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Genetic characterization of novel reassortant H1N2 influenza A viruses isolated from pigs in southeastern China

Brief Report

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Summary. In December 2004, three influenza H1N2 viruses were isolated from lung samples of pigs that had died from respiratory disease on a farm in southeastern China. To determine the genetic characterization and probable origin, one of the three isolates, A/Swine/Zhejiang/1/2004 (Sw/ZJ/1/2004), was genetically analyzed. Sw/ZJ/1/2004 was a reassortant with an NA gene most closely related to the corresponding gene from a human-like H3N2 virus circulating in 1995. The remaining seven genes were most closely related to those from the classical swine H1N1 virus. Sw/ZJ/1/2004 appeared to be a novel reassortant H1N2 virus that was genetically distinguishable from other H1N2 viruses found in pigs worldwide. The isolation of Sw/ZJ/1/2004 provided further evidence for pigs serving as a "mixing vessel" for the generation of new reassortant genotypes of influenza viruses and emphasizes the importance of reinforcing influenza virus surveillance in pigs in China.

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Swine influenza is an economically important respiratory disease of swine resulting from infection with influenza A virus. Currently, three predominant subtypes of influenza virus 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 [1, 28]. Subtype H1N7, H4N6, H9N2, and H3N3 viruses have also been isolated sporadically from pigs but have not become established [1, 15, 16, 25]. Therefore, although aquatic birds are generally believed to be the principal reservoirs for influenza A virus, with sixteen HA and nine NA avian-derived subtypes so far been reported [6], pigs may also play an important role in the evolution and ecology of influenza A virus [1, 29]. Due to their susceptibility to both avian and human viruses, pigs have

been postulated to serve as an intermediate host for the interspecies transmission of avian influenza viruses to humans or as mixing vessels for the generation of human, avian, and/or swine 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 [1, 16].

H1N2 isolates from pigs have been previously demonstrated in Japan from 1978 to 1992 [12, 18, 24], in France from 1987 to 1988 [7], in the United Kingdom since 1994 [2, 3], and in the United States since 1999 [14, 28]. Antigenic and/or genetic characterization indicated that the viruses from Japan and France were reassortants derived from classical (Japan) or avian-like (France) swine H1N1 and human-like H3N2 viruses. In contrast, H1N2 viruses originally described in the United Kingdom but which have since spread to pigs in continental Europe after 1994, resulted from multiple reassortment events initially involving human H1N1 and H3N2 viruses, followed by reassortment with avian-like swine viruses [3]. H1N2 viruses first reported in the United States and transmitted to pigs in South Korea in 2003 were the result of reassortment between co-circulating classical H1N1 and swine-human-avian triple reassortant H3N2 viruses [13]. Viruses of similar genotype were thereafter recovered from turkey and wild duck in North America [23]. The French H1N2 viruses have not been found since their initial isolation, but those in Japan, the UK, and the US became widely distributed among the pig populations of those countries and have often been associated with respiratory disease and even (in the US) with abortions among sows. In this study, three H1N2 viruses were isolated in 2004 from pigs suffering from respiratory disease on a farm in southeastern China. One isolate has been subjected to genetic characterization and phylogenetic analysis in order to determine its genetic origin.

In late November 2004, a severe outbreak of respiratory disease occurred on a pig farm with 100 breeding sows in Zhejiang province in southeastern China, causing five sows to abort and causing the death of twenty finishing pigs over a six-week period. Clinical signs in pigs included temperature up to 42 °C, lack of appetite, lethargy and coughing. This herd had been vaccinated for porcine reproductive and respiratory syndrome (PRRS) but not for swine influenza. Three viruses with hemagglutination activity were isolated from lung samples taken from three dead 3-month-old pigs using both embryonic eggs and MDCK cells, and genetic analyses were conducted on the MDCK cell isolates.

The subtype of the isolates was identified using two multiplex RT-PCR assays described by Choi et al. [5] and subsequently, sequencing. Amplified H1 (1006 bp) and N2 (502 bp) bands, but not H3 (663 bp) and N1 (754 bp) bands, were detected from the three isolates by 0.8% agarose electrophoresis. When BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for homology, the nucleotide sequence of the RT-PCR products showed the highest degree of similarity with the HA sequences of the swine H1N1 subtype (95%) and with NA sequences of the human H3N2 subtype (96%), respectively. In addition, the nucleotide similarities of H1 and N2 among the three isolates were 99–100% and

100%, respectively. Therefore, the three isolates were determined to belong to the H1N2 subtype, and may have originated from identical progenitor viruses.

A two-step RT-PCR assay, using a set of universal primers developed by Hoffmann et al. [10], was performed to amplify the entire genomic sequences of the isolates. Since genome sequencing ultimately revealed the three isolates to be virtually identical genetically, isolate A/Swine/Zhejiang/1/2004 (Sw/ZJ/1/2004) was selected hereafter as representing all three and subjected to further genetic analysis. The sequences of other two isolates are not shown in this paper. The nucleotide sequences of the entire genome of Sw/ZJ/1/2004 obtained from this study were deposited in GenBank under accession numbers DQ139320 to DQ139327. The genetic origin of Sw/ZJ/1/2004 was initially inferred from BLAST analysis and pairwise comparisons of each gene segment to the corresponding sequences of reference viruses. Since many of the reference sequences available in GenBank are only partial, the sequence of each Sw/ZJ/1/2004 gene was selected for similarity comparisons. Sequence comparisons revealed that the NA gene of Sw/ZJ/1/2004 was most closely related to the corresponding gene from a human H3N2 influenza virus, A/Nanchang/933/95, circulating in 1995 (95.9% sequence similarity), while the other seven genes had the highest sequence similarity with genes of the classical swine H1N1 viruses circulating in China in 1993 and 1994 (93.3% for PB1 gene to 97.6% for M gene) (Table 1). These results demonstrated that Sw/ZJ/1/2004 most likely arose from the reassortment between the classical swine H1N1 viruses and the human-like H3N2 viruses.

To characterize more precisely the genetic origin of the gene segments of Sw/ZJ/1/2004, we constructed phylogenetic trees using the majority of the sequence of NA, NP, M, and NS, a portion of the PB2, PB1, and PA, and the entire HA1 region of the HA gene. In the HA1 tree, the HA1 gene of Sw/ZJ/1/2004

Table 1. Genetic similarity between A/Swine/Zhejiang/1/2004 and reference strains available in GenBank

Gene	Region compared (nt)[a]	Virus with the greatest similarity[b]	Lineage	Similarity (%)
PB2	977–1304	A/Swine/Hong Kong/273/94(H1N1)[U48287]	Swine	93.9
PB1	1251–1490	A/Swine/Hong Kong/273/94(H1N1)[U48282]	Swine	93.3
PA	27–362	A/Swine/Hong Kong/273/94(H1N1)[U48851]	Swine	94.6
HA	84–1064	A/Swine/Hong Kong/273/94(H1N1)[U45452]	Swine	94.6
		A/Swine/Hong Kong/172/93(H1N1)[U45451]	Swine	94.5
NP	45–1460	A/Swine/Hong Kong/273/94(H1N1)[U49092]	Swine	96.3
NA	20–1402	A/Nanchang/933/95(H3N2)[AJ457945]	Human	95.7
M	26–975	A/Swine/Hong Kong/273/94(H1N1)[U49115]	Swine	97.6
NS	27–846	A/Swine/Hong Kong/273/94(H1N1)[U49490]	Swine	96.1

[a] *nt* nucleotide

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

clustered with contemporary classical swine H1N1 viruses and H1N2 viruses found in North America. Phylogenetic analysis of the NA gene showed that Sw/ZJ/1/2004 clustered with human H3N2 viruses circulating in the mid-1990s (Fig. 1). All six internal genes of Sw/ZJ/1/2004 belonged to the classical swine H1N1 virus lineage (data not shown). Early studies showed that Swine H1N2 viruses from Europe [1, 7] and North America [14] contained the gene segments

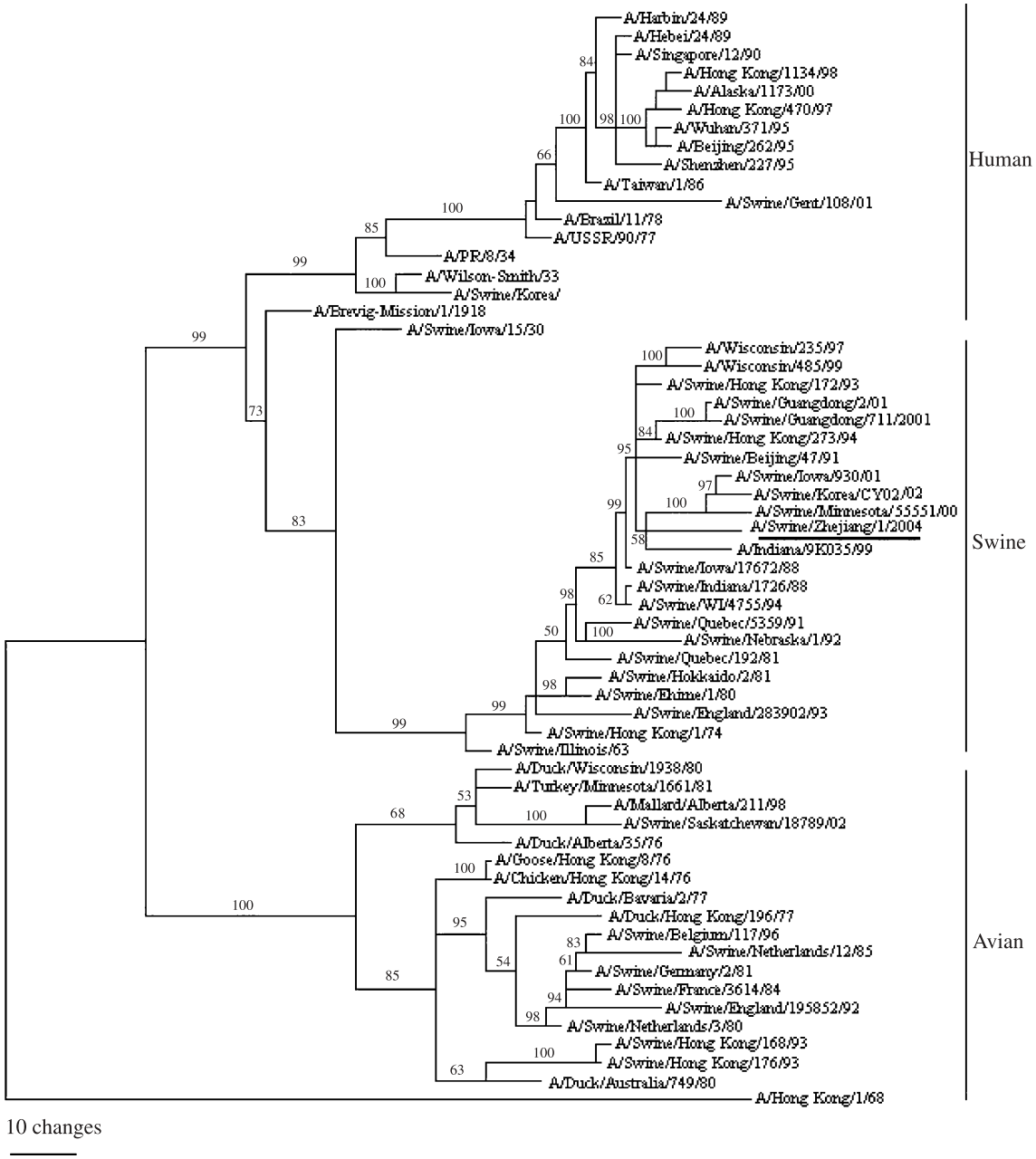

Fig. 1 (*continued*)

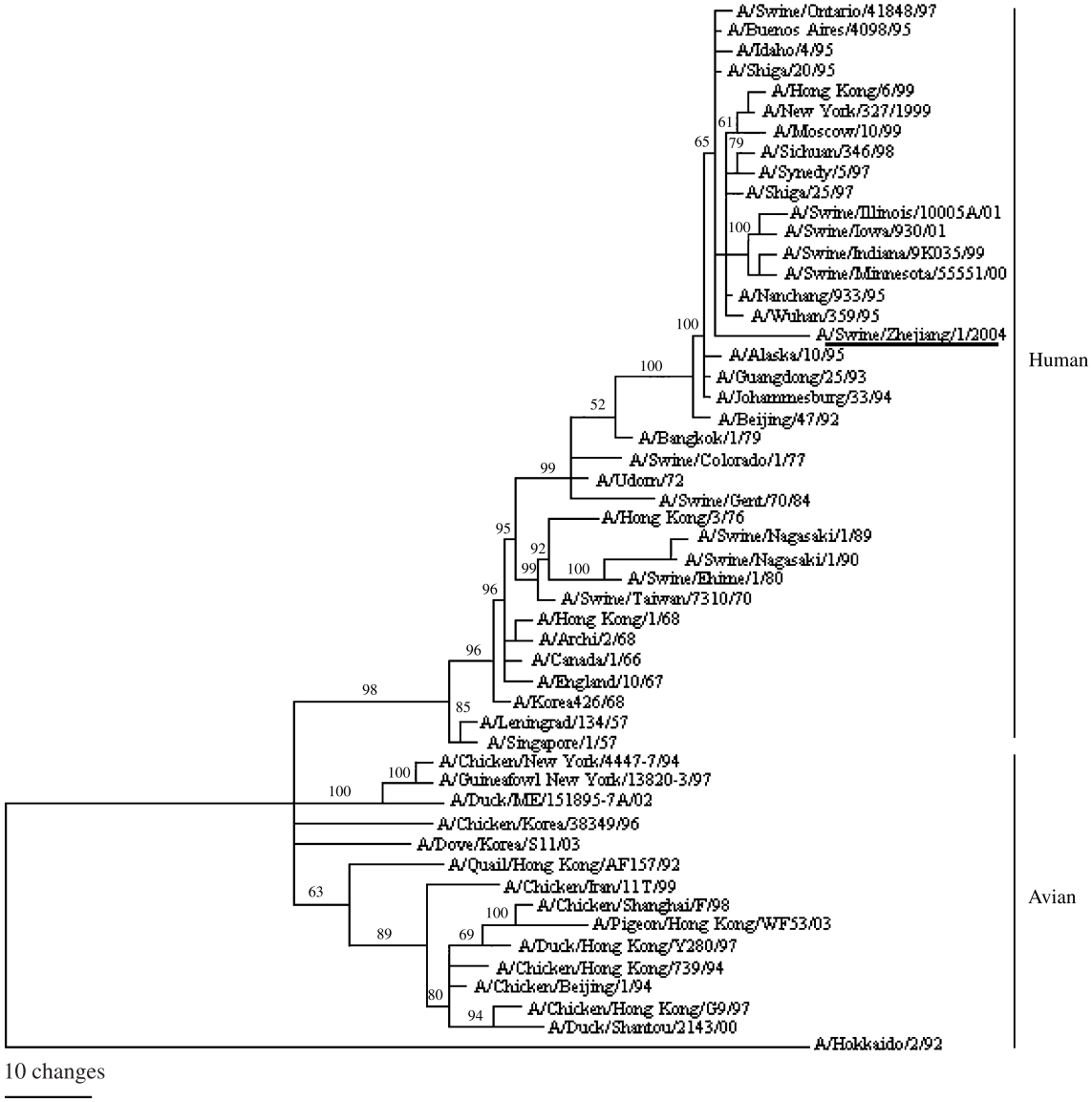

Fig. 1. Phylogenetec trees for the HA1 and the NA gene of A/Swine/Zhejiang/1/2004 and related reference viruses. The evolutionary relationships among these viruses were estimated by the method of maximum parsimony (PAUP software version 4.0b6; David Swofford, Smithsonian Institution, and Sinauer Associates) by using a bootstrap resampling method (500 replications) with a fast heuristic search algorithm, and gaps were treated as "missing". Sequence comparisons to reference viruses were performed by using DNASTAR software (version 4.0 for Win32). Alignments of each influenza virus sequence were created using program ClustalX 1.83. The numbers at the nodes of the phylograms indicate the bootstrap confidence levels. Horizontal line distances are proportional to the minimum numbers of nucleotide changes needed to join nodes and gene sequences. The vertical lines are present simply to space the branches and labels. The sequence regions compared were as follows: HA1 (981 bp), residues 84 to 1064; NA (1383 bp), residues 20 to 1402. The HA tree is rooted to A/Hong Kong/1/68 (H3N2), NA gene tree is rooted to B/Yamagata/16/88

of avian viruses. In contrast, Sw/ZJ/1/2004 resembled swine H1N2 viruses isolated in Japan and most likely originated from reassortment of classical swine H1N1 and human-like H3N2 viruses. The question then is whether the Chinese swine H1N2 viruses are descendants of the Japanese entities or products of the independent genetic reassortment of swine H1N1 and human H3N2 viruses in China. Phylogenetic analyses of HA1 and NA genes, and nucleotide sequence comparison of HA1 genes, supported the second possibility. According to the HA tree, Sw/ZJ/1/2004 and A/Swine/Ehime/1/80 (the H1N2 virus from Japan) are located in distinct clades. Similarly, in the NA tree, the NA gene of A/Sw/ZJ/1/2004 is only distantly related to the corresponding genes of Japanese swine H1N2 viruses (Fig. 1). Furthermore, the sequence similarities of the HA1 and the NA genes were relatively low (89.3% and 84.5%, respectively) between Sw/ZJ/1/2004 and A/Swine/Ehime/1/80. Japanese swine H1N2 viruses have been shown to be reassortants of swine H1N1 and human H3N2 viruses co-circulating in Japan in the late 1970s [12, 18, 24]. Therefore, swine H1N2 viruses isolated in China are distinct from their Japanese counterparts that continue to circulate in Japanese pigs and have formed a stable lineage [12]. In addition, reassortant H1N2 viruses were isolated sporadically from humans in China in the early 1990s, but these viruses arose entirely from co-circulating human H1N1 viruses and H3N2 viruses at that time [9]. These findings further indicate that Sw/ZJ/1/2004 is probably derived from an independent reassortment event between swine H1N1 and human-like H3N2 viruses that were circulating in their natural hosts in China in the middle 1990s. Therefore, Sw/ZJ/1/2004 is genetically distinguishable from other H1N2 viruses in pigs worldwide. The HA protein is the major surface antigen of influenza virus and is subject to a high rate of mutation. It is well known that the HA sequence variation contributes to antigenic drift, and this property confers a selective advantage on the virus, allowing it to escape host immunity. Host immune pressure on influenza viruses is not so marked in pigs as in humans because of the continual availability of young pigs lacking protective immunity [1, 21], and antigenic drift among swine viruses is limited compared to that existing among human viruses. In this study, we also investigated putative amino acid changes in the HA1 protein of Sw/ZJ/1/2004 compared to four genetically related swine H1N1 viruses, A/Swine/Beijing/47/91 (Sw/BJ/47/91), A/Swine/Hong Kong/172/93 (Sw/HK/172/93), A/Swine/Hong Kong/273/94 (Sw/HK/273/94), and A/Swine/Guangdong/1/01 (Sw/GD/1/01), all of which were isolated in China from 1991 to 2001 (Fig. 2). Amino acid sequence comparison revealed that amino acid similarities between Sw/ZJ/1/2004 and the other four H1N1 viruses ranged between 91.2 and 93.0%, while high homologies (95.5 to 97.9% sequence similarities) were found among the four H1N1 viruses. When Sw/ZJ/1/2004 was compared to Sw/HK/172/93 and Sw/HK/273/94, 19- and 22-amino-acid differences, respectively, were detected in the HA1 protein sequence. Even more differences were found when Sw/ZJ/1/2004 was compared with Sw/BJ/47/91 and Sw/GD/1/01 (29 and 25 amino acids, respectively). Interestingly, these differences in amino acid composition between Sw/ZJ/1/2004 and the four swine H1N1 viruses included nine that were located in previously defined antigenic sites

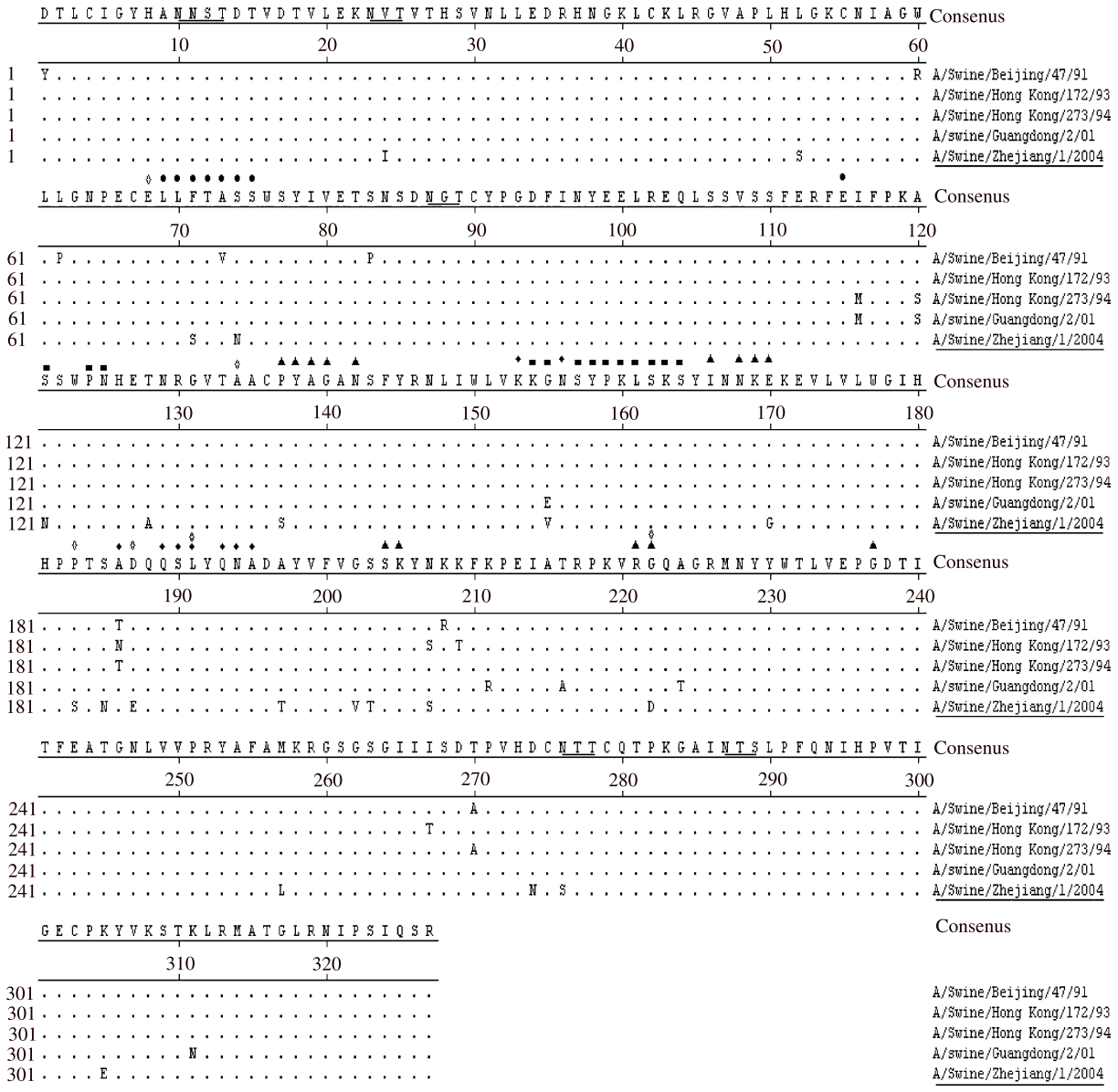

Fig. 2. Amino acid sequence comparison of the HA1 proteins of Sw/ZJ/1/2004 and four swine H1N1 viruses from China. Only the amino acids different from those in the consensus sequence are indicated. Numbering starts at the N terminus of HA1. Amino acid residues mapped in previously defined antigenic sites are indicated: site Sa (■), site Sb (◆), site Ca (▲), site Cb (●). Receptor-binding sites are shown as open diamonds (◊). Some of the receptor-binding residues are also in known antigenic sites: for these sites, symbols for both are shown. Underlined residues are potential glycosylation sites

[4, 22, 31] and three in receptor-binding sites [11, 17, 30] (one is also located at an antigenic site) of the HA1 protein (Table 2). Moreover, more changes were evident in the amino acid sequence of the HA1 protein of Sw/ZJ/1/2004 compared to the

Table 2. Amino acid changes in antigenic and receptor-binding sites of HA1 proteins of Sw/ZJ/1/2004 and four swine H1N1 viruses from China

Virus	Amino acid position in HA1 protein[a]										
	Antigenic site									Receptor-binding site	
	Site Sa		Site Sb	Site Cb			Site Ca				
	121	155	186	71	73	74	137	170	222[b]	183	187
Sw/BJ/47/91	S	G	T	F	V	S	P	E	G	P	D
Sw/HK/172/93	S	G	N	F	A	S	P	E	G	P	D
Sw/HK/273/94	S	G	T	F	A	S	P	E	G	P	D
Sw/GD/1/01	S	E	A	F	A	S	P	E	G	P	D
Sw/ZJ/1/2004	N	V	A	S	A	N	S	G	D	S	E

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

[b]Also in receptor-binding sites

four H1N1 viruses, which is inconsistent with the known low immune pressure in pigs. However, it is possible that Sw/ZJ/1/2004 appeared to be subjected to greater immunity pressure compared to the swine H1N1 viruses as a result of the NA gene introduced from human virus evolving intimately with the HA gene of swine origin. Since several changes in the amino acid sequence of the HA1 protein of Sw/ZJ/12004 were unique to antigenic and receptor-binding sites, this might offer the virus a selective advantage. 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 virus. Data indicated that avian viruses had fewer glycosylation sites than human and swine viruses because they are subject to less immunologic pressure [26]. Six potential glycosylation sites (Asn-X-Ser/Thr) were conserved at positions 10, 11, 23, 87, 276, and 287 in the HA1 protein of four swine H1N1 viruses, and five of them were conserved in the HA1 protein of the Sw/ZJ/1/2004 isolate (Fig. 2). Sw/ZJ/1/2004 lost one potential glycosylation site at position 276 owing to substitution of Ser for Asn at this position. HA1 proteins in all avian H1N1 viruses share four glycosylation site [11, 17]. The receptor-binding property of the HA protein of influenza virus is an important molecular determinant of host-range restrictions. Most avian influenza viruses preferentially bind to receptors in which sialic acid is linked to galactose by α2-3 linkage (SAα2-3Gal), whereas human viruses prefer the α2-6 linkage (SAα2-6Gal). A shift from SAα2-3Gal to SAα2-6Gal binding appears to be essential in the adaptation of an avian virus to the mammal host [26, 29]. Amino acid mutations at position 187 (Glu→Asp) (190 according to H3 number) are critical for adaptation of the avian HA to swine hosts [11, 17]. However, Sw/ZJ/1/2004 had Glu rather than Asp at position 187, which is typical of avian H1 virus and is postulated

to enhance binding to NeuAC 2, 3 Gal receptors. These results implied that Sw/ZJ/1/2004 probably had potential interspecies transmission from pigs to birds. Furthermore, the effect of these amino acid changes on the genetic and receptor-binding characterization of the HA protein of Sw/ZJ/12004 remains to be further studied.

To our knowledge, this is the first reported isolation of natural reassortant H1N2 viruses from pigs in China. It was well documented that both classical swine H1N1 viruses and human H3N2 viruses were present in pigs in China [8, 19, 25, 27]. Early studies on influenza viruses from pigs in southern China from 1976 to1982 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 [27]. These reassortant viruses had not become established, and no any other reassortant viruses were found in pigs in China thereafter. The isolation of Sw/ZJ/1/2004 provides further evidence that pigs serve as a "mixing vessel" for the generation of new reassortant genotypes of influenza viruses.

Swine influenza virus is one of the major pathogens associated with swine respiratory disease. In this study, the application of PCR methodology detected porcine circovirus type II (PCV-II) but not PRRSV (data not shown). We cannot say whether other pathogens associated with pocine respiratory disease complex, such as pseudorabies virus (PRV), porcine respiratory corona virus (PRCV), *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae*, or *Bordetella bronchioseptica*, were present in the pigs on the farm where Sw/ZJ/1/2004 was isolated. Swine reproductive disorders were reported in association with reassortant swine H3N2 and H1N2 influenza viruses in North America [14, 28]. Abortions also occurred on the farm from which Sw/ZJ/1/2004 originated. Therefore, the virulence and pathogenesis (especially respiratory disease and abortigenic potentials) of Sw/ZJ/1/2004 need to be determined experimentally. Furthermore, whether the SW/ZJ/1/2004-like virus spread within the regional or national swine population of China remains to be further investigated.

Currently, the dominant influenza viruses circulating in European and North American pig populations have a variety of genes of avian origin [1, 28]. Interestingly, the 1957 and 1968 human pandemic viruses also contained genes of avian viruses [29]. These data showed that reassortant viruses containing genes of avian origin might have a selective advantage in mammals. Although no reassortant viruses derived from avian, swine, and/or human have yet been found in China, there is evidence suggesting that avian H1, H9, and H5 had been transmitted to pigs in southeastern China [8, 20, 25], the region regarded as an epicenter for the emergence of pandemic influenza viruses. Introduction of avian viruses into pigs co-infected with human H3N2 or swine H1N1 and H1N2 viruses provide a favorable opportunity for the generation of reassortants containing avian genes and would thereby pose a significant pandemic threat. This should raise concerns about the genetic evolution of influenza virus in pigs and reinforce influenza virus surveillance in pigs in China.

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