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Antigenic and Genetic Characterization of Swine Influenza A (H1N1) Viruses Isolated from Pneumonia Patients in The Netherlands

G. F. Rimmelzwaan,*[,1] J. C. de Jong,* T. M. Bestebroer,* A. M. van Loon,† E. C. J. Claas,*[,2] R. A. M. Fouchier,* and A. D. M. E. Osterhaus*

*Institute of Virology, National Influenza Center, Erasmus Medical Center Rotterdam, 3000 DR Rotterdam, The Netherlands; and †Department of Clinical Microbiology, Academic Hospital Utrecht, Utrecht, The Netherlands

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It is generally believed that pigs can serve as an intermediate host for the transmission of avian influenza viruses to humans or as mixing vessels for the generation of avian–human reassortant viruses. Here we describe the antigenic and genetic characterization of two influenza A (H1N1) viruses, which were isolated in The Netherlands from two patients who suffered from pneumonia. Both viruses proved to be antigenically and genetically similar to avian-like swine influenza A (H1N1) viruses which currently circulate in European pigs. It is concluded that European swine H1N1 viruses can infect humans directly, causing serious disease without the need for any reassortment event.

INTRODUCTION

Influenza A virus infections are a major cause of disease in several animal species including humans, pigs, and birds. In humans, three major influenza pandemics caused by newly introduced subtypes of influenza A viruses, which continued to circulate after these pandemics, have caused significant mortality during the 20th century. It is generally believed that birds are the principle reservoir from which new subtypes of influenza A virus are introduced in mammalian species. Influenza A viruses can be introduced into humans from the bird reservoir through several mechanisms. First, avian influenza A viruses can be transmitted directly from birds to man. The infection of people in the Hong Kong area in 1997 with an avian influenza virus (H5N1) is an example of this transmission route (De Jong *et al.,* 1997; Claas *et al.,* 1998; Subbarao *et al.,* 1998). This virus, however, which caused six casualties, failed to be transmitted efficiently from human to human (Buxton Bridges *et al.,* 2000; Katz *et al.,* 1999). Second, pigs can be infected with both avian and human influenza viruses, enabling these viruses to reassort (Webster *et al.,* 1992). Avian viruses may also adapt in pigs to mammalian hosts without exchanging gene segments and may subsequently be transmitted from pigs to humans (Ito *et al.,* 1998). It is believed that during adaptation of avian influenza viruses in pigs the receptor-binding site of these viruses acquires specificity for the *N*-acetylneuraminic acid 2,6-galactose (NeuAcα2,6Gal) linkage on sialyloligosaccharides, which is present on mammalian cells. In doing so, it loses the specificity for the NeuAcα2,3Gal linkage, which is present on avian cells (Ito *et al.,* 1998). Several reports on the transmission of "classical" swine H1N1 viruses, which predominantly circulate in the United States, to humans illustrate the latter possibility. In some cases, human infections with these swine influenza viruses proved to be fatal (Gaydos *et al.,* 1977; Patriarca *et al.,* 1984; Wells *et al.,* 1991; Wentworth *et al.,* 1994). Besides classical swine influenza A viruses, also avian-like swine influenza A (H1N1) viruses, prevalent in Europe since 1979 (Donatelli *et al.,* 1991; Scholtissek *et al.,* 1983; Campitelli *et al.,* 1997) have been shown to be transmitted to humans (De Jong *et al.,* 1986, 1988). Here we describe the antigenic and genetic characterization of such a previously identified H1N1 virus, A/Netherlands/386/86 (De Jong *et al.,* 1986) and of another, more recent, H1N1 isolate, A/Netherlands/477/93.

RESULTS

Antigenic properties of A/Netherlands/386/86 and A/Netherlands/477/93

First it was demonstrated that A/Netherlands/386/86 and A/Netherlands/477/93 were not related to human and swine influenza H3N2 viruses, including two swine-like H3N2-viruses isolated from humans in 1993 (Claas *et al.,* 1994) (data not shown). As shown in Table 1, both A/Netherlands/386/86 and A/Netherlands/477/93 were antigenically related to swine H1N1 viruses, isolated in

[1] To whom reprint requests should be addressed at Institute of Virology and WHO National Influenza Center, Erasmus Medical Center Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Fax: +31-10-4089485. E-mail: rimmelzwaan@viro.fgg.eur.nl.

[2] Present address: Department of Virology, University Medical Center, Leiden, The Netherlands.

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TABLE 1

Hemagglutination Inhibition (HI) Titers with Influenza A (H1N1) Viruses

Virus strain	Ferret antisera directed against											
	PR/34	FM/47	Chile/83	Sing/86	Tex/91	Sw/NL/80	Sw/NL/81	Sw/NL/85	Sw/NL/87	Sw/NL/96	NL/386/86	NL/477/93
A/PR/8/34	1280	—	—	30	—	—	—	—	—	—	—	—
A/FM/1/47	—	2560	—	—	—	—	30	40	—	—	—	—
A/Chile/1/83	—	—	1280	—	—	—	—	—	—	—	—	—
A/Singapore/6/86	—	—	—	960	160	—	—	—	—	—	—	—
A/Texas/36/91	—	—	—	640	1280	—	30	—	—	—	—	—
A/Sw/Neth/25/80	30	—	—	—	—	3840	1920	480	640	240	1920	640
A/Sw/Neth/101/81	—	—	—	—	—	480	960	80	120	80	160	80
A/Sw/Neth/12/85	—	—	—	—	—	160	480	160	—	80	60	80
A/Sw/Neth/1/87	—	—	—	—	—	640	640	160	1280	80	480	480
A/Sw/Neth/1743/93	—	—	—	—	—	—	640	160	1280	1280	640	640
A/Sw/609/96	—	—	—	—	—	160	480	40	320	160	160	160
A/Neth/386/86	—	—	—	—	—	640	640	80	320	40	640	160
A/Neth/477/93	—	—	—	—	—	320	640	80	1280	160	1280	480

Note. Homologous antibody titers are underlined; — indicates HI titers <10.

The Netherlands between 1980 and 1996. No reactivity was observed with ferret antisera raised against a number of reference human H1N1 influenza viruses, including A/Singapore/6/86 and A/Texas/36/91, prototypes of the viruses isolated in the same period as A/Netherlands/386/86 and A/Netherlands/477/93.

Genetic characterization of A/Netherlands/386/86 and A/Netherlands/477/93

Comparison of the nucleotide sequences of the HA and the NP genes of A/Netherlands/386/86 and A/Netherlands/477/93 with those of swine H1N1 influenza viruses isolated in The Netherlands and France in the same period and human, avian, and swine influenza A (H1N1) viruses from the influenza virus sequence database (Los Alamos National Laboratory, http://www-flu.lanl.gov) revealed that the two swine-like human viruses show the highest degree of similarity with the European swine influenza A (H1N1) viruses (Fig. 1). Indeed, at amino acid positions of the HA molecule showing the highest degree of variability, A/Netherlands/386/86 was found to share amino acids with old (positions 54, 185, and 271) and more recent swine influenza A (H1N1) viruses (positions 47, 96, 121, 138, 207, 267, 276, and 311), whereas A/Netherlands/477/93 was found to share amino acids only with recent swine influenza viruses (positions 54, 185, and 271) (Table 2). The selected regions of the eight gene segments were found to have the highest nucleotide sequence homologies predominantly with European swine influenza A (H1N1) viruses, available from the influenza sequence database (Table 3). Due to the limited number of sequences available, relative low homologies (as low as 88.6%) were found with polymerase genes of influenza A viruses. However, high sequence homologies were found for all genes, including the polymerase genes, with swine H1N1 viruses isolated in The Netherlands in the same year as A/Netherlands/1/87 and A/Netherlands/477/93 ranging from 92 up to 100% (Table 3). These results indicate that these viruses were directly transmitted from pigs without a previous reassortment event with human or avian influenza A viruses.

DISCUSSION

The antigenic and genetic characterization of the human influenza virus isolates A/Netherlands/386/86 and A/Netherlands/477/93, obtained from patients who suffered from severe viral pneumonia, show that these viruses were avian-like swine H1N1 influenza viruses. Since all eight genes originated from these viruses, it was concluded that they were transmitted from pigs without a previous reassortment event with human or avian influenza A viruses. Phylogenetic analysis showed that these two human isolates had the highest homology with European swine influenza A H1N1 viruses that were isolated in the same years. These data confirm the place of the two viruses within the genetic chronology of European swine influenza A H1N1 viruses. Since a leucine at position 142 of HA1 was associated with specificity for the NeuAcα2,6Gal linkage present on mammalian cells (Ito *et al.,* 1998), both swine influenza viruses isolated from the two patients were expected to have this amino acid at position 142. However, only A/Netherlands/477/93 was found to contain a leucine at position 142, whereas A/Netherlands/386/86 and more recent swine influenza viruses did not. This is in contrast with the data reported by others (Ito *et al.,* 1998). Little information is available on the incidence of transmissions of these avian-like H1N1 influenza viruses from pigs to humans (Campitelli *et al.,* 1997). At present, there is no evidence

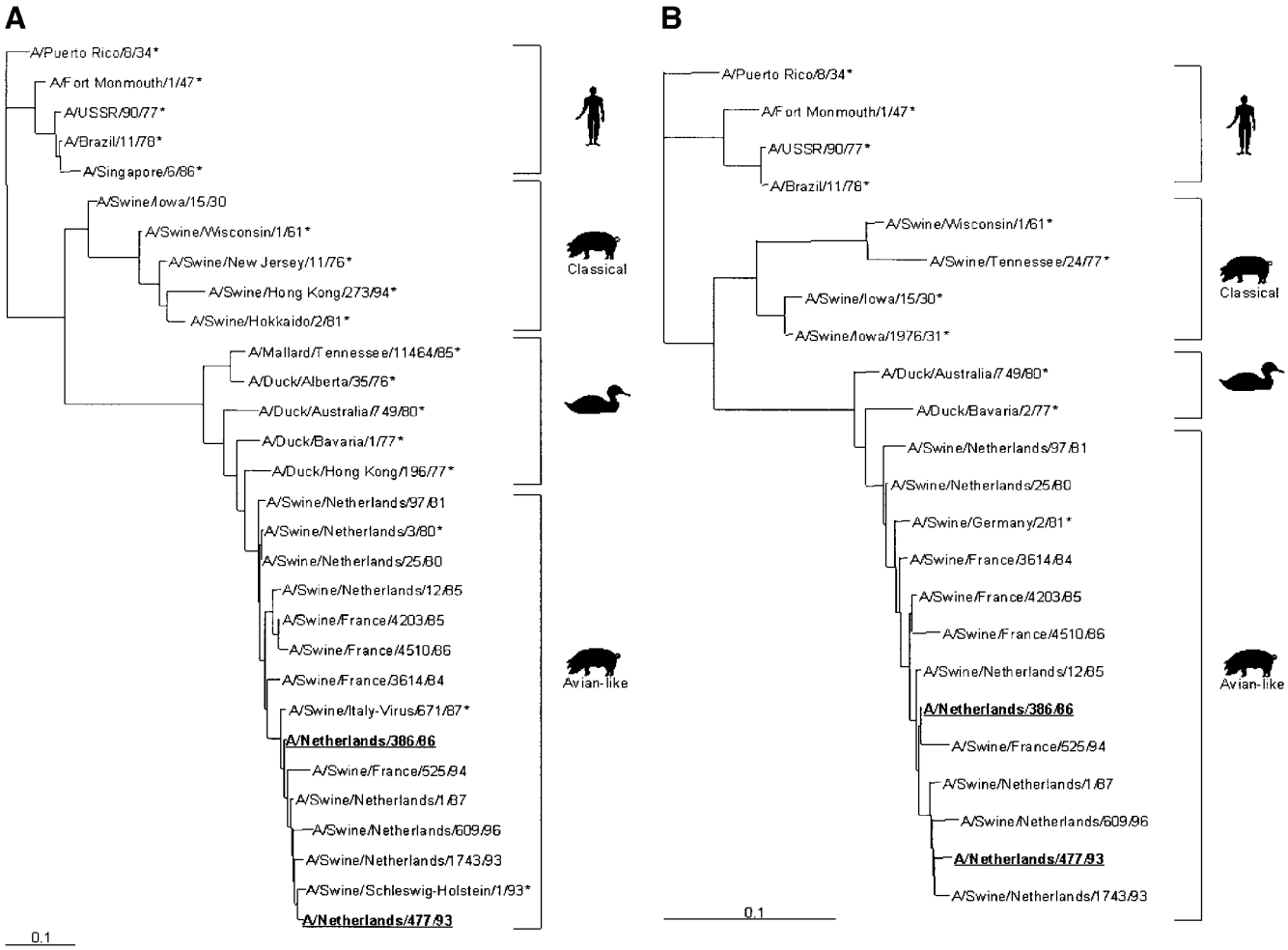

FIG. 1. Maximum likelihood tree based on the nucleotide sequence of (A) the HA1 gene and (B) the NP gene. The HA1 coding region and a part of the NP gene (nt 720–1175) of A/Netherlands/386/86 and A/Netherlands/477/93 and a selection of European swine influenza viruses (H1N1) were sequenced and subjected to phylogenetic analysis. For reference, some influenza (H1N1) viruses were obtained from the influenza sequence database and are indicated with an asterisk (*).

that these viruses can spread within the human population. However, since these viruses and also avian H3N2 reassortant viruses continue to circulate among pigs in Europe (Campitelli *et al.,* 1997), it should be emphasized that new transmissions of these viruses to which little or no immunity exists in the human population may take place more frequently than the isolated cases identified so far (Claas *et al.,* 1994; De Jong *et al.,* 1988).

In conclusion, the data presented demonstrate that European avian-like swine influenza A (H1N1) viruses can infect humans directly, without the need for a reassortment event, and that this may result in serious disease. Although so far swine influenza viruses have not been shown to transmit efficiently within the human population and have not caused epidemics, these viruses may be considered to constitute a continuous threat. After further adaptation to the human host or after reassortment with other influenza A viruses, they may form the basis for newly emerging pandemic influenza viruses. These data stress the importance of annual surveillance for influenza viruses in pigs, birds, and humans.

MATERIALS AND METHODS

Virus strains

Influenza virus A/Netherlands/386/86 was isolated in 1986 from a 29-year-old farmer, who suffered from severe pneumonia. Shortly before his illness, this patient had been in contact with pigs showing respiratory disease. This virus was identified as a swine-like influenza A (H1N1) virus serologically (De Jong *et al.,* 1986, 1988). Influenza virus A/Netherlands/477/93 was isolated in the summer of 1993 from a 5-year-old girl, who also suffered from severe pneumonia. This girl, who lived on a pig farm, had also been in close contact with pigs; it is unknown whether these pigs suffered from respiratory disease. Three days after the onset of clinical symptoms a bronchoalveolar aspirate was obtained from which the virus was isolated after passage in tertiary monkey kid-

TABLE 2

HA Variability among Avian-like Swine Influenza Viruses (H1N1)

Strain	Amino acid position in HA1												
	47	54	96	121	132	138	142	185	207	267	271	276	311
Sw/Netherlands/25/80	I	K	I	N	V	Y	S	T	N	T	P	D	K
Sw/Netherlands/101/81	I	K	I	N	V	Y	N	T	N	T	P	D	K
Sw/France/84	M	K	I	N	V	Y	R	T	N	M	P	D	K
Sw/Netherlands/12/85	V	K	I	N	I	Y	R	T	N	T	P	D	K
Sw/France/85	V	K	I	N	I	Y	R	T	N	T	P	D	K
Sw/France/86	V	K	I	N	T	Y	R	T	H	T	P	D	K
A/Netherlands/386/86	**I**	**K**	**A**	**T**	**T**	**H**	**H**	**T**	**Y**	**M**	**P**	**T**	**Q**
Sw/Netherlands/1/87	I	N	A	T	A	H	L	N	Y	M	Q	T	Q
Sw/Netherlands/1743/93	I	N	A	T	A	H	R	N	Y	M	Q	T	Q
A/Netherlands/477/93	**I**	**N**	**A**	**T**	**A**	**H**	**L**	**N**	**Y**	**M**	**Q**	**T**	**Q**
Sw/France/94	I	D	A	T	A	H	R	N	Y	M	P	T	Q
Sw/Netherlands/609/96	I	N	A	T	V	H	R	S	Y	M	Q	T	Q

Note. Influenza viruses isolated from human patients are indicated in boldface. Sequence identity of A/Netherlands/386/86 with older swine influenza viruses at variable positions in the HA1 is indicated by light gray boxes. Sequence identity of A/Netherlands/477/93 with more recent swine influenza viruses is indicated by dark gray boxes. Only those positions in the HA1 at which at least three strains contain an amino acid change are shown.

ney cells. At that time, the virus could not be identified with reference antisera for human influenza viruses, although negative contrast electron microscopic analysis identified it as an orthomyxovirus. The virus was further identified as an influenza A virus by RT-PCR, using primers based on conserved sequences in the matrix gene (Fouchier *et al.,* 2000), and by immunofluorescence of infected MDCK cells using a FITC-labeled monoclonal antibody, specific for influenza A viruses (Dako, Imagen Influenza A and B) (Rimmelzwaan *et al.,* 1998). Several swine H1N1 viruses isolated in The Netherlands and France (the latter kindly provided by Prof. M. Aymard, University of Lyon, France) were included in the study for comparison: A/Swine/Netherlands/25/80, A/Swine/Netherlands/101/81, A/Swine/Netherlands/12/85, A/Swine/Netherlands/1/87, A/Swine/Netherlands/1743/93, A/Swine/Netherlands/609/96, A/Swine/France/3614/84, A/Swine/France/4203/85, A/Swine/France/4510/86, and A/Swine/France/525/94.

Hemagglutination inhibition assay

For the antigenic characterization of A/Netherlands/386/86 and A/Netherlands/477/93, hemagglutination in-

TABLE 3

Sequence Homology of A/Netherlands/386/86 and A/Netherlands/477/93 with Swine Influenza H1N1 Viruses

Virus strain	Gene	Virus from database with highest homology	%	% Homology with A/Swine/Netherlands/1/87	% Homology with A/Swine/Netherlands/1743/93
A/Netherlands/386/86	PB2	A/Swine/Germany/2/81	98.0	99.0	98.5
	PB1	A/Duck/Nanchang/668/98	92.8	98.2	96.5
	PA	A/Swine/Hong Kong/81/78	89.1	99.2	96.1
	HA	A/Swine/Italy/671/87	98.5	98.7	97.2
	NP	A/Swine/Netherlands/12/85	99.3	98.6	98.1
	NA	A/Swine/England/195852/92	95.3	96.1	93.0
	M	A/Swine/Germany/2/81	100	100	98.4
	NS	A/Swine/Italy/671/87	98.9	98.7	97.4
A/Netherlands/477/93	PB2	A/Swine/Germany/2/81	96.6	97.5	98.0
	PB1	A/Swine/Hong Kong/126/82	91.5	98.8	97.6
	PA	A/Swine/Hong Kong/81/78	88.6	97.1	96.8
	HA	A/Swine/Schleswig-Holstein/1/93	98.1	98.4	97.3
	NP	A/Swine/Germany/8533/91	99.3	98.2	98.2
	NA	A/Swine/England/195852/92	95.8	95.3	92.2
	M	A/Swine/Germany/2/81	100	99.2	99.2
	NS	A/Swine/Germany/8533/91	99.8	96.5	98.7

TABLE 4

Primers Used for PCR and Nucleotide Positions of the Regions That Were Sequenced from the Respective Gene Segments

Gene	Forward primer	Reverse primer	Positions of nucleotides sequenced[a]
PB2	ATGGAAGTTGTTTTCCC	TCATCGTCAATGATGTGGGA	967–1575
PB1	AGGGACAACATGACCAAGAAAATG	GTTTGAATTTGTGTGTCACCTCTGTC	610–1349
PA	TTCTCATTCACTGGGGAGGAAATGGC	GGCAGCGCCTCATTTCCATTCCCC	1134–1726
HA	AGCAAAAGCAGGGG	CCATACCATCCATCTATCA	1–1032
NP	AGCAAAAGCAGGGT	AGTAGAAACAAGGGTATTTTTC	687–1432
NA	GGGTTTAAGATGAATCCAAATC	TAGGTAATGATTCTTTGGTTGC	14–141
M	CTTCTAACCGAGGTCGAAACG	AGGGCATTTTGGACAAAKCGTCTA	97–222
NS	AGCAAAAGCAAGGGTG	AGTAGAAACAAGGGTGTTTTTTA	258–720

[a] The first nucleotide of the coding region of the respective gene segments was used to identify position 1.

hibition assays were performed with four viral hemagglutinating units and turkey erythrocytes and with a panel of ferret antisera directed against human and swine influenza viruses, according to standard procedures (Palmer *et al.,* 1975).

Sequence analysis

Selected regions of all gene segments of A/Netherlands/386/86 and A/Netherlands/477/93 and of a number of European swine H1N1 influenza viruses (see above) were amplified by RT-PCR and subsequently sequenced as previously described (Voeten *et al.,* 2000). The primers used for PCR, and the regions of the respective gene segments that were sequenced, are indicated in Table 4. The nucleotide sequences of the HA1 region of these viruses were submitted to GenBank (accession numbers: AF320056–AF320067). Phylogenetic analysis (DNAMLK version 3.5c) was performed after multiple alignment of the sequences (ClustalW).

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