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Infection of Children with Avian–Human Reassortant Influenza Virus from Pigs in Europe

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Pigs have been proposed to act as the intermediate hosts in the generation of pandemic human influenza strains by reassortment of genes from avian and human influenza virus strains. The circulation of avian-like H1N1 influenza viruses in European pigs since 1979 and the detection of human–avian reassortants in pigs raises the question of whether these viruses actually have the potential to transmit and cause disease in humans. We now report the serologic and genetic characterization of two human influenza A viruses (A/Netherlands/5/93 [H3N2] and A/Netherlands/35/93 [H3N2]) that caused influenza in children in The Netherlands in 1993. The results show that these viruses are human–avian reassortants that were generated and currently still are circulating in European swine. This shows the pivotal role that pigs can play in the generation and transmission of avian influenza virus genes to humans and their potential to generate a new human pandemic strain.

The influenza viruses responsible for the 1957 and 1968 human pandemics originated in Southern China and contained genes from both human- and avian-like origins (*1–4*). Pigs have been suggested as the hosts (mixing vessels) for reassortment events involving human and avian influenza virus strains (*2, 5, 6*). Since 1979 avian-like H1N1 viruses have been dominant in the European pig population (*7, 8*), which also harbors virus strains antigenically related to the human H3N2 viruses that caused the 1968 influenza pandemic (*9–12*). Recent studies indicate only minor antigenic drift among these early human H3N2 strains while circulating in pigs (*13–15*). Thus, conditions within the European pig population favor the emergence of influenza virus reassortants and most importantly, they offer the possibility of transferring avian influenza genes to humans. Indeed, Castrucci *et al.* (*16*) have reported evidence for reassortment between a human-like H3N2 virus and an avian-like H1N1 virus during the period 1983–1985. The H3N2 reassortant believed to have been generated by this event (e.g., A/Swine/Italy/809/89) possesses avian-like genes encoding internal proteins and human-like H3HA and N2NA genes, and is now circulating in Italian pigs. Here we report the transmission of analogous H3N2 reassortants from pigs to children living in The Netherlands.

In the 1992/1993 season human influenza A/H1N1, A/H3N2, and B subtypes circulated, and in Europe over 80% of the isolations were influenza B viruses. Two strains of H3N2 viruses were isolated. The major part of these isolates in Europe and the U.S.A. was antigenically similar to A/HongKong/34/90-like strains, a strain represented in the 1993/1994 vaccine by A/Beijing/32/92. Only a minor part, in The Netherlands only 5 of 40 H3N2 isolates, resembled the A/Beijing/353/89-like vaccine strain of that season. In addition two aberrant viruses, A/Netherlands/5/93 (H3N2) and A/Netherlands/35/93 (H3N2), were isolated in The Netherlands and in the present paper we describe the results of their serologic and genetic analysis. The two viruses were isolated in different regional laboratories from young children with mild respiratory symptoms who were living in geographically distinct areas. The first child, a 1-year-old girl, resided in the southern region of The Netherlands in Oisterwijk, while the second, a 2-year-old boy, resided in the eastern region in Laren. Examination of sera collected from the infant girl 2 months after the onset of influenza symptoms revealed a high haemagglutination-inhibition (HI) antibody titer (1:2172) to the A/Netherlands/5/93 isolate. A relatively high titer was also found in the serum from the girl's father (1:272), but not in the serum from her mother (1:34). Similar serologic results were obtained with an older H3N2 virus (A/Port Chalmers/1/73). Titers to recent H3N2 isolates (e.g., A/Beijing/32/92 [H3N2]) were less than 1:10 in each family member. Both isolates produced only low titers with ferret antisera against recently isolated human H3N2 strains (A/Beijing/353/89, A/Beijing/32/92, and A/Netherlands/3/93), but they reacted strongly with antisera against older human H3N2 strains (A/Port

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TABLE 1

HAEMAGGLUTINATION-INHIBITION (HI) TITERS WITH INFLUENZA A (H3N2) STRAINS[a]

Antigens (H3N2)	Ferret antisera to: A/Port Chalmers/1/73	A/Victoria/3/75	A/Swine/Utrecht/85	A/Beijing/353/89	A/Beijing/32/92	A/Netherlands/3/93	A/Netherlands/5/93	A/Netherlands/35/93
A/Port Chalmers/1/73	**1280**	640	640	<10	<10	<10	80	1280
A/Victoria/3/75	640	**5120**	1280	<10	<10	<10	160	2560
A/Swine/Utrecht/85	640	640	**1280**	80	<10	10	640	2560
A/Beijing/353/89	<10	<10	<10	**5120**	40	20	40	<10
A/Beijing/32/92	<10	<10	<10	160	**2560**	640	<10	10
A/Netherlands/3/93	<10	<10	<10	10	320	**320**	<10	<10
A/Netherlands/5/93	320	320	2560	20	<20	<10	**1280**	2560
A/Netherlands/35/93	640	640	2560	40	<20	<10	1280	**2560**

[a] Homologous titers are in bold print. The HI assay was performed according to standard procedures (2).

Chalmers/1/73 and A/Victoria/3/75) and a swine H3N2 virus (A/Swine/Utrecht/85) (Table 1). By contrast, a representative H3N2 human isolate from 1993 (A/Netherlands/3/93) reacted only with an antiserum to a recent human virus (A/Beijing/32/92).

To establish the phylogenetic relationships of the A/Netherlands/5/93 and A/Netherlands/35/93 strains, we sequenced the entire HA1 region and 400 to 500 nucleotides of each of the remaining genes. The same parts were sequenced from A/Swine/Utrecht/85 (H3N2). In addition, we obtained a recent H3N2 swine virus isolate, A/Swine/Netherlands/93 (H3N2), from Oisterwijk, the residence of the infant girl, of which the NP gene was sequenced. These sequences were compared with published sequences.

Figure 1 shows a phylogenetic tree constructed from nucleoprotein (NP) gene sequences. Viruses isolated from the same animal species formed clusters (species-specific lineages) in the tree, allowing us to identify the origins and interspecies transfer of individual genes. Thus, the NP genes of viruses isolated from European pigs (denoted by asterisks) belong to the avian lineage (17). More specifically, the NP genes of the two 1993 swine-like human isolates belong to this European swine lineage and are closely related to A/Swine/Italy/809/89 and A/Swine/Utrecht/85, the avian–human reassortants found in an Italian and Dutch pig, respectively. A/Swine/Netherlands/93, isolated in Oisterwijk, was phylogenetically most closely related to the isolate from the girl, strongly supporting the transmission of the avian–human reassortant from pigs to humans. In addition, the sequences of the PB2, PB1, PA, M, and NS genes also clustered with avian-like virus sequences, whereas the HA and NA clustered with human-like virus sequences (Table 2). This clearly shows the transmission to humans of a human–avian reassortant virus which is generated and currently still circulating in European pigs.

Corresponding genes of the two human isolates differed by at least 1.3%, suggesting independent transmission of the viruses from pigs to humans and independent circulation of the two viruses in their local swine population.

For genetic reassortment to occur for the generation of a pandemic strain (i.e., avian–human reassortants), a host has to be infected with both avian and human viruses. That pigs can serve as "mixing vessels" for genetic

FIG. 1. Phylogenetic tree for the NP genes of influenza A viruses. The viruses whose NP genes were sequenced for this study are shown in boxes; the others have been described (17). Swine viruses that form the avian-like European swine lineage are noted with asterisks. The tree is rooted to the A/Equine/Prague/56 NP gene. Roman numerals indicate species-specific lineages: I, recent equine; II, gull; III, avian; IV, classic swine; V, human. SWUTR85, A/Swine/Utrecht/85 (H3N2); NETH35-93, A/Netherlands/35/93 (H3N2); NETH5-93, A/Netherlands/5/93 (H3N2); SWNETH93, A/Swine/Netherlands/93 (H3N2). Abbreviations for other viruses have been described previously (17). Phylogenies were determined with PAUP Software Version 2.4 (David Swofford, Illinois National History Survey), which uses maximum-parsimony algorithm to find the shortest trees. The horizontal distances are proportional to the minimum number of nucleotide differences needed to join the gene sequences. The vertical lines merely space branches and labels.

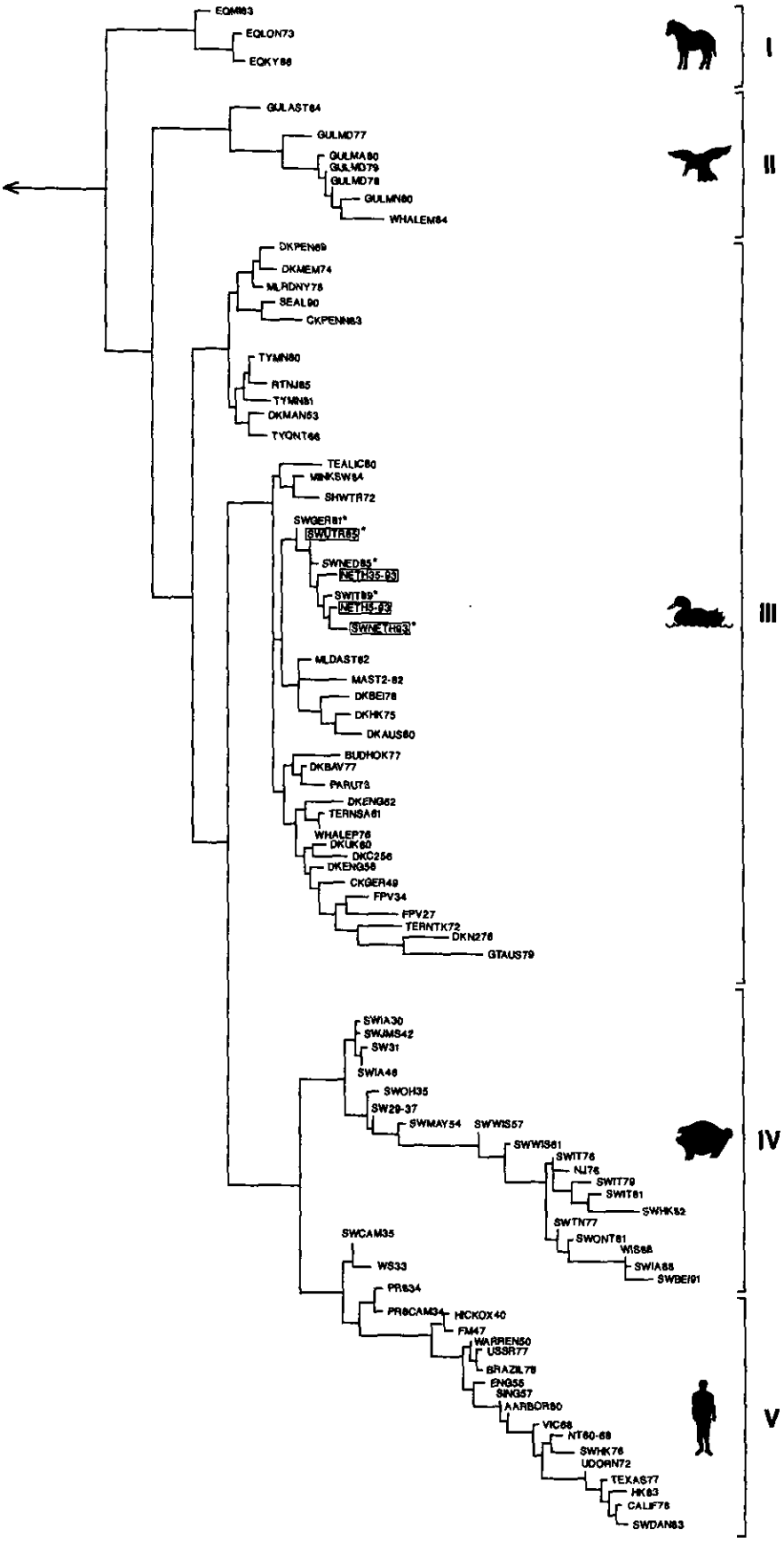
EQM93
EQLON73
EQKY86
GULAST84
GULMD77
GULMA80
GULMD79
GULMD78
GULMN80
WHALEM84
DKPEN69
DKMEM74
MLRDNY78
SEAL90
CKPENN83
TYMN80
RTNJ85
TYMN81
DKMAN53
TYQNT66
TEALIC80
MINKSW84
SHWTR72
SWQER81*
SWUTR82*
SWNED85*
NETH35-93
SWIT89*
NETH5-93
SWNETH93*
MLDAST82
MAST2-82
DKBEI78
DKHK75
DKAUS80
BUDHOK77
DKBAV77
PARU73
DKENG82
TERNSA81
WHALEP76
DKUK80
DKC256
DKENG58
CKGER49
FPV34
FPV27
TERNTK72
DKN278
GTAUS79
SWIA30
SWJMS42
SW31
SWIA46
SWOH35
SW29-37
SWMAY54
SWWS57
SWWS61
SWIT76
NJ76
SWIT79
SWIT81
SWHK52
SWTN77
SWONT81
WIS88
SWIA88
SWBEI91
SWCAM35
WS33
PR834
PR8CAM34
HICKOX40
FM47
WARREN50
USSR77
BRAZIL78
ENG55
SING57
AARBOR60
VIC68
NT60-68
SWHK76
UDORN72
TEXAS77
HK83
CALF78
SWDAN83
I
II
III
IV
V

TABLE 2

ORIGINS OF THE GENES OF THE A/NETHERLANDS/5/93 (H3N2) AND A/NETHERLANDS/35/93 (H3N2) VIRUSES[a]

Gene	No. of nucleotides sequenced[b]	Virus		
		A/Swine/Italy/89[c]	A/Netherlands/5/93	A/Netherlands/35/93
HA	984	H	H	H
NA	474	H	H	H
PB2	455	A	A	A
PB1	440	A	A	A
PA	502	A	A	A
NP	404	A	A	A
M	459	A	A	A
NS	522	A	A	A

[a] Determined by direct nucleotide sequencing of PCR-amplified DNA fragments (Fmol sequencing kit, Promega, Madison, WI) and phylogenetic analysis using the PAUP software (*16*). A, genes belonging to the European swine virus branch of the Eurasian avian lineage; H, genes belonging to the human lineage.

[b] The whole HA1 part of the HA gene was determined. The exact positions of the sequences of the other genes are: N2 (770–1034 and 1128–1338), PB2 (967–1232 and 1411–1601), PB1 (378–588 and 1247–1486), PA (38–284 and 358–614), NP (1048–1452), M (99–316 and 624–866) and NS (24–265 and 583–864).

[c] The origins of the genes were determined previously and are similar to those of A/Swine/Utrecht/85 (H3N2).

reassortment between avian and human influenza A viruses was first proposed by Scholtissek and Naylor (*5*). Direct support for this hypothesis was recently presented by Castrucci *et al.* (*16*), but examples of pig-derived human–avian viral reassortants circulating in humans were not available when we began our study. Interspecies transmission of influenza viruses between humans and pigs has been described regularly (*18–21*). The genetic and serologic findings presented here indicate that both of The Netherlands isolates are close homologues of avian–human reassortant viruses circulating in Italian pigs. This evidence for interspecies transmission of the reassortant viruses, together with the earlier observation (*16*), establishes the role of pigs as vehicles for the generation and transmission of potentially dangerous influenza A viruses.

The exact mode of virus transmission in our two cases is not clear, although the data presented here indicate a pig-to-person spread of both isolates. Person-to-person transmission must have occurred as well because neither child had been in close contact with pigs. However, the father of the boy in whom A/Netherlands/35/93 was found was regularly working on a pig farm, and serum from the father of the infant girl cross-reacted in an HI assay with her A/Netherlands/5/93 (H3N2) isolate. The absence of influenza signs and symptoms in both fathers and other family members could be interpreted as evidence for partial immunity to H3N2 strains due to previous exposure of the adults to H3N2 strains. Resistance to infection could be expected in persons over 20 years old, since the two reassortants are antigenically related to H3N2 strains that circulated in humans in 1973–1975. However, the proportion of people who are susceptible to such viruses will increase each year, thus increasing the likelihood of a major influenza outbreak.

These studies demonstrate that avian influenza viruses from European pigs, in combination with suitable surface glycoproteins, have the potential to replicate and cause disease in humans. The presence of an avian influenza virus in the intermediate host with the potential to replicate in humans may increase the possibility of introduction of new influenza virus genes into humans. It is now 26 years since the H2 influenza viruses disappeared from humans. The possibility exists that this subtype could be reintroduced from the aquatic bird reservoir through the pig. Alternatively, one of the other haemagglutinin subtypes (H4 through H14) may have the possibility of being introduced into humans by reassortment with the avian influenza virus in pigs that is now known to have the capacity to transmit to and replicate in humans.

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