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Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype

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Novel H1N2 influenza A viruses which were first detected in pigs in Great Britain in 1994 were examined antigenically and genetically to determine their origins and establish the potential mechanisms for genetic reassortment. The haemagglutinin (HA) of all swine H1N2 viruses examined was most closely related to, but clearly distinguishable both antigenically and genetically from, the HA of human H1N1 viruses which circulated in the human population during the early 1980s. Phylogenetic analysis of the HA gene revealed that the swine H1N2 viruses formed a distinct branch on the human lineage and were probably introduced to pigs shortly after 1980. Following apparent transfer to pigs the HA gene underwent genetic variation resulting in the establishment and cocirculation of genetically and antigenically heterogeneous virus populations. Genetic analyses of the other RNA segments of all swine H1N2 viruses indicated that the neuraminidase gene was most closely related to those of early 'human-like' swine H3N2 viruses, whilst the RNA segments encoding PB2, PB1, PA, NP, M and NS were related most closely to those of avian viruses, which have been circulating recently in pigs in Northern Europe. The potential mechanisms and probable progenitor strains for genetic reassortment are discussed, but we propose that the swine H1N2 viruses examined originated following multiple genetic reassortment, initially involving human H1N1 and 'human-like' swine H3N2 viruses, followed by reassortment with 'avian-like' swine H1N1 virus. These findings suggest multiple reassortment and replication of influenza viruses may occur in pigs many years before their detection as clinical entities.

Introduction

Influenza A viruses are widespread in nature and cause disease in a number of species including humans, pigs and birds (reviewed by Webster *et al.*, 1995). The periodic exchange of influenza virus genes or whole virus between species can give rise to pandemics of disease in humans, lower animals and birds. The causative agent of the 1918 'Spanish' flu pandemic appears to have derived from classical swine H1N1 viruses (Taubenberger *et al.*, 1997). In contrast, the viruses that caused the 1957 and 1968 influenza pandemics resulted from genetic reassortment of human and avian influenza viruses (Webster & Laver, 1972; Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989).

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The nucleotide sequence data reported in this paper will appear in the GenBank and EMBL nucleotide sequence databases, accession nos AF085413–AF085417.

Influenza viruses do not pass readily between humans and birds (Beare & Webster, 1991), possibly because humans do not possess the NeuAc 2,3Gal receptors required for virus attachment to epithelial cells. Transmission between humans and other animals has been demonstrated, leading to the suggestion that one mechanism for the reassortment of human and avian viruses may be in an intermediate animal with subsequent transference to the human population. Pigs have been considered a logical intermediate because they may serve as hosts for productive infections of both avian and human viruses (Kundin, 1970; Schultz *et al.*, 1991; Kida *et al.*, 1994) and because they possess both NeuAc 2,3Gal and 2,6Gal receptors (Ito *et al.*, 1996). In addition, there is good evidence that they have been involved in interspecies transmission of influenza viruses (reviewed by Webster *et al.*, 1995).

Influenza virus subtypes H1N1 and H3N2 circulate in pigs worldwide being largely enzootic but are periodically introduced from humans and birds. Cocirculation of influenza A viruses in pigs can result in the production of new reassortant

Table 1. H1 influenza A virus strains used in phylogenetic analyses of the HA gene

Virus	Subtype	Abbreviation	Accession no.	Reference
A/swine/Ehime/1/80	H1N2	swEhm80	X57494	Sugita *et al.* (1991)
A/swine/Germany/2/81	H1N1	swGer81	Z30276	Ludwig *et al.* (1994)
A/swine/Weybridge/117316/86	H1N1	swEng86	U72666	Brown *et al.* (1997*b*)
A/swine/Indiana/1726/88	H1N1	swInd88	M81707	Luoh *et al.* (1992)
A/swine/England/195852/92	H1N1	swEng92	U72667	Brown *et al.* (1997*b*)
A/swine/England/283902/93	H1N1	swEng93	U72668	Brown *et al.* (1997*b*)
A/swine/Schleswig-Holstein/1/93	H1N1	swGer93	U72669	Brown *et al.* (1997*b*)
A/swine/Scotland/410440/94	H1N2	swScot94	AF085413	This report
A/swine/England/438207/94	H1N2	swEng94	AF085414	This report
A/swine/England/690421/95	H1N2	swEng95	AF085415	This report
A/swine/England/17394/96	H1N2	swEng96a	AF085416	This report
A/swine/England/72685/96	H1N2	swEng96b	AF085417	This report
A/Puerto Rico/8/34	H1N1	PR34	J02143	Winter *et al.* (1981)
A/Fort Monmouth/1/47	H1N1	FM47	U02085	Smeenk & Brown (1994)
A/USSR/90/77	H1N1	USSR77	K01330	Concannon *et al.* (1984)
A/Kiev/59/79	H1N1	Kiev79	M38353	Beklemishev *et al.* (1986)
A/England/333/80	H1N1	Eng80	X00031	Raymond *et al.* (1986)
A/India/6263/80	H1N1	Ind80	X00030	Raymond *et al.* (1986)
A/Chile/1/83	H1N1	Chile83	–	Raymond *et al.* (1986)
A/Taiwan/1/86	H1N1	Tai86	D00407	Robertson (1987)
A/Finland/73/88	H1N1	Fin88	L33753	Pyhala *et al.* (1995)
A/Harbin/1/89	H1N2	Hbn89	L19006	Guo *et al.* (1992)
A/Massachusetts/1/90	H1N1	Mass90	L19027	Xu *et al.* (1993)
A/Singapore/12/90	H1N1	Sing90	L20117	Rocha *et al.* (1993)
A/Netherlands/813/91	H1N1	Neth91	L33744	–
A/duck/Alberta/35/76	H1N1	dkAlb76	D10477	Austin *et al.* (1990)
A/duck/Hong Kong/196/77	H1N1	dkHK77	D00839	Kanegae *et al.* (1994)

viruses, as occurred in Italian pigs during the 1980s, when there was genetic mixing between 'avian-like' H1N1 and 'human-like' H3N2 viruses (Castrucci *et al.*, 1993). In Great Britain (GB) since 1994, influenza viruses of H1N2 subtype have been isolated from pigs with symptoms of acute respiratory disease. These viruses have become widespread within the national pig population and have been associated increasingly with respiratory disease. Antigenic studies on the initial isolates indicated that the haemagglutinin (HA) gene was derived from a human H1N1 virus which was circulating in humans during the early 1980s, whilst the neuraminidase (NA) gene appeared most similar to those from 'human-like' swine H3N2 viruses (Brown *et al.*, 1995).

In the present study, we further examined the antigenic characteristics of the HA of swine H1N2 viruses isolated between 1994 and 1998, using comparisons with H1 viruses from pigs, humans and birds. To investigate these relationships more precisely and to determine the origin of the HA gene of swine H1N2 viruses, the nucleotide sequences for HA1 were determined and compared to published data to examine their genetic relatedness. In order to identify the genotype(s) of these viruses, partial sequences for the other genes encoding the internal proteins and NA were obtained and compared to existing data to determine their origins and establish the likely mechanisms for genetic reassortment.

Methods

■ **Virus strains.** A total of 14 H1N2 influenza viruses from pigs was used for antigenic analyses. Each virus was derived from a separate disease outbreak in GB between 1994 and 1998. Antigenic comparisons were done in haemagglutination inhibition (HI) tests with six H1N1 influenza viruses from pigs and humans. Five swine H1N2 viruses were selected from which to sequence the HA1 coding region. These complemented the HA1 sequences of 22 other H1 viruses from database sources. A summary of the viruses used for genetic analyses of the HA is given in Table 1, which includes their abbreviations used in the text. To identify the origins of the other genes, we sequenced partially the genes encoding the internal proteins and NA, for all swine H1N2 viruses (plus the HA of nine swine H1N2 viruses not listed in Table 1).

■ **Virus growth and RNA isolation.** The viruses were propagated in the allantoic/amniotic cavities of 10 to 11-day-old embryonated fowls' eggs for 48 to 72 h at 35 °C. The viruses were purified and viral RNA extracted using standard procedures (Wood *et al.*, 1994).

■ **Antigenic characterization.** Swine H1N2 viruses and selected H1N1 viruses isolated from pigs and humans during 1977 to 1998 were examined in HI tests according to standard methods, with polyclonal antisera raised against reference antigens.

Table 2. Haemagglutination inhibition tests with swine H1N2 viruses and selected strains of human and swine influenza A viruses

HI titres expressed as the reciprocal of the dilution of antisera inhibiting four haemagglutinating doses of virus. < = < 40.

		Post-infection sera*							
Virus†	Subtype	USSR/ 90/77[a]	Eng/ 333/80[a]	Chile/ 1/83[a]	Taiwan/ 1/86[a]	sw/Wey/ 117316/86[b]	sw/Eng/ 195852/92[b]	sw/Eng/ 438207/94[b]	sw/Eng/ 17394/96[b]
USSR/90/77	H1N1	320	80	80	<	<	<	320	320
England/333/80	H1N1	160	640	640	40	<	<	320	<
Chile/1/83	H1N1	40	80	640	40	<	<	160	<
Taiwan/1/86	H1N1	<	<	80	640	<	<	320	<
sw/Eng/117316/86[c]	H1N1	<	<	<	<	640	80	<	<
sw/Eng/195852/92[d]	H1N1	<	<	<	<	80	1280	<	<
sw/Scot/410440/94[1]	H1N2	160	160	640	160	<	<	640	40
sw/Eng/438207/94[1]	H1N2	40	40	320	160	<	<	1280	40
sw/Eng/463180/94[1]	H1N2	160	320	640	640	<	<	1280	640
sw/Eng/690421/95[1]	H1N2	40	160	640	320	<	<	1280	160
sw/Eng/17394/96[2]	H1N2	<	40	80	<	<	<	160	1280
sw/Eng/40280/96[1]	H1N2	80	160	320	320	<	<	1280	320
sw/Eng/637659/96[2]	H1N2	80	160	640	160	<	<	640	1280
sw/Eng/72685/96[2]	H1N2	80	80	80	<	<	<	320	640
sw/Eng/645913/96[2]	H1N2	80	80	80	<	<	<	160	320
sw/Eng/88761/97[1]	H1N2	160	160	640	160	<	<	320	640
sw/Eng/88762/97[1]	H1N2	320	320	640	320	<	<	1280	80
sw/Eng/661264/97[1]	H1N2	80	160	160	160	<	<	640	<
sw/Eng/161916/97[1]	H1N2	<	40	160	160	<	<	320	<
sw/Eng/181412/98[2]	H1N2	<	40	160	40	<	<	160	640

* Produced in ferrets (*a*) and chickens (*b*).
† *c*, Classical swine H1N1 virus; *d*, avian-like swine H1N1 virus; 1, antigenic group 1; 2, antigenic group 2.

■ **RT–PCR.** The HA1 portion of the HA gene was amplified from purified viral RNA by RT–PCR according to standard methods (Wood *et al.*, 1994) using specific primers. For use in partial sequence determination, all RNA segments were amplified by RT–PCR (Adeyefa *et al.*, 1994) followed by a nested PCR on purified product using segment-specific primers. Primer pairs used in nested PCR and sequencing were as follows: PB2, V1 8–31 (5′) GCAGGTCAAATATATTCAATATGG and V1 484R CTGCATGGCCCGGGTTTATGTC (3′); PB1, V2 18–41 (5′) CATTTGAATGGATGTCAATCCGAC and V2 359R TCCATCGTTTCAAGACA (3′); PA, V3 8–32 (5′) GCAGGTACTGATCCAAAATGGAAGA and V3 620R TCTTCGCCTCTYTCGGACT (3′); H1.1 (5′) AGCAAAAGCAGGGGAAAATAA and H1 720R CTGGGGTGAATCTCCTGTTAT (3′); NP, V5 8–35 (5′) GCAGGGTAGATAATCACTCACTGAGTGA and V5 551R CCYTGCATCAGAGAGCACAT (3′); NA, V6 8–32 (5′) GCAGGAGTGAAGATGAATCCAAATC and NA2 548R CTATACACACTTGCCTGGTTC (3′); M, V7 8–30 (5′) GCAGGTAGATATTGAAAGATGAG and V7 564R TCATGCCTGATTAGTGG (3′); NS, V8 8–31 (5′) GCAGGGTGACAAAAGCATAATGGA and V8 519R AGTATGTCCTGGAAGAGA (3′).

■ **Nucleotide sequence determination.** The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham). Newly prepared (sequences of HA primers available on request) or existing panels of synthetic oligonucleotide primers were annealed to template DNA. The DNASTAR software package was used for the assembly, analysis and translation of nucleotide sequence data.

■ **Sequence analyses of all gene segments.** Partial nucleotide sequence data were assembled and analysed by a 'best-local-homology' rapid search procedure, using a gene database, to generate the 50 most homologous scores and reveal the relationship of the virus gene to others in the database (Adeyefa *et al.*, 1994).

■ **Phylogenetic analyses of the HA gene.** Phylogenetic analyses were done with the software package PHYLIP, version 3.5c (Felsenstein, 1991). The HA1 portion of the HA gene of a total of 27 H1 influenza A viruses from pigs, birds and humans was analysed. Following sequence alignment using MEGALIGN and conversion to the PHYLIP format with READSEQ, phylogenies of gene nucleotide sequences were estimated by maximum likelihood using the program DNAML (Felsenstein, 1981). The program DRAWTREE was used to produce the phylogenetic tree diagram.

Results

Antigenic reactivity of swine H1N2 viruses in HI tests with selected H1 viruses

There was low or no reactivity with all swine H1N2 viruses or their antisera in HI tests with classical swine H1 and 'avian-

Table 3. Genotype of selected influenza A viruses from pigs in Great Britain

Partial nucleotide sequences of each gene were analysed by a 'best-local-homology' rapid search procedure using a gene database. A, genes belonging to the European swine virus branch of the Eurasian avian lineage; H, genes belonging to the human lineage.

		Gene†							
Virus	Subtype	PB2	PB1	PA	HA	NP	NA	M	NS
sw/Eng/163266/87	H3N2	H	H	H	H	H	H	H	H
sw/Eng/195852/92	H1N1	A	A	A	A*	A	A	A	A
sw/Scot/410440/94	H1N2	A	A	A	H*	A	H	A	A
sw/Eng/438207/94	H1N2	A	A	A	H*	A	H	A	A
sw/Eng/463180/94	H1N2	A	A	A	H	A	H	A	A
sw/Eng/690421/95	H1N2	A	A	A	H*	A	H	A	A
sw/Eng/17394/96	H1N2	A	A	A	H*	A	H	A	A
sw/Eng/40280/96	H1N2	A	A	A	H	A	H	A	A
sw/Eng/637659/96	H1N2	A	A	A	H	A	H	A	A
sw/Eng/72685/96	H1N2	A	A	A	H*	A	H	A	A
sw/Eng/645913/96	H1N2	A	A	A	H	A	H	A	A
sw/Eng/88761/97	H1N2	A	A	A	H	A	H	A	A
sw/Eng/88762/97	H1N2	A	A	A	H	A	H	A	A
sw/Eng/661264/97	H1N2	A	A	A	H	A	H	A	A
sw/Eng/161916/97	H1N2	A	A	A	H	A	H	A	A
sw/Eng/181412/98	H1N2	A	A	A	H	A	H	A	A

† The whole (*) or part (positions 31–428) of HA1 of the HA gene was determined. The positions of the sequences of the other genes are PB2 (58–435), PB1 (45–439), PA (71–564), NP (46–361), NA (68–489), M (51–320) and NS (46–393).

like' swine H1 influenza viruses and their respective antisera, in contrast to a broad spectrum of reactivity with human H1N1 viruses (and their respective sera) representative of antigenic drift from 1977 to 1986. The highest reactivity to human H1 virus was generally obtained in HI tests with Chile83, suggesting that the HA of swine H1N2 viruses from GB were most closely related antigenically to human H1 influenza viruses from the early 1980s. Reactivities of the swine H1N2 isolates with antisera to two of the H1N2 strains (swEng94 and swEng96a) could be divided broadly into two groups based on differing antibody titres. The results are summarized in Table 2.

Genotypic characterization of swine H1N2 viruses

Swine H1N2 viruses were examined to determine the host of origin of all the gene segments (Table 3). The HA gene of all isolates was related most closely to those of human H1 viruses which were circulating in humans in the early 1980s (Eng80 and Ind80), with nucleotide identities ranging from 93·1 to 95·4%. The NA gene of all isolates was related most closely to those of human H3N2 viruses which were circulating in humans in the early 1970s and in pigs since that time (nucleotide identities ranging from 89·9 to 93·6%). Nucleotide identities with the GB prototype 'human-like' swine H3N2 strain (A/sw/England/163266/87) ranged from 90·5 to 97·1%. The genes encoding the internal proteins of all swine H1N2 viruses examined had the highest identity to those of European 'avian-like' swine viruses. The genotype of representative GB strains of 'avian-like' swine H1NI and 'human-like' swine H3N2 was entirely avian and human respectively.

The sequences of the genes encoding the internal protein genes of selected GB swine viruses were compared to sequences available in GenBank and of swEng92. When aligned with sequences from virus groups of different host origin (Table 4), the genes of representative swine H1N2 viruses had the highest identity to those of recent European 'avian-like' swine viruses (97·0 to 99·3%), suggesting a recent origin. Lower identities were obtained in comparisons with the polymerase genes of Italian swine viruses of both H1N1 and H3N2 subtypes isolated between 1976 and 1989 (data not shown). Viruses of the latter group isolated since 1985 in Italy contain genes encoding internal proteins of avian origin (Castrucci *et al.*, 1993).

Sequence analyses of the HA genes of swine H1N2 viruses

To investigate further the genetic similarity to human influenza strains and the molecular basis for observed antigenic variations, the region of RNA segment 4 encoding the HA1

Table 4. Comparison of nucleotide sequence identities of selected GB swine influenza A viruses with representative viruses of avian, swine and human origin

Representative virus strains used for comparison were A/sw/England/195852/92 (recent avian-like swine), A/sw/Germany/2/81 (early avian-like swine), A/oystercatcher/Germany/87 (recent European avian), A/FPV/Rostock/34 (avian), A/sw/Iowa/15/30 (classical swine), A/WSN/33 (human H1N1) and A/NT/60/68 (human H3N2). ND, Not done. Highest identities are in bold. *a*, *b*, *c*, Comparison with A/sw/Tennessee/26/77, A/WS/33 and A/Udorn/72.

				Virus groups (% identity to test strain)						
Virus	Gene	Position	Subtype	Recent avian-like swine	Early avian-like swine	Recent European avian	Avian	Classical swine	Human H1N1	Human H3N2
sw/Eng/163266/87	PB2	58–435	H3N2	81·0	80·2	ND	82·5	87·3	92·1	**94·7**
sw/Eng/195852/92	PB2	58–435	H1N1	–	**96·0**	ND	86·0	84·7	85·2	82·0
sw/Eng/438207/94	PB2	58–435	H1N2	**98·7**	94·7	ND	85·4	84·1	84·7	81·7
sw/Eng/17394/96	PB2	58–435	H1N2	**97·4**	95·5	ND	84·9	83·9	83·9	80·2
sw/Eng/163266/87	PB1	45–349	H3N2	89·8	91·8	ND	ND	85·2[a]	84·9	**96·1**
sw/Eng/195852/92	PB1	45–349	H1N1	–	**96·7**	ND	ND	87·2[a]	87·2	92·1
sw/Eng/438207/94	PB1	45–349	H1N2	**99·3**	96·1	ND	ND	87·2[a]	87·2	91·5
sw/Eng/17394/96	PB1	45–349	H1N2	**97·7**	94·8	ND	ND	87·9[a]	87·5	90·5
sw/Eng/163266/87	PA	386–582	H3N2	81·2	81·7	ND	85·8	86·8	90·9	**93·4**
sw/Eng/195852/92	PA	386–582	H1N1	–	**97·0**	ND	87·3	81·2	82·2	84·3
sw/Eng/438207/94	PA	386–582	H1N2	**99·0**	97·0	ND	88·3	81·2	81·7	83·8
sw/Eng/17394/96	PA	386–582	H1N2	**97·0**	95·9	ND	88·8	81·7	81·7	84·8
sw/Eng/163266/87	NP	46–439	H3N2	82·5	83·0	79·9	80·2	86·5	90·6[b]	**93·7**
sw/Eng/195852/92	NP	46–439	H1N1	–	**97·0**	91·6	89·8	85·5	83·5[b]	83·5
sw/Eng/438207/94	NP	46–439	H1N2	**99·7**	96·7	91·4	89·6	85·3	83·2[b]	83·2
sw/Eng/17394/96	NP	46–439	H1N2	**99·7**	96·7	91·4	90·1	85·0	83·0[b]	83·0
sw/Eng/163266/87	M	51–320	H3N2	92·2	92·2	91·9	91·5	88·5	93·7	**97·8**[c]
sw/Eng/195852/92	M	51–320	H1N1	–	**97·4**	92·6	93·7	89·6	93·0	93·3[c]
sw/Eng/438207/94	M	51–320	H1N2	**99·3**	98·1	91·9	93·0	88·9	92·2	92·6[c]
sw/Eng/17394/96	M	51–320	H1N2	**99·6**	97·0	93·0	93·3	89·3	92·6	93·0[c]
sw/Eng/163266/87	NS	46–393	H3N2	76·4	81·0	81·3	79·9	78·2	83·0	**90·8**[c]
sw/Eng/195852/92	NS	46–393	H1N1	–	**94·0**	91·7	87·9	85·1	85·6	80·5[c]
sw/Eng/438207/94	NS	46–393	H1N2	**99·1**	93·1	90·8	87·1	85·3	84·8	79·6[c]
sw/Eng/17394/96	NS	46–393	H1N2	**98·3**	93·1	90·8	87·6	84·5	85·1	81·0[c]

polypeptide chains of the HA gene of five viruses was sequenced. These viruses were representative of the group under study. There was high nucleotide (92·5 to 94·7%) and amino acid (92·6 to 95·7%) sequence identities in HA1 of selected swine H1N2 viruses with those of human H1 viruses isolated between 1980 to 1983. Sequence comparisons of HA1 of swine H1N2 viruses revealed variable identities at nucleotide (92·9 to 99·8%) and amino acid (92·3 to 99·7%) level, indicating genetic heterogeneity within the group. The deduced amino acid sequences were compared to determine the number of amino acid substitutions in HA1 of selected swine and human viruses. SwEng94 differed from swEng96a and Eng80 by 25 and 23 amino acids respectively, whilst swEng96a differed from swEng96b and Eng80 by 1 and 22 amino acids respectively. The mutations in the swine viruses were distributed randomly across HA1 and not associated specifically with regions that have been determined or proposed to influence antigenic properties of the influenza virus HA (Wiley *et al*., 1981; Raymond *et al*., 1986; Wiley & Skehel, 1987). Five potential glycosylation sites (N-X-S/T) were observed on HA1 of swine H1N2 viruses examined, two less than were present in the apparent progenitor human strains. Numbering was aligned with the H3 viruses (Wiley & Skehel, 1987), with the amino acid residues for the primary translation product given in parentheses. Four glycosylation sites were conserved at positions 27/28 (20/21), 40 (33), 288 (273) and 304 (289) in all strains analysed. A fifth glycosylation site was present at positions 144 (130) and 172 (158) in antigenic groups characterized by swEng96a and swEng94 respectively. Both of these sites were present on HA1 of human H1 viruses 1980 to 1983 in addition to a seventh potential glycosylation site at position 104 (94).

Phylogenetic analysis of the HA gene

A phylogenetic tree of the nucleotide sequences of the HA1 portion of the HA gene of a total of 27 H1 influenza A

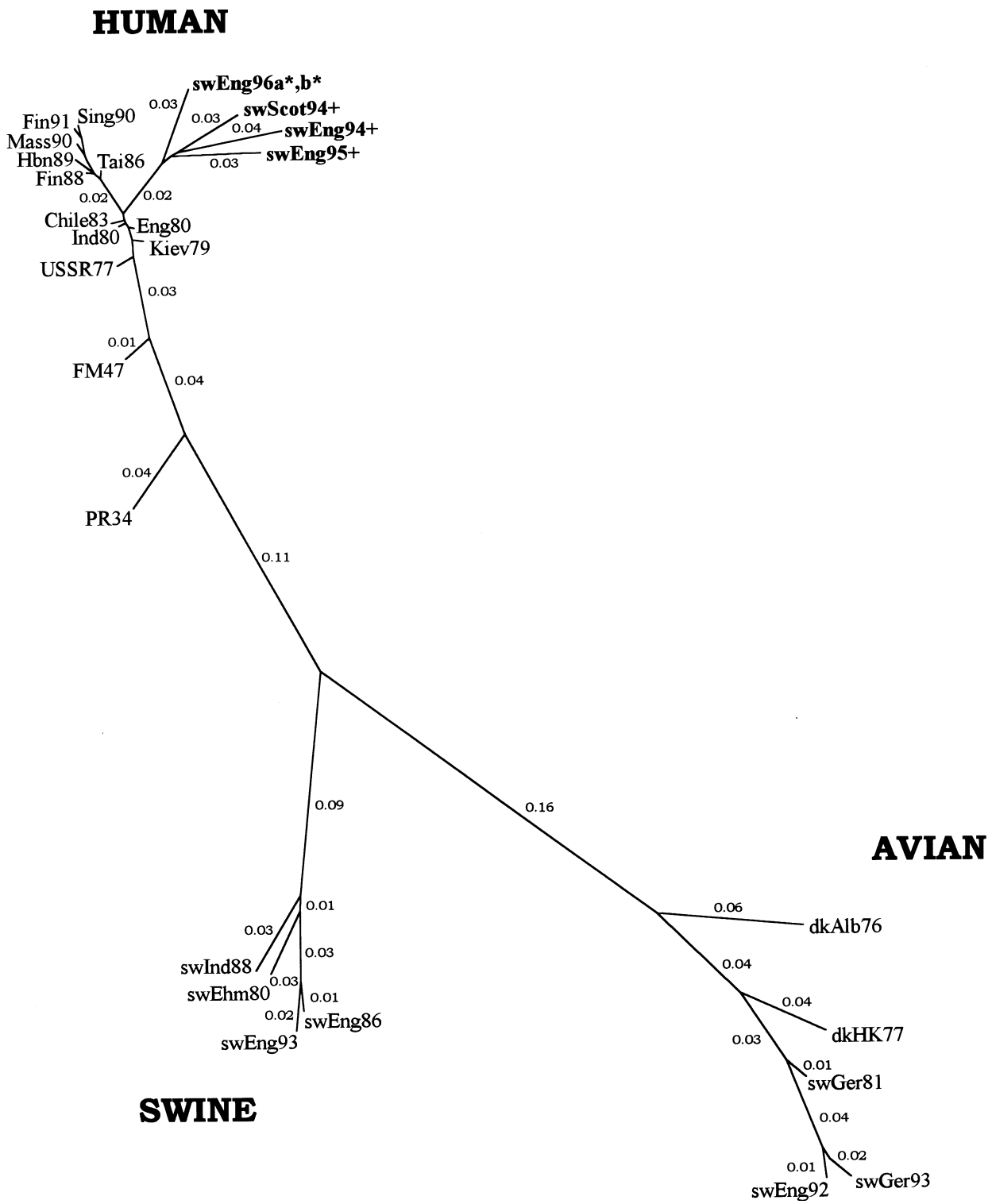

Fig. 1. Unrooted phylogenetic tree for the HA1 portion of the HA gene of selected H1 influenza A viruses from pigs, humans and birds analysed using maximum likelihood. The numbers are arbitrary units relative to nucleotide sequence differences. Antigenic groups 1(+) and 2(*).

viruses from pigs, birds and humans is shown in Fig. 1. Viruses grouped into three distinct evolutionary lineages based on the evolution of influenza virus HA genes in host-specific pathways, namely avian, swine and human. The HA genes of swine H1N2 viruses from pigs in GB formed a distinct subgroup in the human lineage, appearing to branch off from the main

lineage shortly after 1980, and have evolved independently since that time. Further divergence within this sublineage has also occurred, resulting in the five swine H1N2 viruses analysed being located in four distinct branches.

Discussion

The H1N2 viruses isolated from pigs in GB during 1994 to 1998 were characterized antigenically in HI tests with polyclonal antisera to selected influenza A viruses. The H1N2 viruses could be distinguished clearly from swine H1N1 viruses and were most closely related to, but clearly distinguishable from, human H1N1 viruses which circulated in the human population during the early 1980s. All swine H1N2 viruses examined could be divided broadly into two antigenic groups based on clearly distinguishable reactivities in HI tests, but which were not associated with the year of isolation of the respective viruses, suggesting antigenic diversity in cocirculating viruses of the same genotype.

Sequence analyses of the HA1 regions of the HA genes of five selected swine H1N2 viruses confirmed that the HA gene was derived from a human H1N1 virus. The HA gene of these viruses was most similar to those of Eng80 and Ind80 suggesting introduction to pigs in the early 1980s. The overall degree of amino acid sequence identity of the HA of swine and human viruses was lower than that implied by the antigenic data, ranging from 92·6 to 95·7%. The relatively small number of amino acid substitutions in regions of the molecule known to be associated with antigenicity are consistent with genetic analyses of the HA gene of other viruses following transmission from humans (Castrucci *et al*., 1994) and birds (Brown *et al*., 1997*b*) to pigs. The functional importance of these changes is unknown but may be related to host adaptation. Host immune pressure is not so marked in pigs as in humans because of the continual availability of young pigs without protective immunity, thereby offering less selection advantage to antigenic variants of influenza viruses.

The reduction in the number of glycosylation sites on the HA1 subunits of swine H1N2 viruses compared to the apparent progenitor strains from humans is consistent with earlier findings (Neumeier & Meier-Ewert, 1992; Inkster *et al*., 1993; Brown *et al*., 1997*b*) and supports the concept of reduced immune pressure in pigs since carbohydrate side-chains can mask antigenic sites sterically contributing to antigenic conservation or variation (Skehel *et al*., 1984; Air & Laver, 1986). The close genetic relationship between the HAs of human H1 and swine H1N2 viruses was emphasized by the presence of a conserved motif (Gln-Glu-Gly) at residues 226 to 228 in the sialic acid-binding site (Wilson *et al*., 1981; Naeve *et al*., 1984; Weis *et al*., 1988), which binds preferentially to α2,6-galactose sialic acid linkages in cells lining the trachea of both pigs and humans (Ito *et al*., 1996).

Genetic analyses by nucleotide sequencing of all the RNA segments of selected swine viruses revealed that all swine H1N2 viruses examined contained a novel genotype comprising two genes, HA and NA, of human origin and six genes of avian origin. In contrast, the genotypes of prototype GB isolates of swine 'avian-like' H1N1 and 'human-like' H3N2 viruses were entirely avian and human respectively, suggesting that they were not derived following recent genetic reassortment. The NA gene appeared to derive from early human H3N2 viruses, which have been maintained in pig populations worldwide since the early 1970s. It would appear therefore that the swine H1N2 viruses associated currently with disease outbreaks in GB were derived from genetic reassortment of human and avian influenza A viruses. Although genetic reassortment involving human H1N1 and avian influenza A viruses resulted in the emergence of the 1957 Asian H2N2 pandemic strain (Gething *et al*., 1980; Scholtissek *et al*., 1978; Kawaoka *et al*., 1989), recombinant viruses from similar progenitor strains have not been reported in pigs previously, but 'human-like' H3N2 viruses circulating in Italian pigs since 1985 have contained internal protein genes of avian origin (Castrucci *et al*., 1993) and these viruses have formed a stable lineage within pigs (Campitelli *et al*., 1997).

Influenza viruses of H1N2 subtype have been described in pigs previously from both Europe (Gourreau *et al*., 1994) and Asia (Sugimura *et al*., 1980), with increasing prevalence in Japan (Ouchi *et al*., 1996). All of these viruses are derived from cocirculating swine strains of H1N1 and 'human-like' H3N2 viruses, and are therefore clearly distinguishable both antigenically and genetically from the swine H1N2 viruses isolated recently in GB. In addition, H1N2 viruses were isolated sporadically from humans in China during the late 1980s, but these strains were derived entirely from the prevailing strains cocirculating in the human population at the time (Guo *et al*., 1992).

To identify the potential progenitor viruses, the mechanisms and the time-period for genetic reassortment of swine H1N2 viruses, we further examined the genetic data in comparison to that for all gene segments of other influenza A viruses and by phylogenetic analyses of the HA gene. It would appear that the HA gene of human H1N1 influenza A viruses was transferred to pigs shortly after 1980, since at this time the lineage containing the HA gene of swine H1N2 diverged from the human lineage and evolved independently forming a distinct branch. At this time 'human-like' H3N2 viruses (most similar to early human H3N2 strains) and 'avian-like' H1N1 were cocirculating in European pigs, although the latter was not present in pigs in GB until 1992 (Brown *et al*., 1993). Human and avian reassortant viruses of H3N2 subtype were not detected in Italian pigs until 1985, whilst in GB the earliest swine H3N2 virus, isolated in 1987, derived all of its genes from a human virus. It would appear therefore, that following reassortment of human H1N1 and 'human-like' swine H3N2 viruses in the early 1980s progeny viruses would not contain any genes of avian origin. Furthermore, the possibility of reassortment between human and avian H1N1 viruses at this

time appears unlikely since the genes of avian origin of swine H1N2 viruses were genetically most closely related to those of recent 'avian-like' swine H1N1 viruses from North European pigs. Transmission of human H1N1 viruses to pigs occurs infrequently and is usually reflective of the prevailing strains in the human population (Kanegae *et al.*, 1994; Katsuda *et al.*, 1995). However, in contrast to human H3N2 viruses there is no evidence suggesting that human H1N1 viruses are able to persist in pigs independently of the human population, unless some of the viral genes including the HA gene are maintained following genetic reassortment (Brown *et al.*, 1997*a*). It would appear less likely that the progenitor strain of the HA gene of swine H1N2 viruses could have persisted in pigs for a sufficient period of time in order to reassort with human–avian H3N2 virus. We propose therefore that swine H1N2 viruses from pigs in GB arose following a multiple reassortment event. Firstly, genetic reassortment of human H1N1 and 'human-like' swine H3N2 may have occurred shortly after 1980 producing progeny virus of H1N2 phenotype, followed several years later by a second reassortment event with 'avian-like' swine H1N1 viruses, resulting in progeny virus of H1N2 phenotype but containing genes encoding internal proteins of avian origin.

The emergence of an apparently stable lineage of avian–human H1N2 influenza virus in pigs in GB contributes further to the complex aetiology of influenza in European pigs, raising further concerns about the potential of swine viruses to infect humans. All influenza pandemics since 1918 were caused by viruses that contain genes from avian viruses (reviewed by Webster *et al.*, 1995) and transmission of avian–human H3N2 viruses to humans, presumably from pigs, appears to occur regularly (Claas *et al.*, 1994; Campitelli *et al.*, 1997). The recently emerged H1N2 viruses spread rapidly in the pig population of GB and were frequently associated with respiratory epizootics in pigs. Pigs can therefore act as reservoir for influenza viruses containing genes from human strains long after their disappearance from the human population, in addition to viruses of novel genotype whose composition may constitute that of a pandemic strain should a susceptible human population become available. The current studies demonstrate that such viruses may replicate and spread within pigs many years before their detection and reinforce the need for continued surveillance of pig populations worldwide for evidence of novel influenza A viruses.

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