analyze the swine viruses. The two isolates were antigenically similar to the CS H1N1 influenza viruses, and their reactivity profile showed no notable differences. A/swine/Hebei/10/2006, A/swine/Tianjin/1/2007, A/swine/Heilongjiang/44/2009, and A/swine/Guangxi/12/2005 showed greater cross-reactivity, but they were rather poorly inhibited by the A/swine/Henan/11/2005 serum. The antigenic properties of the two swine H1N2 influenza viruses demonstrated they were related to CS H1N1 influenza viruses (Table 1).

3.3. Homology analysis of nucleotide sequences

Eight gene segments of the two isolates were sequenced, and their homology was determined by comparison with the sequences available in GenBank. The nucleotide sequences obtained in this study have been

Table 1

Hemagglutination inhibition titers with swine influenza A (H1) viruses of different lineage isolated in China.

Virus strain	Lineage	Chicken antisera directed against					
		HeB/10/06	TJ/1/07	HLJ/44/09	GX/12/05	HN/11/05	TJ/1/04
A/swine/Hebei/10/2006	Triple-reassortant H1N2	<u>512</u>	512	512	512	<	512
A/swine/Tianjin/1/2007	Triple-reassortant H1N2	1024	<u>512</u>	1024	512	<	512
A/swine/Heilongjiang/44/2009	Pandemic H1N1	512	512	<u>1024</u>	512	<	512
A/swine/Guangxi/12/2005	Classical H1N1	512	512	512	<u>512</u>	<	512
A/swine/Henan/11/2005	Eurasian avian-like H1N1	64	32	128	32	<u>512</u>	32
A/swine/Tianjin/1/2004	Double-reassortant H1N2	512	512	256	512	<	<u>512</u>

Note. Homologous antibody titers are underlined; < indicates hemagglutination inhibition titer <2.

deposited in the GenBank database under accession numbers HQ541664–HQ541671, HQ541640–HQ541647. For the A/swine/Hebei/10/2006 (H1N2) isolate, seven gene segments including HA, NA, PB2, PB1, NP, M, and NS shared the highest nucleotide sequence identity with a TRIG H1N2 influenza virus, A/swine/Hong Kong/NS623/2002 (H1N2), and the PA shared the highest nucleotide sequence identity with another TRIG H1N2 influenza virus isolated in Hong Kong. For the A/swine/Tianjin/1/2007 (H1N2) isolate, surface gene segments including HA and NA showed a close relationship with A/swine/Hong Kong/NS246/2004 (H1N2), which is a double-reassortant virus of CS H1N1 that acquired the NA gene from the human H3N2 virus, and six internal genes shared the highest homology with EA swine H1N1 viruses isolated in Hong Kong and mainland China during 2005 and 2011, with a homology rate ranging from 98.2% to 99.3%. The M gene segment most closely related to the EA H1N1 viruses was from A/Jiangsu/1/2011(H1N1), which was isolated from a human case of swine influenza (Table 2). The sequence identity of each gene between the two H1N2 strains ranged from 83.0% to 92.7%.

3.4. Genetic and phylogenetic analyses of the H1N2 isolates

A phylogenetic analysis of the HA gene previously revealed that all swine H1 viruses isolated in the world can be classified into four lineages, including human, CS, EA swine, and avian cluster (Vijaykrishna et al., 2011). Phylogenetic analysis of the nucleotide sequences of the HA genes of the two viruses of the present study indicated that they were related to swine viruses from the classical H1 lineage, which include swine viruses isolated in North America and Asia (Fig. 1a). A genetic relationship analysis showed that the eight genes of influenza A/swine/Hebei/10/2006 were more closely related to those of the TRIG H1N2 swine viruses (Fig. 1). A genetic relationship analysis of the HA nucleotide sequences for the viruses showed that the HA gene of influenza A/swine/Tianjin/1/2007 was more closely related to that of the CS H1N1 swine viruses

(Fig. 1a). The N2 gene of influenza A/swine/Tianjin/1/2007 also appeared to be more closely related to the H1N2 swine virus circulating in China (A/swine/Zhejiang/1/2004) (Qi and Lu, 2006), with a homology of 98.6%. The nucleotide sequences for the six internal genes of A/swine/Tianjin/1/2007 were compared with sequences available on public databases and were found to have the greatest degree of similarity to the internal genes of EA H1N1 viruses circulating in Eurasian swine.

3.5. Molecular analyses of swine H1N2 influenza viruses isolated in China

Residues 190 and 225 (H3 numbering) are critical determinants of the receptor-binding specificity of A/H1N1 HA. It was previously demonstrated in 1918 A(H1N1) HA that 190D and 225D are key determinants of effective binding to human-like a (2,6)-linked receptors and consequently impart high infectivity of the virus among the human population (Reid et al., 2003). Both viruses had the same D at positions 190 and 225 of HA as the 1918 human strain, suggesting that these strains have high potential to infect humans. Glycosylation is an important process in the generation of a new virus because of the effects of host-specific glycosylation on receptor binding, fusion activity, and antigenic properties of the virus. Some glycosylation sites have a significant effect on the antigenic and receptor-binding properties of the influenza virus HA protein (Schulze, 1997). The available data indicate that the three H1N2 viruses from China have six glycosylation sites in their HA1 proteins; sites 13, 14, 26, 90, and 290 (H3 numbering) were conserved in all isolates. The potential glycosylation sites of Sw/HeB/10/06 are identical to those of the TRIG H1N2 A/swine/Minnesota/5763/2003, whereas the potential glycosylation sites of Sw/TJ/1/07 are similar to those of the double-reassortant swine H1N2 virus A/swine/Zhejiang/1/2004. Several mutations were found in the antigenic sites of the two isolates: G158E and Q166K at site Sa; S188N at site Sb; I169T, S206T, and R208K at site Ca; and F74S at site Cb (Fig. 2).

Table 2

Nucleotide homology of genes of influenza viruses to the closest related sequences available in GenBank.^a

Virus	Gene	Closest related virus strain	Nucleotide identity (%)	Accession no.
A/swine/Hebei/10/2006	PB2	A/swine/Hong Kong/NS623/2002(H1N2)	97.8	GQ229369
	PB1	A/swine/Hong Kong/NS623/2002(H1N2)	98.1	GQ229364
	PA	A/swine/Hong Kong/1111/2004(H1N2)	98.2	CY085661
	HA	A/swine/Hong Kong/NS623/2002(H1N2)	97.6	GQ229365
	NP	A/swine/Hong Kong/NS623/2002(H1N2)	98.5	GQ229366
	NA	A/swine/Hong Kong/NS623/2002(H1N2)	98.1	GQ229370
	M	A/swine/Hong Kong/NS623/2002(H1N2)	98.6	GQ229367
	NS	A/swine/Hong Kong/NS623/2002(H1N2)	98.1	GQ229363
A/swine/Tianjin/1/2007	PB2	A/swine/Hong Kong/NS376/2006(H1N1)	99.1	CY085779
	PB1	A/swine/Hong Kong/434/2006(H1N1)	98.9	CY085772
	PA	A/swine/Hong Kong/421/2006(H1N1)	98.2	CY085765
	HA	A/swine/Hong Kong/NS246/2004(H1N2)	98.6	CY085638
	NP	A/swine/Hong Kong/170/2006(H1N1)	98.9	CY085759
	NA	A/swine/Hong Kong/NS246/2004(H1N2)	99.1	CY085640
	M	A/Jiangsu/1/2011(H1N1)	99.3	KF057133
	NS	A/swine/Henan/11/2005(H1N1)	98.9	HQ541676

^a PB, polymerase basic subunit; PA, polymerase acidic subunit; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural.

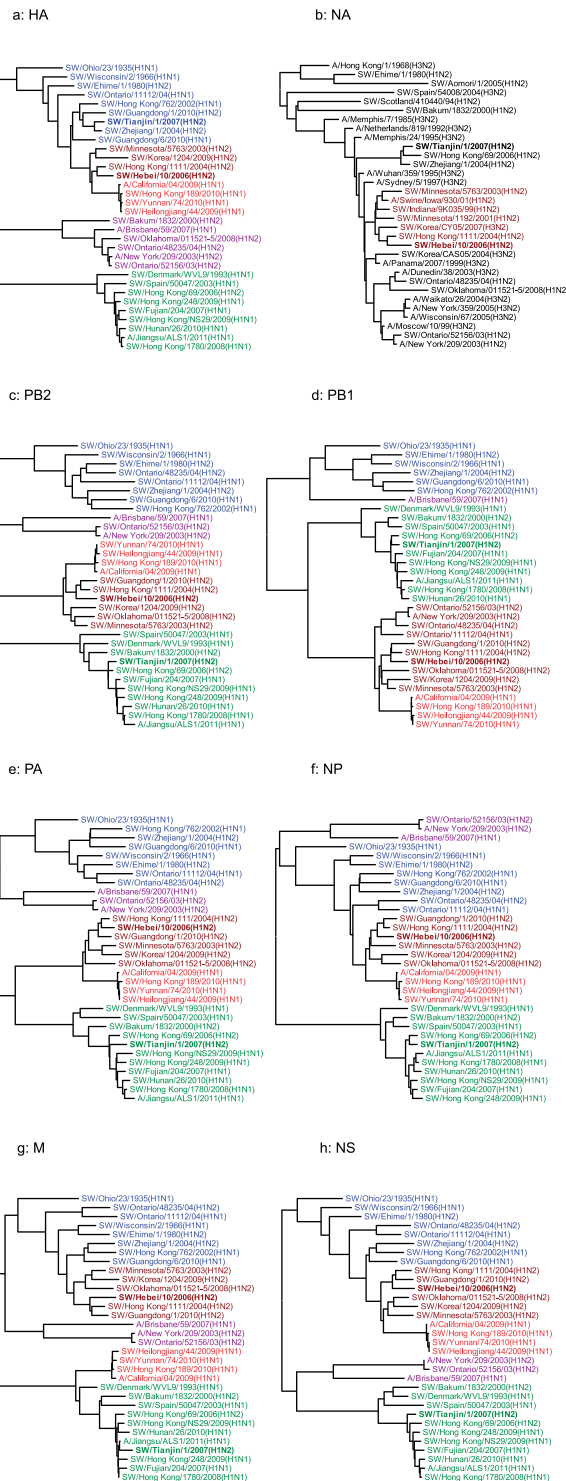


Fig. 1. Phylogenetic trees of the eight genes of the H1N2 influenza viruses isolated from pigs in China compared with genes of selected swine and human influenza virus strains: (a) HA; (b) NA; (c) PB2; (d) PB1; (e) PA; (f) NP; (g) M; (h) NS. The tree was created using the neighbor-joining method (MEGA5.10) based on the nucleotide sequence lengths of each gene segment for nucleotides (nt) 33–1733 for HA, 21–1430 for NA, 28–2307 for PB2, 25–2298 for PB1, 25–2175 for PA, 46–1542 for NP, 26–1007 for M, and 27–864 for NS. Bootstrap values were calculated using 1000 replications. Different lineages are marked with different colors, CS H1

The presence of specific amino acids in the PB2 gene is known to affect the virulence and transmission of some viruses. In addition to the E627K mutation, the PB2 mutation, T271A, enhances polymerase activity in human cells (Bussey et al., 2010). The D701N mutation not only improves viral growth in mammalian cells but also enhances transmission between guinea pigs when PB2 627 is an E residue (Steel et al., 2009). The Sw/HeB/10/06 isolate contained 271A, 591R, 627E, and 701D, and the Sw/TJ/1/07 isolate contained 271T, 591Q, 627E, and 701N in the PB2 polymerase subunit. In influenza viruses of the N2 subtype, E119V, R292K, and N294S NA mutations are most frequently associated with NA inhibitor resistance; no such amino acid substitution was observed in these residues in the present study, suggesting that the viruses isolated in this study remain susceptible to NA inhibitors.

3.6. Replication and pathogenicity of the viruses in mice

Body weight changes were calculated as percentages of the baseline body weight. Infected mice exhibited weight loss after infection and then regained their initial body weight toward the end of the follow-up. Mice infected with Sw/HeB/10/06 and Sw/TJ/1/07 viruses exhibited an average weight loss of 12.8% and 8.1%, respectively (Fig. 3a). On day 3 post-infection, tissues were collected for virus titration. Both viruses caused severe lesions in the lungs of infected mice. Viral replication in nasal turbinates and lung tissues harvested from experimentally inoculated mice was efficient for the two viruses; virus titers in the lung tissues were higher than those in the nasal turbinates, ranging from $3.33 \log_{10}$ TCID₅₀/ml to $4.17 \log_{10}$ TCID₅₀/ml. No virus was detected in the kidney, brain, and spleen (Table 3). All negative controls were negative for influenza viruses in the lung, nasal turbinate, and other tissues. The remaining five mice survived the infection over the course of a 14-day follow-up.

3.7. Experimental inoculation in swine

We did not observe any considerable changes in the clinical presentation of the pigs. The general condition of the animals and food intake remained unchanged in all treatment groups during the experiment. In addition, no significant changes in the amount of sneezing and coughing between animals in the virus-challenged groups and control animals were observed (data not shown). Viral shedding in nasal swabs occurred from 1 to 7 days after challenge in the two virus-challenged groups. Virus shedding from each of the inoculated pigs was detected from 1 to 7 days postinfection, with peak shedding occurring at days 2 and 3 (5.33 and $4.5 \log_{10}$ EID₅₀/ml swab material, respectively). These are currently no significant difference in the presence of the viruses among the pigs (Fig. 3b). At necropsy, the prevalence of lung

(blue); TRIG H1N2 (dark red); EA H1 (green); Human & swine (black). Viruses isolated in the present study are marked by bold font. The scale represents the number of substitutions per nucleotide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

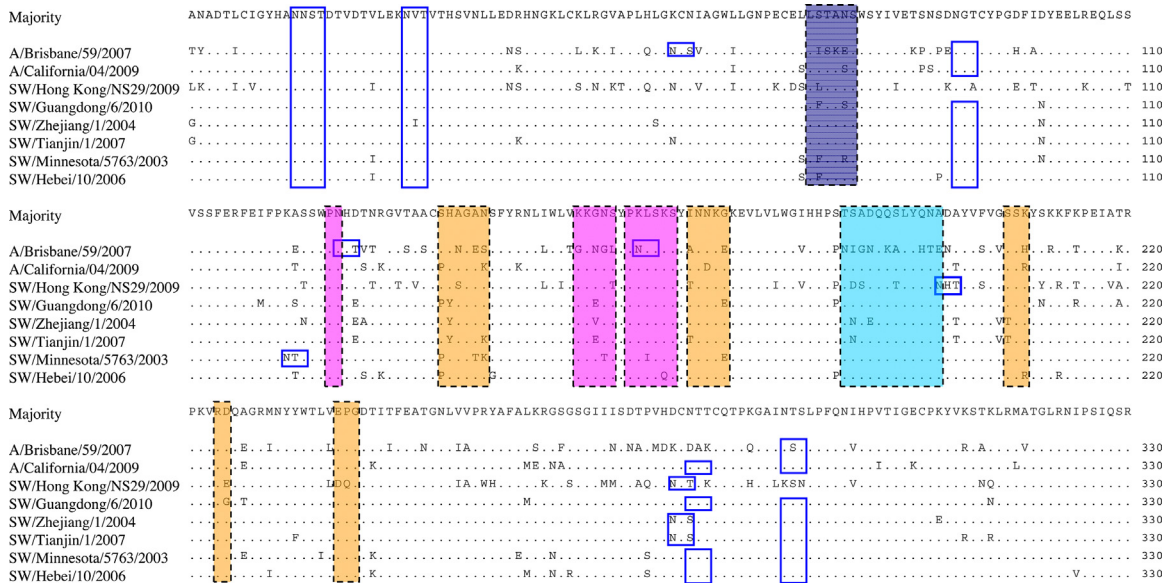


Fig. 2. Molecular analysis of HA1 amino acid sequences of the two H1N2 swine influenza viruses and reference strains. Potential glycosylation sites are indicated in blue boxes. Previously defined antigenic sites are indicated: site Sa (magenta), site Sb (cyan), site Ca (orange), and site Cb (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lesions in challenged pigs was lower than 5%, whereas the lungs of all control pigs had no macroscopic lesions (data not shown). No virus was detected in any of the organs, and

the mean virus titers in the BALF of the Sw/HeB/10 and Sw/TJ/1/07 groups were 1.39 and 1.72 $\log_{10}$ EID₅₀/ml, respectively. These findings suggest that the two H1N2 viruses of the present study have low pathogenicity in pigs because infection under experimental conditions resulted in no apparent clinical signs.

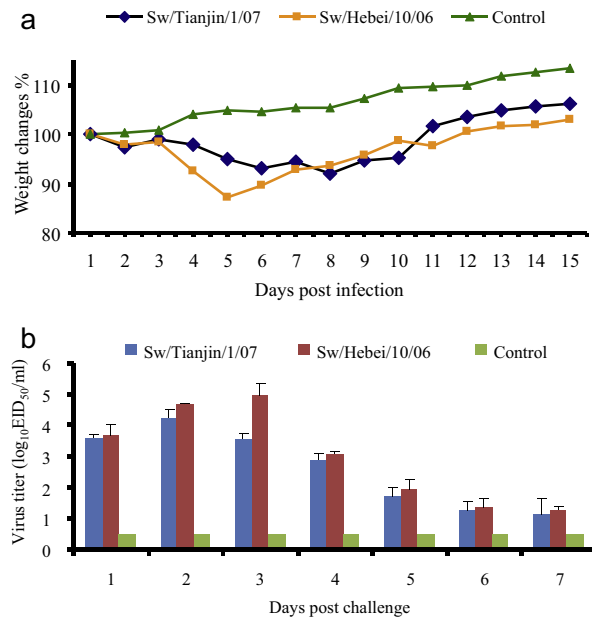


Fig. 3. (a) Mean changes in the body weight of mice infected with H1N2 viruses. BALB/c mice were inoculated intranasally with each of two H1N2 viruses at a dose of 10^6 TCID₅₀. The body weight was monitored daily during a 14-day follow-up and expressed as a percentage of the baseline value. The data represent the mean of five mice in each group. (b) Virus shedding in pigs after challenge with the two H1N2 swine influenza viruses. Pigs were infected intranasally with each of the two H1N2 viruses at a dose of 10^7 TCID₅₀. Nasal swabs were collected every day for two weeks after challenge and titrated in embryonated chicken eggs ($\log_{10}$ EID₅₀/ml). The limit of virus detection was set at 0.5 $\log_{10}$ EID₅₀/ml.

4. Discussion

In Southern China, frequent contact between wild birds, human, poultry, and pigs creates an opportunity for interspecies transmission and generation of new reassortment influenza viruses. Moreover, the cold and dry weather in northern China may contribute to rapid transmission of SIVs (Lowen et al., 2007). In the present study, A/swine/Hebei/10/2006 appeared to be derived from North American-lineage triple-reassortant H1N2 strains. The isolation of A/swine/Tianjin/1/2007 provides further evidence that pigs can serve as a “mixing vessel” for the generation of new reassortant genotypes of influenza viruses. The A/swine/Tianjin/1/2007 isolate possessing CS H1N1 HA, H1N2 SIV-like NA, and EA swine H1N1 internal genes represents a novel triple-reassortant H1N2 SIV. Pandemic influenza may occur through at least two mechanisms: reassortment between animal and human influenza viruses that yields a new virus, and direct spread and adaptation of a virus from animals to humans (Belshe, 2005). Incidents of direct transmission of EA swine H1N1 viruses from infected domestic pigs to humans have been reported in China (Yang et al., 2012). These incidents of direct swine-to-human transmission may result in gradual adaptation of swine viruses and their spread among humans.

The pathogenicity of the viruses in mice indicates that the viruses can cause rapid weight loss and replicate effectively in the respiratory organs. The results of our

Table 3

Mouse infectivity and tissue viral titers on day 3 p.i.

Virus strain	Viral titers ^a				
	Spleen	Kidney	Brain	Nasal turbinate	Lung
A/swine/Hebei/10/2006	<0.5 ^b	<0.5	<0.5	3.3 ± 0.3	3.8 ± 0.4
A/swine/Tianjin/1/2007	<0.5	<0.5	<0.5	3 ± 0.3	3.6 ± 0.1

^a Virus endpoint titers are expressed as the mean log₁₀TCID₅₀/ml plus the standard deviation of 3 mice per tissue.^b <0.5, not detected. The limit of virus detection was 0.5 log₁₀TCID₅₀/ml.

experimental inoculation in swine also indicated that the two triple-reassortant H1N2 viruses replicated effectively in pigs, and the levels of replication and shedding of the two H1N2 viruses were similar. However, no major clinical symptoms were observed, although mild lesions were observed on the surface of the lungs on day 3 post infection.

H1N2 viruses from China are of increasing concern and appear to have more complex genotypes than H1N1 viruses in the swine population in China. Multiple reassortments of H1N2 SIVs have included genetic segments from swine and human influenza viruses and have resulted in the generation of multiple genotypes of H1N2 SIVs with gene constellations changing over time (Vijaykrishna et al., 2011). A previous study indicated that some H1N2 viruses with high transmissibility and virulence may pose a potential threat to human and animal health (Barman et al., 2012; Pascua et al., 2012). Further research and epidemiological studies are needed to fully understand the role of H1N2 SIVs in the overall ecology of IAV between humans and pigs. Pigs have been shown to serve as reservoirs for influenza viruses. Therefore, to prevent a potential influenza pandemic, in-depth studies on SIV reassortment are required.

Conflict of interest

None of the authors have any personal or financial conflicts of interest relating to the outcomes of this study.

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