Genetic characterization of H1N1 swine influenza A viruses isolated in eastern China

X. Qi · B. Pang · C. P. Lu

Abstract Three influenza H1N1 viruses were isolated in 2005 from pigs with respiratory disease on a farm in eastern China. The three isolates were characterized to determine their probable origin. Each of the eight genes of the isolates was most closely related to the corresponding gene from the classical swine H1N1 virus. Also, phylogenetic analysis further confirmed that each of the eight genes of the isolates was closely related to the classical swine H1N1 viruses, especially those isolated in China. The HA1 proteins of the three isolates were identical to that of A/Swine/Guangdong/1/01, a virus isolated in 2001 in China, even though three nucleotide differences were observed. These results further support the concept that swine can serve as a reservoir of genetically stable influenza viruses.

Keywords Influenza A virus · H1N1 · Swine · Genetic characterization

Swine influenza is an economically important respiratory disease of pigs resulting from infection with influenza A virus. Influenza A viruses can infect a variety of animals, including poultry, human, pigs, horses, marine mammals, and carnivore animals (i.e., dog and cat), and they are classified into different subtypes on the basis of the antigenic properties of the two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA) [1, 2]. Wild aquatic birds are the primary natural reservoir of influenza A viruses, which harbor all currently known 16 HA and nine NA subtypes [2, 3].

Currently, three predominant subtypes of influenza viruses are prevalent in pig populations worldwide: H1N1, H3N2, and H1N2, and these include classical swine H1N1, avian-like H1N1, human-like H3N2, reassortant H3N2, and various genotype H1N2 viruses [4–7]. Pigs may also play an important role in the evolution and ecology of influenza A viruses [2, 4]. The tracheal epithelium of pigs contain both SA α 2,6 Gal and SA α 2,3 Gal receptors and can be infected with swine, human, and avian viruses; therefore, pigs have been hypothesized to serve as an intermediate host for the adaptation of avian influenza viruses to humans or as mixing vessels for the generation of genetically reassortant viruses. The probability that pigs pose a threat as sources for zoonotic transmission of influenza viruses is supported by reports of several sporadic human infections caused by swine-like viruses, some of which resulted in serious disease [8]. In March 2009, a novel influenza A (H1N1) virus emerged in human population, and continues to spread in many countries, especially North American. Further study has shown that this new virus is a "quadruple reassortant" with two genes from flu viruses that normally circulate in pigs in Europe and Asia and avian genes and human genes (http://www.who.int/csr/disease/swineflu/en/index.html). This outbreak further highlight that swine influenza viruses pose a significant threat to human health.

The classical swine virus (H1N1) that evolved from the 1918/19 pandemic was first isolated in 1930, and since then its progeny have been detected in swine populations from many parts of the world [4]. Unlike human viruses, swine viruses have different epizootiological patterns in different areas of world. Since 1979, the previously dominant classical H1N1 swine viruses have been replaced by the

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avian-like H1N1 viruses which antigenically and genetically were closely related to avian H1N1 viruses in Europe [4, 5]. Prior to 1998, the classical H1N1 viruses were only isolated from pigs in North America, and at present, the predominant H1N1 swine influenza viruses are reassortants carrying PA and PB2 genes of avian origin [4, 6]. Occasionally, human-like H1N1 viruses are identified from pigs but have not become established [4, 8]. In this study, we recovered three H1N1 viruses in 2005 from pigs suffering from respiratory disease on a farm in eastern China. Each of the three isolates has been subjected to genetic characterization and phylogenetic analysis to determine their relationship to previously characterized isolates.

In late July 2005, a severe outbreak of respiratory disease occurred on a pig farm with 100 breeding sows in Shanghai city in eastern China, causing the death of 30 finishing pigs over a six-week period. Clinical signs in pigs included a temperature of up to 42°C, loss of appetite, lethargy, and coughing. Three lung samples were collected from the dead pigs. We used Madin-Darby canine kidney (MDCK) cells for the primary isolation of swine influenza virus. 10% [w/v] tissue homogenates of the three specimens (200 µl per egg) were inoculated allantoically in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs and into MDCK cell cultures in Eagle's minimum essential medium with TPCK (L-tosylamido-2-phenylethyl chloromethyl ketone)-trypsin (2 µg/ml). After incubation at 35°C for 72 h, the allantoic fluids were harvested and tested for hemagglutinin (HA) activity with a 0.5% suspension of chicken erythrocytes. The cell cultures were incubated at 37°C for 7 days and examined for cytopathic effect. The isolates were classified by hemagglutinin inhibition (HI) and neuraminidase inhibition (NI) assays [9]. Virus-containing allantoic fluid was stored at –80°C.

In our study, genetic analyses were conducted on the MDCK cell isolates. Viral RNAs were extracted from infectious allantoic fluid by the use of Trizol LS reagent (Invitrogen) as specified by the manufacturer. PCR was performed with a set of primers specific for each gene segment of influenza A virus [10]. The cDNA was amplified by using the Expand High-Fidelity PCR system (Roche Diagnostics, Mannheim, Germany) according to the protocols provided. PCR products were purified with the TaKaRa agarose gel DNA purification kit (TaKaRa). Sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Sequences were compiled with the Lasergene sequence analysis software package (DNAStar, Madison, WI, USA).

The nucleotides BLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify related reference viruses, and the reference sequences were obtained from GenBank. The nucleotide sequences were compared initially with the Megalign program (DNASTAR). Pairwise

sequence alignments were also performed with the Megalign program to determine nucleotide and amino acid sequence similarities. To understand the evolutionary origins of viruses isolated in this study, phylogenetic and molecular evolutionary analyses for eight gene segments were conducted by the distance-based neighbor-joining method using MEGA version 4 [11], Bootstrap values were calculated based on 1,000 replicates. Alignments of each influenza virus sequence were created using program Clustal X 1.83 [12].

Three viruses with hemagglutination activity were isolated from the lung samples taken from three dead pigs using both embryonic eggs and MDCK, and named A/swine/Shanghai/1/2005, A/swine/Shanghai/2/2005, and A/swine/Shanghai/3/2005, respectively. The isolates were determined to be H1N1 subtype by HI and NI assays, which was confirmed by genomic sequencing and the nucleotides BLASTn analysis.

To elucidate the genotype and genetic origin of the isolates, we completely sequenced the eight gene segments of the three isolates. The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers EU502884-EU502892 and FJ789824-FJ789838. The nucleotide sequence identities among the eight genes of the three isolates were 99–100%, suggesting that the three isolates come from the same progenitor viruses. The genotype and genetic origin of isolates were initially inferred from BLAST analysis and pairwise comparisons of each gene segment to the corresponding sequences of reference viruses. Given the high levels of sequence identity between the three isolates, isolate A/swine/Shanghai/1/2005 was used as the representative virus. Each of the eight gene segments of A/swine/Shanghai/1/2005 showed the highest nucleotide sequence identities to those of classical H1N1 swine influenza viruses (Table 1). The HA1 gene of the isolate shared the highest sequence identity with that of A/Swine/Guangdong/2/01 (99.7%). The NA gene shared the highest identity with that of A/Swine/Maryland/23239/1991 (96.0%). NP gene had the highest identity with that of A/Swine/Beijing/94/1991 (97.0%). PB2 and PB1 genes had the highest identities with those of swine H1N1 viruses in North America (A/swine/Memphis/1/1990 96.3% and A/swine/Iowa/24297/1991 96.7%, respectively). The PA, M, and NS genes shared the highest identity with those of three human-like H3N2 viruses (A/Shandong/02/2005 99.0%, A/Shandong/01/2005 99.0%, and A/Shandong/3/2005 99.9%, respectively). The three H3N2 viruses were reassortants with PA, M, and NS genes originated from classical swine H1N1 virus [13, 14]. Only a few gene sequences of swine H1N1 viruses are available to date in public databases, particularly sequences of viruses originating from China. Therefore, the possible origins of the

Table 1 Nucleotide sequence identity between A/Swine/Shanghai/1/2005 and reference strains available in GenBank

Gene	Region compared (nt)[a]	Virus with the greatest similarity[b]	Lineage	Similarity (%)
PB2[EU502892]	25–2322	A/swine/Memphis/1/1990 (H1N1)[CY035077]	Swine	96.3
PB1[EU502891]	25–2321	A/swine/Iowa/24297/1991 (H1N1)[CY027161]	Swine	96.7
PA[EU502890]	18–2212	A/swine/Guangdong/02/2005 (H3N2)[EU620727]	Swine	99.0
HA[EU502884]	84–1064	A/Swine/Guangdong/2/01(H1N1)[DQ058215]	Swine	99.7
NP[EU502887]	1–1565	A/swine/Beijing/94/1991 (H1N1)[U49091]	Swine	97.0
NA[EU502888]	15–1349	A/swine/Maryland/23239/1991 (H1N1)[CY022479]	Swine	96.0
M[EU502886]	1–1027	A/swine/Guangdong/01/2005 (H3N2)[EF455563]	Swine	99.0
NS[EU502889]	1–890	A/Swine/Shandong//3/2005 (H3N2)[EU116044]	Swine	99.0

[a] nt, nucleotide

[b] The numbers in brackets are the GenBank accession numbers for the reference virus sequences

genes of the isolates remain unclear. However, it can be concluded that all three isolates belong to the classical swine H1N1 viruses circulating in pig populations in China and North America.

To characterize more precisely the genetic origin of the gene segments of the three isolates, we constructed phylogenic trees, with reference viruses consisting of H1N1 viruses isolated from poultry, human, and swine. In the HA1 tree, there are three distinct clades, including human viruses dating from 1918 to the present, classical swine viruses dating from 1930 to the present, and avian viruses or viruses of avian origin (Fig. 1). The three isolates clustered with classical swine H1N1 viruses, within a subclade comprising swine H1N1 viruses isolated in China from 1991 to 2001. Three lineages of human, swine, and avian also were observed in the phylogenetic trees of the other seven genes (NA, NP, M, NS, PB2, PB1, and PA) (results not shown) and the isolates grouped with swine lineage in each of the seven trees. Therefore, phylogenetic analysis further confirmed that each of eight genes of the isolates was closely related to the classical swine H1N1 viruses, especially those isolated in China.

The HA protein is responsible for binding to receptors on host cells and initiating infection, and it is also the principal target of the host's immune system [2, 15, 16]. In this study, we also investigated putative amino acid changes in the HA1 protein of A/Swine/Shanghai/1/2005 compared to four genetically related swine H1N1 viruses, A/Swine/Beijing/47/91, A/Swine/Hong Kong/172/93, A/Swine/Hong Kong/273/94, and A/Swine/Guangdong/1/01, all of which were isolated in China from 1991 to 2001 (Table 2). Sequence comparison revealed that the nucleotide sequence and amino acid identities between the HA gene of A/Swine/Shanghai/1/2005 and those of four reference viruses were 95.9–99.7% and 95.4–100%, respectively. When A/Swine/Shanghai/1/2005 was compared to A/Swine/Beijing/47/91, A/Swine/Hong Kong/172/93, and the A/Swine/Hong Kong/273/94, 19, 10, and 6 amino acid

changes, respectively, were detected in the HA1 protein sequence. Compared to A/Swine/Beijing/47/91, A/Swine/Hong Kong/172/93, and A/Swine/Hong K/273/94, A/Swine/Shanghai/1/2005 isolate had three (V73A, G155E, and T186A), two (G155E and N186A), and two (G155E and T186A) amino acid changes, respectively, which were located in previously defined antigenic sites [15]. In contrast, the HA1 protein of A/Swine/Shanghai/1/2005 was identical to that of A/Swine/Guangdong/1/01, a virus isolated in 2001, even though three nucleotide differences were observed. Antigenic variability resulting from antigenic drift is one of the principal biological properties of influenza A viruses, especially human viruses [1, 2, 4]. Antigenic drift confers a selective advantage on the virus, allowing it to escape host immunity. Compared with human viruses, swine viruses are subject to slow drift as a result of the short life span of pigs and year-round production of piglets lacking protective immunity [4]. In this study, compared to human H1N1 virus, antigenic variants of HA proteins of the three isolates were not marked, and these results further support the concept that swine can serve as reservoir of influenza virus strains that maintain genetic and antigenic stability.

Based on the deduced amino acid sequence, the isolates contained an amino acid motif PSIQSR↓G at their HA cleavage sites, which is a characteristic of low pathogenic influenza viruses. Some glycosylation sites have a significant effect on the antigenic and receptor-binding properties of the influenza virus HA protein, and glycosylation is therefore an important process in the generation of new viruses [16, 17]. Compared to four reference swine H1N1 viruses, six potential glycosylation sites (Asn-X-Ser/Thr) were conserved at positions 10, 11, 23, 87, 276, and 287 in the HA1 protein of A/Swine/Shanghai/1/2005. The receptor-binding property of the HA protein of influenza virus is an important molecular determinant of host range [2, 16]. The A/Swine/Shanghai/1/2005 isolate maintains amino acids typical of swine viruses at residues 68E, 134A, 183P,
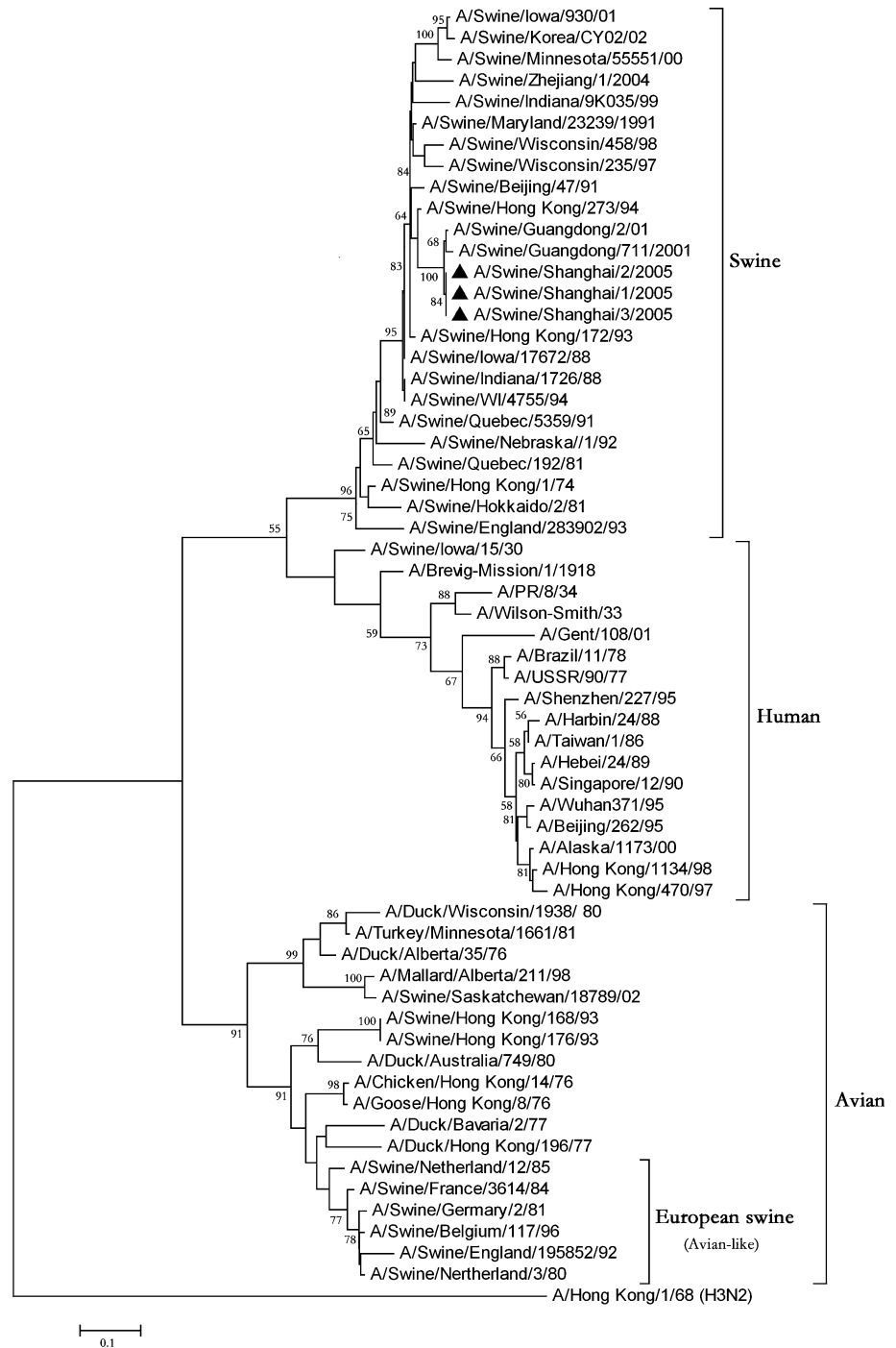
187D, 191L, and 222G (according to H1 number), which are previously defined receptor-binding sites [16, 18].

A balance between the activity of HA in virus attachment and NA in virus release is crucial for optimal viral replication [2]. Recent avian H5N1 viruses have a 20 amino acid deletion in the stalk of NA [1], and deletions of 11–16 amino acids were also found in some early human H1N1 viruses [19]. The three isolates had a full-length stalk of NA, which is conserved in all classical swine H1N1 viruses.

Antivirals are essential for the treatment and prevention of influenza infections. The substitutions H274Y and N294S of NA have been reported to confer resistance to oseltamivir in both human and influenza H5N1 viruses [20]. No amino acid substitutions were observed at the conserved residues in the NA proteins of the three isolates,

Table 2 Amino acid sequence comparison of sites of HA1 of A/Swine/Shanghai/1/2005 and four swine H1N1 viruses from China

Viruses	Amino acid position in HA1[a]																	
	1	60	62	73[b]	83	116	120	155[c]	186[d]	207	208	209	211	216	224	267	270	311
A/Swine/BeiJing/47/91	Y	R	P	V	P	I	A	G	T	N	R	K	K	T	A	I	A	K
A/Swine/Hong Kong/172/93	D	W	L	A	S	●[e]	●	●	N	S	K	T	●	●	●	T	T	●
A/Swine/Hong Kong/273/94	D	W	L	A	S	M	S	●	●	●	K	●	●	●	●	●	●	●
A/Swine/Guangdong/1/01	D	W	L	A	S	M	S	E	A	●	K	●	R	A	T	●	T	N
A/Swine/Shanghai/1/2005	D	W	L	A	S	M	S	E	A	●	K	●	R	A	T	●	T	N

[a] The amino acids were numbered with the N-terminal asparagines of the HA1 protein designated as amino acid 1

[b] 73, [c] 155, and [d] 186 are located in antigenic site Cb, Sa, and Sb, respectively

[e] ●, Amino acid is conserved relative to A/Beijing/47/91

which suggests that they are sensitive to NA inhibitors. Anti-influenza drugs amantadine and rimantadine target the M2 protein, and the substitutions S31N have been reported to confer resistance to these drugs in recent European swine viruses, including H1N1, H1N2, and H3N2 viruses [5]. The three isolates contained H rather than Y at position 31, which suggests that they are sensitive to this class of antiviral drugs.

PB1-F2 is a protein encoded by an alternative ORF of the PB1 gene in many influenza A viruses, which was first observed in the A/Puerto Rico/8/34 (H1N1) isolate [21]. PB1-F2 has been shown to localize to the mitochondria and induce apoptosis, which is an important pathogenic mechanism in influenza A virus infection and, in turn, shown to enhance viral virulence in a mouse model [21, 22]. The complete ORF for PB1-F2 in A/Puerto Rico/8/34 isolate is from positions 119 to 379, which encodes an 87-residue protein [21]; however, most of the avian and human H3N2 viruses contained the entire PB1-F2 ORF of 90 residues [21, 23]. The truncated PB1-F2 (with <87 or 90 aa residues) does not contain the mitochondrial translocation signal at the C-terminal end and may therefore lose its PB1-F2 function. Recently a study showed that classic swine influenza viruses and human H1N1 isolates collected since 1950 contained a truncated PB1-F2. While PB1-F2 of human H1N1 viruses terminates after 57 aa, classic swine H1N1 sequences have stop codons after 11, 25, and 34 codons [23]. In this study, similar to most classical swine H1N1 viruses, the three isolates contained truncated

PB1-F2 proteins because of in-frame stop codon after residue 11 (Table 3). This possibly plays a role in their decreased virulence although the virulence and pathogenesis of isolates remain to be further determined experimentally.

Internal proteins also harbor determinants for host range and virulence, as demonstrated by genetic studies on avian/human reassortant viruses [1]. The amino acid at 627 of PB2 has previously been shown to be a determinant of host range, and the majority of avian influenza viruses have E at this position, whereas all human influenza viruses (H1N1, H2N2, and H3N2) have K [24]. In this study, each of the three isolates possessed a K at position 627 of PB2 which is characteristic of mammalian influenza viruses (Table 3).

The NS1 protein is also important in determining the pathogenicity of influenza A virus in different hosts, especially H5N1 virus. A five amino acid residue deletion at positions 80–84 was found in the NS1 molecule of the clade 1-9 H5N1 viruses, which may contribute to increased virulence [1]. No deletions were observed at positions 80–84 of NS1 proteins in the three isolates. A previous study has shown that a mutation at position D92E may increase the virulence of H5N1 viruses in pigs [25]. In this study, all three isolates possessed D rather than E at position 92 of NS1.

Large-scale sequence analysis of avian influenza viruses indicated that the four C-terminal residues of the NS1 protein of avian influenza viruses is a potential PDZ ligand binding (PL) motif of the X-S/T-X-V type [26]. The PL

Table 3 Molecular characterization of the HA, NA, PB2, PB1-F1, M2, and NS1 of A/Swine/Shanghai/1/2005

HA	NA			PB2	PB1-F2	M2	NS		
Cleavage site	Stalk region	274[a]	294[a]	627		31	80–84	92	PL motif
PSIQSR↓G	+	H	N	K	MGLEQDTLWIL	H	+	D	−

[a] N2 numbering

+ No amino acid deletion

− Deletion

motifs with ESEV or EPEV of NS1 from highly pathogenic H5N1 viruses isolated in 1997 and 2003 as well as the 1918 pandemic virus (all of avian origin) are able to bind cellular PDZ-containing proteins involved in host cellular signaling pathways. However, NS1 in most low pathogenic human influenza viruses contain a different motif (RSKV or RSEV), which cannot bind PDZ. A recent study showed that the PL motif of NS1 was a new virulence factor of influenza A viruses [27]. Sequence analysis indicated that the NS1 protein of the early human H1N1 strains was 230 aa in length, but in the 1940s the C-terminal PL motif was lost because of the extension of NS1 protein to 237 aa [27]. This seven amino acid extension was retained (with a few exceptions) in human H2N2, H3N2, and H1N1 viruses until the 1980s when both the co-circulating H1N1 and H3N2 viruses reverted back to encoding a 230-aa NS1 protein, and the PL motif was regained [27]. Unlike the NS1 of human viruses, interestingly, the NS1 proteins of classical swine H1N1 viruses have different evolutional patterns. We analyzed the NS1 proteins of classical swine H1N1 viruses isolated from 1930 to the present and deposited in GenBank (Table 4). We found that the NS1 protein of the early swine H1N1 viruses contained RSEV motif which is a characteristic of human viruses with 230 aa until 1940s. However, the C-terminal PL motif became GSEI as a result of nucleotide changes in about 1950s–60s. From 1960s, swine H1N1 viruses lost PL motif resulting from a stop codon after 219 residue, which may be a characteristic of the swine H1N1 viruses from 1960s. Whether the truncated C-terminal of the NS1 has an impact

on virulence remains unknown. In this study, like most of the swine H1N1 viruses, each of the isolates lost PL motif at the NS1 C-terminal region because of a stop codon after 219 residue.

Influenza virus infection in pigs was first described in 1918 in China, and it coincided with the so-called Spanish pandemic in humans [4]. In China, it was documented that four subtypes (H1N1, H1N2, H3N1, and H3N2) of swine influenza viruses were present in pig populations [7, 28–31]. Early studies on influenza viruses from pigs in southern China from 1976 to 1982 revealed co-circulation of swine H1N1 and human-like H3N2 viruses, and detected three reassortant H3N2 viruses containing the surface genes HA and NA from human virus origin and the internal genes from swine H1N1 virus [31–33]. In 1993–1994, avian-like H1N1 viruses were detected in pigs and co-circulated with classical H1N1 viruses in Southern China [28]. In 2004, we isolated three reassortant H1N2 viruses that contained the NA gene of a human H3N2 virus origin and the other seven genes from classical H1N1 virus [7]. Recently, interspecies transmission of human H1N1, avian H5N1, and avian H9N2 to pigs has been reported in China [29, 34, 35]. Since 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have become endemic in poultry in China and have posed serious threat to poultry, mammalian animals, and human health [1]. In this study, we failed to detect avian H5N1 or H9N2 viruses on the farm where three isolates were recovered, although retrospective epidemiology survey showed that chicken death was observed in some neighboring backyard farms at that time.

Table 4 Molecular characterization of the four C-terminal residues of the NS1 proteins of A/Swine/Shanghai/1/2005 and reference viruses

Viruses	GenBank accession no.	Four C-terminal residues	Location[a]	PL motif	Lineages
swine/Iowa/15/1930	U49484	RSEV	230	+	Classical swine
A/swine/1976/1931	M55482	RSEV	230	+	Classical swine
A/swine/1931	CY009632	RSEV	230	+	Classical swine
A/swine/Jamesburg/1942	CY026431	RSEV	230	+	Classical swine
swine/Wisconsin/1/1957	CY026287	GSEI	230	+	Classical swine
A/swine/Wisconsin/1/1961	M80951	GSEI	230	+	Classical swine
A/swine/Wisconsin/2/1966	CY026303	PEQK	219	−	Classical swine
swine/Wisconsin/1/1967	CY026295	PEQK	219	−	Classical swine
swine/Iowa/1/1976	CY022073	PEQK	219	−	Classical swine
swine/Tennessee/49/1977	CY009920	PEQK	219	−	Classical swine
Swine/Indiana/9K035/99	AF250128	PEQK	219	−	Classical swine
A/Swine/Shanghai/1/2005	EU502889	PEQK	219	−	Classical swine
A/Puerto Rico/8/34(H1N1)	AAM75163	RSEV	230	+	Human
A/Hong Kong/486/97(H5N1)	AAK49307	EPEV	230	+	Avian
A/Brevig Mission/1/1918(H1N1)	AAK14368	KSEV	230	+	Avian

[a] The amino acids were numbered with the N-terminal asparagines of NS1 protein designated amino acid 1

+ No deletion

− Deletion

Genetic reassortment may be the main mechanism of generating new pandemic strains, which is exemplified by the pandemic influenza viruses of 1957 (H2N2) and 1968 (H3N2) derived from reassortments of avian viruses with the prevailing human viruses [2]. Furthermore, the novel influenza A (H1N1) virus emerging in human population is also reassortment with swine, avian, and human genes. Southeastern China is regarded as an epicenter for the emergence of pandemic influenza viruses, where a variety of influenza viruses co-circulate in humans, pigs, and poultry [1, 36]. Introduction of avian viruses (H5N1 and H9N2) into pigs co-infected with human viruses (H3N2 and H1N1) or swine viruses (H1N1 and H1N2) could provide a favorable opportunity for the generation of re-assortants containing avian genes and would thereby pose a significant threat to human health. This should raise concerns about the genetic evolution of influenza virus in pigs and highlight the need for virus surveillance of pigs in China.

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