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Antigenic and genetic characterization of H9N2 swine influenza viruses in China

Yan L. Cong,[1] Juan Pu,[1] Qin F. Liu,[1] Shuai Wang,[1] Guo Z. Zhang,[1] Xing L. Zhang,[1] Wei X. Fan,[2] Earl G. Brown[3] and Jin H. Liu[1]

Correspondence
Jin H. Liu
ljh@cau.edu.cn

[1]Laboratory of Infectious Diseases, College of Veterinary Medicine, State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100094, PR China

[2]China Animal Health and Epidemiology Center, Qingdao 266032, PR China

[3]Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, ON, Canada

As pigs are susceptible to infection with both avian and human influenza A viruses, they have been proposed to be an intermediate host for the generation of pandemic virus through reassortment. Antigenic and genetic characterization was performed for five swine H9N2 influenza viruses isolated from diseased pigs from different farms. The haemagglutinin (HA) antigenicity of swine H9N2 viruses was different from that of chicken H9N2 viruses prevalent in northern China. Genetic analysis revealed that all five isolates had an RLSR motif at the cleavage site of HA, which was different from those of A/duck/Hong Kong/Y280/97 (Dk/HK/Y280/97)-like viruses established in chickens in China. Phylogenetic analyses indicated that the five swine H9N2 viruses formed novel HA and neuraminidase sublineages that were related closely to those of earlier chicken H9 viruses and were also consistent with the extent of the observed antigenic variation. The six internal genes of the isolates possessed H5N1-like sequences, indicating that they were reassortants of H9 and H5 viruses. The present results indicate that avian to porcine interspecies transmission of H9N2 viruses might have resulted in the generation of viruses with novel antigenic and genetic characteristics; therefore, surveillance of swine influenza should be given a high priority.

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INTRODUCTION

Influenza A viruses are classified into a number of subtypes based on antigenic differences in their two surface glycoproteins, haemagglutinin (HA; 16 subtypes) and neuraminidase (NA; nine subtypes) (Fouchier *et al.*, 2005; Webster *et al.*, 1992). The H9N2 subtype virus is a conspicuous member of the influenza family because it can infect not only chickens, ducks and pigs, but also humans (Guo *et al.*, 2000; Liu *et al.*, 2003; Peiris *et al.*, 1999; Xu *et al.*, 2004). In China, the H9N2 virus was first isolated from a chicken in 1992 in Guangdong province (Chen *et al.*, 1994) and is now the most prevalent subtype of influenza virus in poultry in China. Recent studies have shown that the H9N2 viruses can infect pigs and cause significant morbidity and mortality (Xu *et al.*, 2004). However, the detailed characteristics of the viruses, such as antigenicity and phylogenetic properties, are not well defined.

Pigs are thought to be susceptible to infection with both avian and human influenza A viruses because the cells of their respiratory tract express both sialic acid-α2,3-galactose (SAα2,3Gal) receptors, preferred by avian influenza viruses, and sialic acid-α2,6-galactose (SAα2,6Gal) receptors, preferred by human influenza viruses (Ito *et al.*, 1998). Thus, pigs are proposed to be 'mixing vessels' for the generation of reassortant influenza viruses with pandemic potential (Brown *et al.*, 1998; Castrucci *et al.*, 1993; Scholtissek *et al.*, 1985; Webster *et al.*, 1992). The broad susceptibility of pigs to many influenza viruses emphasizes the importance of surveillance of swine influenza viruses as possible sources of pandemic influenza. This concern is heightened by the presence of not only H1N1 and H3N2 strains from human sources, but also avian influenza virus strains, in both American and Eurasian herds of pigs (Brown, 2000). Coinfections have allowed intermixing of these genomes to produce triple reassortants with genes derived from human, swine and avian influenza strains (Olsen *et al.*, 2006). As porcine viruses become more human-like as well as more avian-like, the probability of generating human-adaptive viruses increases. Here, we describe the genetic composition of swine influenza strains in China that comprise avian H9N2-like and H5N1-like genome segments.

The GenBank/DDBJ/EMBL accession numbers for the nucleotide sequences of the H9N2 influenza viruses analysed in this study are DQ981591–DQ981630.

Swine influenza outbreaks occurred in Shandong province, China, in 2003. At that time, most of the diseased pigs showed typical respiratory signs, such as fever, nasal and ocular discharge, coughing and dyspnoea; however, paralysis associated with fatal disease was also observed. Laboratory diagnosis and virus isolation demonstrated that the disease resulted from infection with subtype H9N2 influenza viruses. To elucidate the antigenic and genetic characteristics of these viruses and the relationship of the swine H9N2 viruses with avian influenza viruses, we analysed five H9N2 swine influenza virus isolates antigenically and genetically.

METHODS

Viruses. Nasal swabs and lung samples were collected from diseased and/or dead swine. Initial isolation of viruses from the samples was performed in 10-day-old specific-pathogen-free embryonated chicken eggs (ECE). Subtype identification of these viruses was performed by standard haemagglutination-inhibition (HI) tests and NA-inhibition tests with a panel of reference antisera recommended by the World Health Organization (http://www.who.int/csr/resources/publications/en/#influenza). Allantoic fluids were harvested for ECE-passaged viruses and used as stock for sequence analysis. The following reference avian influenza viruses were employed for HI analysis: A/quail/Hong Kong/G1/1997 (H9N2) (Qa/HK/G1/97), A/chicken/Hong Kong/G9/1997 (H9N2) (Ck/HK/G9/97) and A/turkey/Wisconsin/1966 (H9N2) (Ty/WI/66), kindly provided by Dr H. Kida, Graduate School of Veterinary Medicine, Hokkaido University. The five virus isolates obtained in this study were named as follows: A/swine/Shandong/FHZ/03 (Sw/SD/FHZ/03), A/swine/Shandong/FJN/03 (Sw/SD/FJN/03), A/swine/Shandong/FLS/03 (Sw/SD/FLS/03), A/swine/Shandong/FNY/03 (Sw/SD/FNY/03) and A/swine/Shandong/FZC/03 (Sw/SD/FZC/03).

Antisera and HI. The viruses were analysed antigenically by HI testing using a panel of chicken hyperimmune antisera. Chicken hyperimmune sera against Qa/HK/G1/97 and Ck/HK/G9/97 were provided by Dr H. Kida. Chicken hyperimmune sera against A/chicken/Hebei/1/1996 (H9N2) (Ck/HB/1/96) and Sw/SD/FJN/03 were prepared in our laboratory. HI tests were performed as described previously (Kendal *et al.*, 1982).

Gene sequencing and phylogenetic analysis. Viral RNA was extracted from allantoic fluids by using TRIzol reagents (Gibco-BRL) and reverse transcription was performed by using oligonucleotide influenza universal primer Uni12: 5′-AGCAAAAGCAGG-3′ (Hoffmann *et al.*, 2001). After reverse transcription, PCR was done as described by Shu *et al.* (1994), using primers (sequences available on request) specific for each of the eight RNA segments. PCR products were purified with a QIAquick PCR purification kit (Qiagen). The purified PCR products were then partially sequenced by using an Amersham ET Dye terminator kit and analysed with an ABI 3730 DNA sequencer (Perkin-Elmer Applied Biosystems).

Assembly of sequences, translation of nucleotide sequences into protein sequences, and initial multiple sequence alignments were performed with the CLUSTAL_V method using MegAlign software version 1.03 (DNAStar Inc.). The phylogenetic relationships were estimated from the nucleotide sequences of each H9N2 swine influenza viral gene relative to selected H9N2 and H5N1 subtype influenza reference strains obtained from GenBank (using the PHYLIP software package; http://clustalw.ddbj.nig.ac.jp/top-e.html). The phylogenetic tree was drawn by using TreeView (version 1.40; Page, 1996). In this study, the nucleotide sequences used for the phylogenetic analysis are as follows: PB2 (nt 1342–2172), PB1 (nt 1140–1762), PA (nt 761–1215), HA (nt 172–1185), NP (nt 1120–1444), NA (nt 67–1398), M (nt 81–560) and NS (nt 18–787).

RESULTS

Antigenic analysis

To investigate the antigenic properties of the swine H9N2 virus isolates from 2003, we performed HI tests with a panel of anti-H9 hyperimmune sera (Table 1). Antigenic analysis demonstrated a diversity of reaction patterns generally corresponding with the phylogenetic relationships determined by sequence comparisons (see 'Phylogenetic analysis' below). All of the tested H9N2 swine viruses reacted well with antiserum of Sw/SD/FJN/03; however, they showed moderate or low reactivity to antisera of Ck/HK/G9/97 and Ck/HB/1/96, indicating that the swine viruses were related to but distinct from the avian

Table 1. Antigenic analysis of H9N2 influenza viruses by HI titre

Abbreviations: Ck, chicken; Qa, quail; Sw, swine; Ty, turkey; HB, Hebei; HK, Hong Kong; SD, Shandong; WI, Wisconsin. Homologous titres are indicated in bold.

Virus	Antisera			
	Qa/HK/G1/97	Ck/HK/G9/97	Ck/HB/1/96	Sw/SD/FJN/03
Sw/SD/FHZ/03	80	640	160	2560
Sw/SD/FJN/03	80	640	320	**2560**
Sw/SD/FLS/03	<20	640	320	1280
Sw/SD/FNY/03	<20	320	160	1280
Sw/SD/FZC/03	80	1280	320	5120
Ck/SD/98	80	5120	2560	640
Ck/HB/1/96	40	5120	**2560**	640
Ty/WI/66	<20	80	40	80
Ck/HK/G9/97	160	**10 240**	2560	640
Qa/HK/G1/97	**1280**	160	80	80

isolates. These H9N2 swine viruses revealed little cross-reaction to antiserum of Qa/HK/G1/97, which is seldom found in the chicken population of mainland China. The present findings indicated that the HAs of prevalent swine H9N2 viruses were distinct from those of H9N2 viruses maintained in chickens in Shandong province in China.

Molecular analysis

To try to identify the possible determinants of interspecies transmission of H9N2 influenza viruses from birds to pigs, the deduced amino acid sequences of viral proteins were analysed. We determined the nucleotide sequences (nt 76–1617, 1542 bp) of the five H9 HAs of swine influenza virus isolates in the present study. Remarkably, amino acid sequences at the cleavage site of the HAs of five Shandong isolates possessed an R**L**SR motif, which had an amino acid difference from the RSSR sequence of Qa/HK/G1/97-like and Dk/HK/Y280/97-like viruses (Table 2), established in Asia. It is not clear whether this amino acid sequence motif at the cleavage site is related to infection, pathogenicity and/or tissue tropism. Amino acids at the receptor-binding sites of HA are associated with differences in receptor-binding specificity (Weis *et al.*, 1988). Table 2 also shows the amino acids at positions 183, 190, 226, 227 and 228, which are components of receptor-binding sites of the HAs of H9N2 viruses (Peiris *et al.*, 2001). All of the five swine isolates possessed asparagine at amino acid position 183 (numbered as for H3 HA), as observed in Dk/HK/Y280/97-like viruses prevalent in chickens in mainland China. Four of the isolates, excluding Sw/SD/FNY/03, had leucine at position 226, which is thought to confer receptor specificity similar to that of human H3N2 viruses (Matrosovich *et al.*, 2001). At position 190, alanine or threonine was observed in the five isolates, and alanine seemed to be more prevalent in recent isolates.

The amino acid residues at the antigenic sites on the globular head of the HA molecule were also analysed. All of the swine viruses had distinctive H58R and V205A mutations that are not seen in A/chicken/Shandong/1998 (Ck/SD/98)-like H9N2 viruses. Interestingly, two sites contained D369E and N488S mutations in HA2, a highly conserved region of HA.

It has been considered that the glycosylation sites might affect the receptor-binding capacity of HA (Ohuchi *et al.*, 1997). Analysis of the potential glycosylation sites in the HAs of the five H9N2 virus isolates revealed seven

Table 2. Amino acids at the cleavage site and the receptor-binding sites of HA of H9N2 viruses

Virus*	Amino acid sequence at cleavage site†	Amino acid residue‡				
		183	190	226	227	228
Ck/BJ/1/94	PARSSR/GL	N	V	Q	Q	G
Ty/WI/66	PA**V**SSR/GL	H	E	–	–	–
Dk/HK/Y439/97	PA**A**S**N**R/GL	H	E	–	–	–
Qa/HK/G1/97	PARSSR/GL	H	E	L	–	–
Ck/HK/G9/97	PARSSR/GL	–	A	L	–	–
Dk/HK/Y280/97	PARSSR/GL	–	T	L	–	–
Ck/SD/98	PARSSR/GL	–	–	L	–	–
Sw/HK/10/98	PARSSR/GL	–	–	L	H	–
GZ/333/99	PARSSR/GL	–	–	M	–	–
Ck/HLJ/48/01	PARSSR/GL	–	A	L	–	–
Ck/HN/43/02	PARSSR/GL	–	A	L	–	–
Sw/SD/na/03	PARSSR/GL	–	G	–	–	–
Dk/HuB/W1/04	PARSSR/GL	–	–	–	–	–
Dk/SH/08/05	PARSSR/GL	–	A	L	–	–
Sw/SD/nc/05	PARSSR/GL	–	A	–	–	–
Sw/SD/FNY/03	P**G**R**L**SR/GL	–	T	–	–	–
Sw/SD/FHZ/03	PAR**L**SR/GL	–	A	L	–	–
Sw/SD/FJN/03	PAR**L**SR/GL	–	A	L	–	–
Sw/SD/FZC/03	PAR**L**SR/GL	–	A	L	–	–
Sw/SD/FLS/03	PAR**L**SR/GL	–	A	L	–	–

*Ck, chicken; Dk, duck; Qa, quail; Sw, swine; Ty, turkey; BJ, Beijing; GZ, Guangzhou; HLJ, Heilongjiang; HN, Henan; HK, Hong Kong; HuB, Hubei; SD, Shandong; SH, Shanghai; WI, Wisconsin.

†Residues differing from those of Ck/BJ/1/94 are indicated in bold.

‡Numbered according to H3 HA numbering. Amino acids at positions H183, E190, Q226, Q227 and G228 of the receptor-binding sites were considered conserved at these positions in the avian virus consensus sequence. Dashes indicate residues identical to Ck/BJ/1/94.

common sites with the NXT/S motif (in which X may be any amino acid except proline) in the sequenced regions of HA genes; five were located in the HA1 portion and two in the HA2 portion of the molecule. The potential glycosylation sites of the five isolates (N11, 123, 200, 280, 287, 474 and 533, respectively) were the same as those of Dk/HK/Y280/97-like viruses.

As shown in Fig. 1, the NA of the five isolates had a deletion of 3 aa at positions 63–65 in the stalk of the protein compared with Ck/HK/G9/97, and lacked the 2 aa deletion at position 38 and 39 seen in Qa/HK/G1/97-like viruses. Although the functional relevance of the observed deletion in the NA of the H9N2 viruses is not known, the deletion will serve as a useful marker for this lineage of H9N2 viruses in China.

Phylogenetic analysis

To determine the evolutionary relationships of swine H9N2 viruses prevalent in Shandong province more fully, HA gene sequences were compared with those of other H9N2 viruses isolated from poultry and pigs in Shandong province and strains in GenBank. Phylogenetic analysis of the H9 HAs showed that four distinct H9N2 virus sublineages have been maintained in chicken populations in mainland China. The four sublineages are represented by A/chicken/Beijing/1/94 (Ck/BJ/1/94), A/chicken/Shanghai/F/98 (Ck/SH/F/98), Dk/HK/Y280/97 and A/Swine/Shandong/1/2003 (Sw/SD/1/03) (Fig. 2a). All of the swine H9N2 viruses tested in this study belonged to the same sublineage as Sw/SD/1/03 and, as a group, formed a lineage (Sw/03-like) distinct from other swine viruses in the Ck/SH/F/98 lineage, such as Sw/SD/na/03 and relatives. A previous chicken isolate, Ck/SD/98, located in the root of the Sw/03-like sublineage, indicated that the HA genes of the swine H9 influenza viruses were derived from the early chicken H9 virus identified in 1998 and, in particular, revealed that the swine H9N2 viruses are related closely to Ck/SD/98. Ck/SD/98 is a relative of both Hong Kong and mainland China swine viruses, suggesting that this virus was a bridging strain between viruses present in these two geographical locations and raising the possibility of bidirectional transmission between chickens and pigs.

The phylogeny of the NA genes paralleled that of the HA genes where the five swine isolates belonged to the same sublineage, which had a sister-group relationship with the NA gene of Ck/SD/98 (Fig. 2b).

The results of previous surveillance and the published data in GenBank revealed that H9N2 influenza viruses were cocirculating with H5N1 influenza viruses during recent years, raising the possibility of genetic exchange between these viruses. Phylogenetic analysis of the internal genes of the swine H9N2 viruses showed that reassortants existed, indicating a comparatively complex genetic relationship among them. The evolutionary trees revealed that each of the internal genes of Sw/SD/FJN/03 belonged to a sublineage or a sister sublineage to that of Sw/SD/2/03, an H5N1 virus cocirculating in the pig population (Fig. 2c–h). PB2 genes, except for that of Sw/SD/FJN/03, had a close relationship with those of H9N2 viruses (Fig. 2c), whereas the PB1 gene of Sw/SD/FJN/03 was located in the same sublineage as that of Ck/SD/98 (H9N2), and the other swine isolates had a close relationship with an H5N1 virus, Dk/SH/35/02 (Fig. 2d). The acidic polymerase (PA) genes of Sw/SD/FNY/03 and Sw/SD/FLS/03 formed a sublineage with those of H9N2 viruses, whereas the remaining three isolates belonged to different sublineages (Fig. 2e), indicating that the PA genes of the tested viruses were intricately derived from multiple virus sources. The nucleoprotein (NP) genes of all five isolates had a close relationship with those of H5N1 viruses maintained in mainland China (Fig. 2f). For the matrix (M) and non-structural (NS) genes, four of the five isolates formed a sublineage together with H9N2 viruses, whilst Sw/SD/FJN/03 was a member of another sublineage with an H5N1 virus, Sw/SD/2/03 (Fig. 2g–h).

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Ck/HK/G9/97          31  TTMTLHFKQNECINSSNNQVVPCEPIIIERNITEIVHLNSTTLEKEICPKVADYRNWSKP  90
Qa/HK/G1/97          31  .......**...T.P....A.....................N..I...S.....E.K.....  88
HK/1073/99           31  .......**...T.P....A.....................N..I...S.....E.K.....  88
Dk/HK//Y439/97       31  ..V.........SIP.........................N..I......G.LE.......  90
Ck/BJ/1/94           31  ............S.P..............***.........I.........E.K.....  87
Dk/HK/Y280/97        31  ............S.P..............***.........I.........E.K.....  87
SG/408/98            31  ............S.P..............***.........I.........E.K.....  87
Sw/HK/9/98           31  .N..........S.P..............***.........I.........E.K.....  87
Sw/SD/FNY/03         31  M...........S.P..............***.........I.........E.K.....  87
Sw/SD/FHZ/03         31  M...........S.P..............***.........I.........E.K.....  87
Sw/SD/FJN/03         31  M...........S.P..............***.........I.........E.K.....  87
Sw/SD/FZC/03         31  M...........S.P..............***.........I.........E.K.....  87
Sw/SD/FLS/03         31  M...........S.P..............***.........I.........E.K.....  87
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Fig. 1. Deletion of amino acids in the stalk of the NAs of H9N2 viruses. Asterisks indicate the positions of deleted amino acids. Dots indicate residues identical to those of the Ck/HK/G9/97 virus. Ck, chicken; Dk, duck; Qa, quail; Sw, swine; BJ, Beijing; HK, Hong Kong; SD, Shandong; SG, Shaoguan.

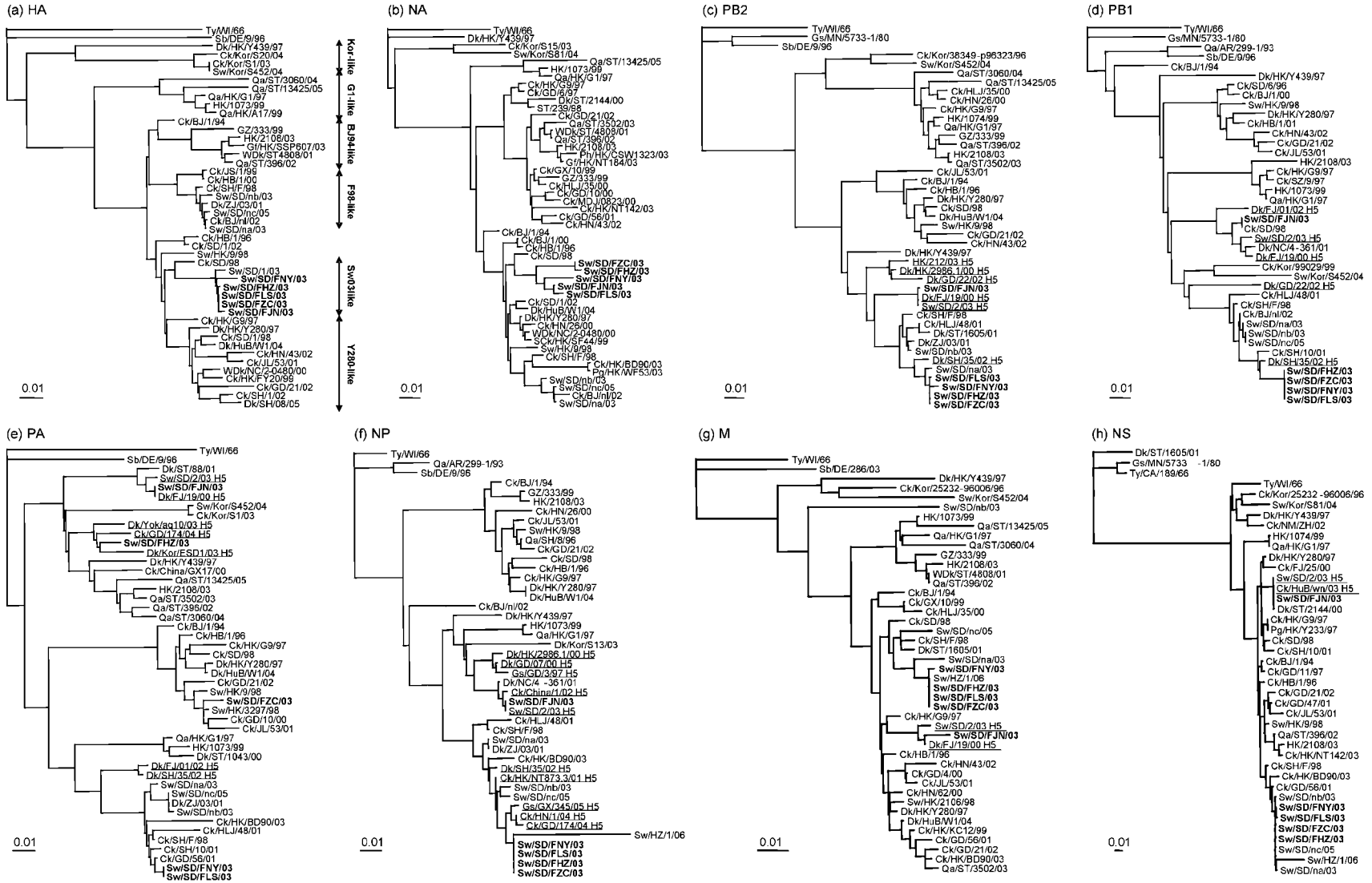

Fig. 2. Phylogenetic trees of eight gene segments of influenza viruses. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Vertical distances are for spacing branches and labels. Viruses isolated in the present study are labelled in bold and H5 viruses are underlined. Ck, chicken; Dk, duck; Gf, guinea fowl; Gs, goose; Pg, pigeon; Ph, pheasant; Qa, quail; SCk, silky chicken; Sb, shorebird; Sw, swine; Ty, turkey; WDk, wild duck; AR, Arkansas; BJ, Beijing; CA, California; DE, Delaware; FJ, Fujian; GD, Guangdong; GX, Guangxi; GZ, Guangzhou; HB, Hebei; HK, Hong Kong; HLJ, Heilongjiang; HN, Henan; HuB, Hubei; HZ, Hangzhou; JL, Jilin; JS, Jiangsu; Kor, Korea; MDJ, Mudanjiang; MN, Minnesota; NC, Nanchang; NM, Neimeng; SD, Shandong; SH, Shanghai; ST, Shantou; SZ, Shenzhen; WI, Wisconsin; Yok, Yokohama; ZJ, Zhejiang.

DISCUSSION

Infection of pigs with H1 and H3 subtype influenza viruses has occurred frequently in Eurasia (Brown, 2000, 2001; Marozin *et al.*, 2002; Reeth *et al.*, 2004). Although instances of H9N2 avian-like viruses in pigs have occurred, interspecies transmission of avian viruses to pigs has not often been documented (Peiris *et al.*, 2001; Xu *et al.*, 2004). Seropositivity to H4, H5 and H9 viruses has been reported among pigs in China (Ninomiya *et al.*, 2002). Peiris *et al.* (2001) were the first to confirm that cocirculation of avian H9N2 and contemporary human H3N2 viruses in pigs had occurred in south-eastern China and predicted that the cocirculation of H9 and H3 viruses in pigs would provide an opportunity for genetic reassortment, leading to the emergence of viruses with pandemic potential. Xu *et al.* (2004) reported that the Sw/SD/1/2003 (H9N2) caused pig disease and death in clinics, and deduced that it probably originated from a reassortant of chicken influenza virus subtype H5N1 and H9N2 subtypes from both chickens and ducks. As pigs can serve as mixing vessels for the reassortment of human and avian influenza viruses, swine influenza infections have been the focus of increasing attention.

In 2003, a characteristic respiratory syndrome in pigs was observed on many pig farms in Shandong province. The diseased pigs showed coughing, ocular discharge, gored skin and high fever. Gross morphology showed swollen, haemorrhagic lungs and tracheas filled with froth. The H9N2 infections in the pigs were diagnosed by virus isolation and serological testing. Although these viruses have caused clinical incidences of disease, genetic analysis showed that these viruses had the characteristic of low-pathogenicity avian influenza virus, for example, the low-pathogenicity sequence motif RLSR at the cleavage site of HA1 and HA2. It is noteworthy that most of the H9N2 influenza viruses persisting in chicken populations in mainland China have the RSSR HA cleavage-site motif. The significance of the S⟶L mutation of the swine influenza viruses at the cleavage site of HA should be assessed further by reverse genetics and animal experiments.

The host range of influenza viruses is associated with different amino acids within and around the HA receptor-binding pocket (Rogers *et al.*, 1983). In the present study, four of the five swine isolates possessed amino acids N, A, and L within the receptor-binding sites of HA at positions 183, 190, and 226, respectively, with Sw/SD/FNY/03 possessing N, T, and Q. Leucine at position 226 is typical of the sequences found in human H2 and H3 isolates, but not in avian viruses (Matrosovich *et al.*, 2000). H3 HA possessing L226 binds to the NeuAcα2,6Gal linkage, whereas that possessing Q226 binds to the NeuAcα2,3Gal linkage (Rogers *et al.*, 1983). It has been shown that avian H9N2 viruses possessing L226 bind preferentially to the NeuAcα2,6Gal linkage, whereas those possessing Q226 bind to the NeuAcα2,3Gal linkage (Matrosovich *et al.*, 2001). These studies also showed that human H9N2 virus possessed L226 and bound preferentially to the α2,6 linkage. The substitution from Q226 to L226 of the H9N2 viruses might be one of the genetic changes that occurred during the adaptation of avian strains to pigs.

At least six different genotypes of H9N2 influenza viruses have been recognized in south-eastern China (Choi *et al.*, 2004). In the present study, phylogenetic analysis of the H9 HAs showed that four distinct H9N2 virus sublineages were maintained in the chicken population in mainland China (Fig. 2a). The five swine H9N2 viruses analysed in this study formed a novel sublineage, corresponding with the antigenic variation. In particular, the previous chicken isolate Ck/SD/98 has a close relationship with these isolates, indicating that the HA and NA genes of the swine H9 influenza viruses might be derived from Ck/SD/98 and further indicating that the swine H9N2 viruses might be transmitted from chickens. It is noteworthy that the six internal genes of Sw/SD/FJN/03 belonged to a sublineage or a sister sublineage of those of Sw/SD/2/03, an H5N1 virus cocirculating in pig populations (Fig. 2c–h). The NP genes of all of the five isolates had a close relationship with those of H5N1 viruses maintained in mainland China (Fig. 2f). The present findings indicated that the H9N2 viruses in the study were reassortants of H9 and H5 viruses. Although we do not know which viral factors are necessary for interspecies transmission, reassortment is likely to increase the chances of generating transmissible viruses. In southern China, at least 2 % of blood donors tested were positive for H9 antibodies (Butt *et al.*, 2005; Guo *et al.*, 1999; Nicholson *et al.*, 2003; Peiris *et al.*, 1999), suggesting that human infection with H9N2 occurs ubiquitously in this area. As H9N2 virus appears to have the potential to cross the species barrier to humans more efficiently than the current H5N1 virus as a result of its predicted affinity for NeuAcα2,6Gal receptors (Butt *et al.*, 2005; Ha *et al.*, 2001; Kaverin *et al.*, 2004; Li *et al.*, 2003; Matrosovich *et al.*, 2004), swine H9N2 viruses, especially reassortants of H9 and H5 viruses, should be highlighted as candidates for human pandemic influenza viruses.

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