Genetic relatedness between influenza A (H1N1) viruses isolated from humans and pigs

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Complete nucleotide sequences were obtained from the nucleoprotein genes of three influenza A viruses and partial nucleotide sequences were obtained from the polymerase, neuraminidase, matrix, and non-structural protein genes of four influenza A viruses that had been isolated between 1931 and 1939 from clinically sick pigs in the United States or Europe. A phylogenetic analysis of the open reading frames of nine nucleoprotein genes showed that the U.S. swine influenza virus isolates from 1931 and 1937 originated from the classic swine viral nucleoprotein lineage, whereas the European swine strains from 1938 and 1939 were placed among the early human influenza virus nucleoprotein lineage. All the partial gene sequences obtained from the two European swine strains from 1938 and 1939 were also more closely related to early human H1N1 reference strains than to the U.S. swine isolates from 1931 and 1937, indicating that none of the four viruses isolated from swine had acquired genes from a heterologous lineage through reassortment.

Influenza A H1N1 viruses appeared simultaneously in humans and pigs in 1918 and have been circulating since then with an intermittent period in humans from 1957 to 1977 (Raymond *et al.*, 1986; Xu *et al.*, 1993). When influenza viruses were first isolated from pigs in 1930 and from humans in 1933, separate swine and human lineages were readily distinguishable by antigenic analysis.

Experimental infection of pigs with human H1N1 influenza viruses was found to produce very mild or no signs of disease (Shope & Francis, 1936), and human H1N1 influenza viruses have not been reported to cause outbreaks of swine influenza. From 1938 to 1940 however, influenza A viruses were isolated from clinically sick pigs during outbreaks of influenza-like illness in the British Isles. The disease, which affected piglets from 8 to 12 weeks old, was characterized by coughing, sneezing and pneumonia; however, no pyrexia was observed. Different mortality rates were reported from the outbreaks in Northern Ireland and England, and cases of chronic cough were observed after recovery from the acute disease (Lamont, 1938; Blakemore & Gledhill, 1941). The strains isolated belonged to the H1N1 subtype but formed a less compact group than the American swine influenza strains and were serologically rather different from them (Lamont, 1938; Blakemore & Gledhill, 1941; Blakemore *et al.*, 1941). The haemagglutinin genes of two strains from these outbreaks, sw38 (A/swine/Northern Ireland/38) and sw39 (A/swine/Cambridge/39), were more closely related both antigenically and genetically to human H1N1 influenza viruses than to classical strains of swine influenza viruses (Gompels, 1953; Jensen & Peterson, 1957; Meier-Ewert *et al.*, 1970; Neumeier & Meier-Ewert, 1992).

In order to investigate whether reassortment of genes other than the haemagglutinin gene could have been responsible for the increased virulence for pigs of the European swine influenza virus strains sw38 and sw39 we sequenced parts of their genomes. These were the nucleoprotein (NP) gene and approximately 200 nucleotides of the neuraminidase (NA), matrix (M), non-structural protein (NS), and polymerase (PB1, PB2, PA) genes. These sequences were compared with those of classic swine and early human influenza virus strains.

Sequencing of the H1N1 swine strains sw31 (A/swine/1976/31), sw37 (A/swine/29/37), sw38 and sw39 was carried out using the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977), modified to utilize total virion RNA, synthetic oligonucleotide primers and reverse transcriptase (Cox *et al.*, 1986; Rocha *et al.*, 1991). The oligonucleotides used to sequence the NP gene started at nucleotide 7, 176, 397, 643, 888 and 1127 respectively. Partial sequences of other genes were obtained with primers PB1-13, PB2-28, PB2-56, PA-38, PA-226, PA-282, NA-692, M-8 and NS-597 (the sequences of these oligonucleotides are available upon request).

The NP sequences of sw37, sw38 and sw39 are available from GenBank under accession numbers U04854, U04855 and U04856.

For sequence alignment and phylogenetic analysis of the NP genes, six additional sequences were selected: wsn33 (A/WSN/33) (Li *et al.*, 1989), pr834 (A/PR/8/34) (Winter & Fields, 1981), ussr77 (A/USSR/90/77) (Altmüller *et al.*, 1989), sw31 (A/swine/1976/31) (Gammelin *et al.*, 1989), sw82 (A/swine/Hong Kong/127/82) (Gammelin *et al.*, 1989) and pa73 (A/parrot/Ulster/73) (Steuler *et al.*, 1985).

The partial sequences of the PB1, PB2, PA, NA, M and NS genes of sw31, sw37, sw38 and sw39 were compared with the corresponding published gene sequences of pr834 (Baez *et al.*, 1980; Fields & Winter, 1982; Fields *et al.*, 1981; Winter & Fields, 1980, 1982), wsn33 (Buonagurio *et al.*, 1986; Hiti & Nayak, 1982; Kaptein & Nayak, 1982; Markusin *et al.*, 1988; Odagiri & Tobita, 1990; Sivasubramanian & Nayak, 1982) and sw30 (A/swine/Iowa/15/30) (Nakajima *et al.*, 1984).

For sequence analysis and alignment the University of Wisconsin Genetics Computer Group software package, version 7.0 was used (Devereux *et al.*, 1984). Phylogenetic analysis was carried out with the software package PHYLIP, version 3.4 (Felsenstein, 1989). Nucleotide sequence distance matrices were produced with DNADIST. Maximum parsimony NP trees were searched for with DNAPENNY and PROTPARS. Branch lengths of nucleotide and amino acid trees were calculated with FITCH. All phylogenetic trees that were generated from NP sequences with one of the PHYLIP programs were rooted in pa73. Phylogenies of partial gene sequences were estimated by maximum likelihood with DNAML.

The NP genes of three swine influenza viruses (sw37, sw38 and sw39) were sequenced. Their single open reading frames (ORFs) consisted of 1497 nucleotides with a coding capacity of 498 amino acids. Although sw38 and sw39 were isolated in Europe only 1 and 2 years, respectively, after sw37 was isolated in the U.S.A., their NP sequences are more closely related to the human H1N1 influenza viruses wsn33 and pr834, which were isolated 3 and 4 years earlier (Table 1). Amino acids that are considered indicators for the human NP lineage, such as 16 D, 61 L, 127 D, 283 P, 313 Y or 375 E (Gammelin *et al.*, 1989; Gorman *et al.*, 1990) were all present in the sw38 and sw39 NPs. Nucleotide sequence differences between sw37 and the European swine influenza strains (sw38, sw39), and between sw37 and the human strains (wsn33, pr834), are in the same order of magnitude (Table 1).

One maximum parsimony nucleotide tree could be generated with DNAPENNY when pa73 was used as a root sequence (Fig. 1*a*). With the protein sequences rooted in pa73, PROTPARS found two maximum parsimony trees of equal length (105 steps) that differed in the branching point of ussr77. Fig. 1(*b*) shows the amino acid tree that corresponds to the nucleotide tree in the position of ussr77. In both amino acid trees, sw82 was placed closer to the ancestral node of the strains analysed in this study than in the nucleotide tree; a similar observation has been made by Gammelin *et al.* (1990). The phylogenetic analysis at both the nucleotide and amino acid levels showed that the NP genes of sw38 and sw39 were not derived from the swine influenza virus lineage, but from human strains that were more similar

Table 1. *Nucleotide and amino acid substitutions between influenza A virus nucleoproteins*

Virus strain	wsn33	pr834	ussr77	sw38	sw39	sw31	sw37	sw82
	Number of nucleotide substitutions							
wsn33		63	99	11	29	116	137	209
pr834	19		100	61	59	124	140	207
ussr77	31	29		99	107	144	160	219
sw38	7	16	30		27	116	135	207
sw39	13	17	31	11		114	133	208
sw31	26	28	41	27	26		57	180
sw37	28	28	41	27	27	9		158
sw82	39	37	45	39	38	22	18	
	Number of amino acid substitutions							

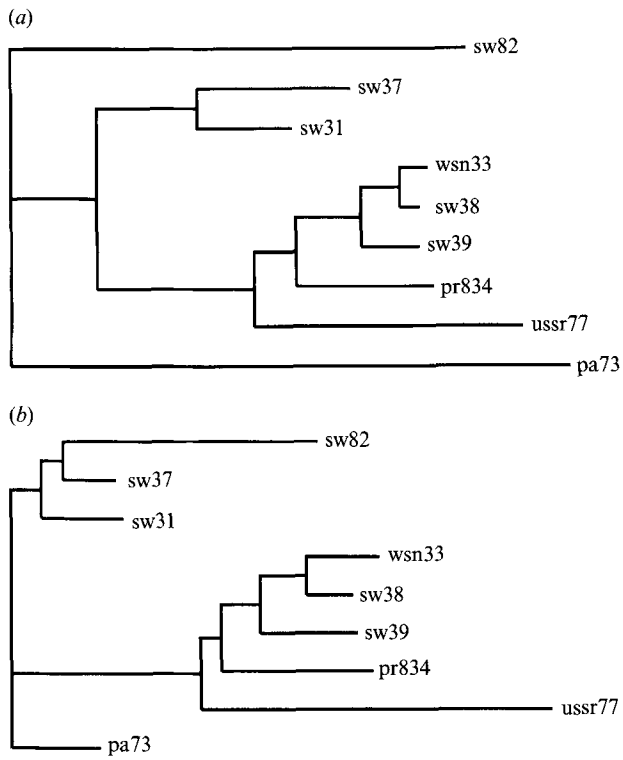

Fig. 1. Phylogenetic trees for the open reading frames of NP genes of influenza A viruses. (*a*) Nucleotide tree; (*b*) amino acid tree. The trees were rooted in pa73. The horizontal lengths of the branches are proportional to the sequence differences; vertical lines are for spacing only.

Table 2. *Genetic distances between partial gene sequences of the European swine influenza strains (sw38, sw39), human and classic swine influenza virus strains*

	PB1 251 nt (10·7%)*		PB2 254 nt (10·85%)		PA 223 nt (9·986%)		NA 290 nt (20·52%)		M 177 nt (17·23%)		NS 120 nt (13·48%)	
	sw38	sw39	sw38	sw39	sw38	sw39	sw38	sw39	sw38	sw39	sw38	sw39
sw38	–	0·0083	–	0·0080	–	0·0092	–	0·0139	–	0·0178	–	0·0084
sw39	0·0083	–	0·0080	–	0·0092	–	0·0139	–	0·0178	–	0·0084	–
wsn33	0·0122	0·0041	0·0040	0·0119	0·0092	0·0232	0·0070	0·0140	0·0178	0·0116	0·0084	0·0000
pr834	0·0289	0·0208	0·0079	0·0159	0·0184	0·0231	0·0389	0·0317	0·0238	0·0174	0·0256	0·0168
sw31*	0·0913	0·0833	0·0724	0·0768	0·1139	0·1134	0·0963	0·0963	0·0431	0·0298	0·1086†	0·1076†
sw37	0·0960	0·0877	0·0897	0·0897	0·1330	0·1269	0·0963	0·0963	0·0373	0·0241	0·0824	0·0729

* The length of the partial sequence analysed and the percentage of the whole gene (in parenthesis) this represents are shown.
† The NS sequence is that of strain sw30.

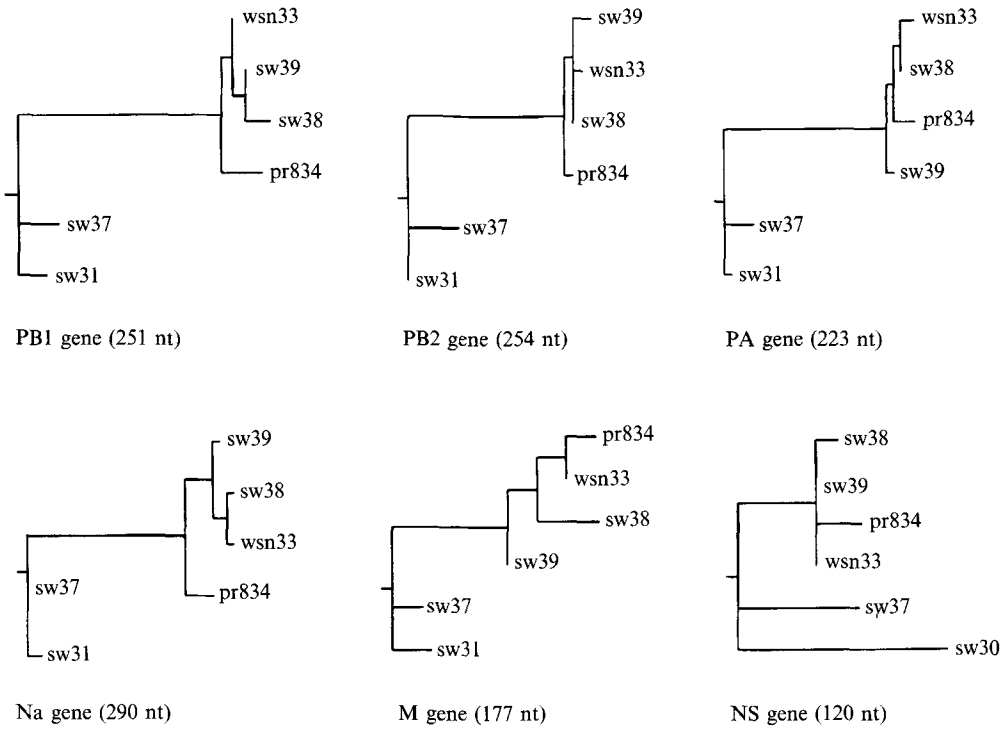

Fig. 2. Phylogenetic trees for gene portions of the polymerase (PB1, PB2, PA), NA, M and NS genes of influenza A viruses. All trees were outgroup-rooted in sw31 or sw30. The horizontal lengths of the branches are proportional to the sequence differences; vertical lines are for spacing only.

to wsn33 than to pr834. The same pattern was found for the sequence relationships of the haemagglutinin genes of these strains (Neumeier & Meier-Ewert, 1992).

Partial sequences between 181 and 325 nucleotides in length were obtained for the NA, M, NS, PB1, PB2 and PA genes of sw31, sw37, sw38 and sw39. The sequences were compared with sequences of human (wsn33, pr834) and swine (sw30) influenza virus strains.

A nucleotide sequence difference matrix that was generated with the DNADIST program from portions representing 9·9% to 20·5% of each gene showed that all genes of sw38 and sw39 analysed were more closely related to those of the human strains (wsn33, pr834) than to the classic swine influenza virus strains (sw30, sw31, sw37) (Table 2). All genes of sw38 and sw39 analysed were more closely related to wsn33 than to pr834. When DNAML was used to estimate phylogenies by maximum likelihood, the European swine isolates were also grouped consistently together with wsn33 and pr834, whereas sw31 and sw37 were on another branch (Fig. 2).

The demonstration of antibodies to human H1N1 viruses in swine sera (Shope, 1938; Arikawa *et al.*, 1979; Tůmová *et al.*, 1980, 1985; Nerome *et al.*, 1982; Shortridge & Stuart-Harris, 1982; Zhang *et al.*, 1988,

1989) and sporadic isolates (Nerome *et al.*, 1982; Hannoun & Gorreau, 1980) indicate that transmission of human H1N1 viruses from humans to pigs occurs readily in nature. However, infection of pigs with human H1N1 strains does not usually produce signs of disease (Shope & Francis, 1936; Nerome *et al.*, 1982; Hannoun & Gorreau, 1980).

The haemagglutinin genes of sw38 and sw39 (Neumeier & Meier-Ewert, 1992) as well as the NP gene of sw39 (Gorman *et al.*, 1991) have been shown previously to be closely related to the early human H1N1 strains wsn33 and pr834 by nucleotide sequence analysis. In this study, partial sequences of the NA gene and the internal genes (PB1, PB2, PA, M, NS), as well as the complete sequence of the ORF of the NP gene of these strains were also found to be more closely related to sequences of the human strains wsn33 and pr834 than to sequences of classic swine influenza strains. Our data therefore indicate that genetic reassortment as a possible factor for the increased virulence of the sw38 and sw39 viruses for pigs is unlikely.

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