

Genetic Reassortment between Avian and Human Influenza A Viruses in Italian Pigs

MARIA R. CASTRUCCI,*† ISABELLA DONATELLI,† LUIGI SIDOLI,‡ GIUSEPPE BARIGAZZI,‡
YOSHIHIRO KAWAOKA,*¹ AND ROBERT G. WEBSTER*

*Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, Tennessee 38101; †Department of Virology, Istituto Superiore di Sanita, 299 v. le Regina Elena, 00161, Rome, Italy; and ‡Istituto Zooprofilattico Sperimentale, Parma, Italy

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Pandemic strains of influenza A virus arise by genetic reassortment between avian and human viruses. To examine the possibility that pigs serve as "mixing vessels" for such reassortment events (Scholtissek *et al.*, *Virology* 147, 287–294, 1985), we phylogenetically analyzed the internal protein genes of classic H1N1, avian-like H1N1, and human-like H3N2 viruses circulating among Italian pigs. The results show that human-like H3N2 strains isolated from 1985 to 1989 contained the internal protein genes of avian-like H1N1 viruses, whereas those isolated in 1977 and 1983 did not. Thus, at some time between 1983 and 1985, genetic reassortment took place between avian- and human-like viruses in Italian pigs. This study provides the first evidence supporting genetic reassortment between human and avian viruses in a natural swine environment. © 1993 Academic Press, Inc.

Three types of influenza A viruses—classic H1N1, avian-like H1N1, and human-like H3N2—circulate in pigs worldwide. The classic H1N1 viruses are descendants of A/swine/Iowa/15/30 and circulate continuously among pigs in the United States (1). Avian-like H1N1 viruses were first introduced into European pigs in 1979 from avian species (2) and have been maintained exclusively in the swine population since then (1). Human-like H3N2 viruses have been isolated repeatedly from pigs in Europe but only sporadically worldwide (3–7). Thus, in contrast to the situation in the United States, avian-like H1N1 and human-like H3N2 viruses cocirculate in European pigs. Classic H1N1 viruses have also been isolated from Italian pigs (8); however, only the HAs and/or NAs of the isolates were identified, so that the origins of the genes encoding the internal proteins remain unknown.

Influenza A viruses responsible for the 1957 and 1968 human pandemics were reassortants between human and avian strains (9, 10). Three of their genes—PB1 (11), hemagglutinin (HA) (10), and neuraminidase (NA) (10)—were acquired from avian viruses; the remainder came from previously circulating human viruses. Reassortment of avian and human influenza virus genes could occur in several ways. One possibility is the direct introduction of an avian virus into humans. The other possibility is that a reassortant is generated in birds after the introduction of a human virus and then transmitted to humans. Alternatively, other

species of animals susceptible to both human and avian viruses might serve as intermediate hosts. Pigs are attractive candidates for this role. They can harbor human as well as avian influenza viruses (12, 13), and the nucleoprotein (NP) gene of some swine viruses can complement the avian NP gene containing temperature-sensitive mutations, whereas that of human viruses cannot (14). Pigs are involved more frequently in interspecies transmission of influenza A viruses than are other animals (1–8, 15–19). Despite its wide appeal, the "pigs-as-mixing-vessels" hypothesis (14) still lacks definitive support, i.e., the demonstration that this species can accommodate genetic reassortment in a natural setting. Thus, we examined phylogenetic relationships among influenza viruses isolated from Italian swine for evidence of reassortment between human and avian viruses *in vivo*.

The classic H1N1, avian-like H1N1, and human-like H3N2 swine viruses in this study, whose HAs and NAs have been characterized antigenically or genetically (4, 8), were isolated at the Istituto Zooprofilattico Sperimentale, Parma, Italy, from pigs with definite symptoms of acute respiratory diseases (4, 8). The HAs and NAs of human-like H3N2 viruses in pigs are antigenically most closely related to human viruses isolated in 1975 (Castrucci *et al.*, manuscript in preparation). Phylogenetic analysis was based on partial nucleotide sequences of the PA (126 nucleotides), PB1 (169 nucleotides), PB2 (189 nucleotides), NP (203 nucleotides), M (145 nucleotides), and NS (213 nucleotides) genes, determined by reverse transcription using viral RNA as a

¹ To whom reprint requests should be addressed.

TABLE 1
ORIGINS OF GENES ENCODING THE INTERNAL PROTEINS OF INFLUENZA VIRUSES IN ITALIAN PIGS^a

Virus	Subtype	Gene							
		HA	NA	PB1	PB2	PA	NP	M	NS
A/Sw/It/425/76	H1N1	S	S	S	S	S	S	S	S
A/Sw/It/151/81	H1N1	S	S	S	S	S	S	S	S
A/Sw/It/547/85	H1N1	A	A	A	A	A	A	A	A
A/Sw/It/594/86	H1N1	S	S	S	S	S	S	S	S
A/Sw/It/768/88	H1N1	A	A	A	A	A	A	A	A
A/Sw/It/835/89	H1N1	A	A	A	A	A	A	A	A
A/Sw/It/1850/77	H3N2	H	H	H	H	H	H	H	H
A/Sw/It/309/83	H3N2	H	H	H	H	H	H	H	H
A/Sw/It/526/85	H3N2	H	H	A	A	A	A	A	A
A/Sw/It/635/87	H3N2	H	H	A	A	A	A	A	A
A/Sw/It/809/89	H3N2	H	H	A	A	A	A	A	A

^a Partial sequences of each gene, together with sequences from GenBank, were analyzed by the maximum parsimony method (30) using a computer software, Phylogenetic Analysis Using Parsimony (PAUP), version 2.4 (David L. Swofford, Illinois Natural History Survey). Nucleotide sequences of PA (positions 76–201), PB1 (positions 66–234), PB2 (positions 45–233), NP (positions 274–485), M (positions 145–289), and NS (positions 610–812) genes were determined by direct RNA sequencing. The origins of the HA and NA genes have been determined by either genetic or antigenic analysis (4, 8). S, classic swine virus; A, avian-like virus; H, human-like virus.

template and oligonucleotide primers (GenBank Accession numbers L05211 through L05272 and L05474 through L05477) (20). The robustness of the resulting phylogenetic trees was tested by comparison with trees constructed from complete sequences (11, 21–25). Although the branching patterns of some lineages differed with respect to the M and PB1 genes, the viruses belonging to each lineage were identical (data not shown). Hence, we considered phylogenetic analysis of partial sequences a valid approach for our purpose.

Table 1 summarizes results of the phylogenetic analysis. Origins of the genes were determined by phylogenetic analysis of the nucleotide sequences. This was possible because previous findings showed that viruses isolated from the same species cluster in the phylogenetic tree and form species-specific lineages (11, 21, 23–26). Evidence of reassortment was not found among viruses isolated in 1976, 1981, or 1986 that contained the H1 HA of classic swine viruses (Table 1), nor was it found among 1985–1989 isolates that contained an avian-like H1 HA. All of the internal protein genes of these viruses have an avian origin. By contrast, human-like H3N2 viruses isolated from pigs from 1985 to 1989 contained the internal protein genes of avian-like viruses, whereas those isolated in 1977 and 1983 did not. This evolutionary pattern is well illustrated by the phylogenetic tree for the NP gene, which is shown as an example (Fig. 1). Our findings indicate that at some time between 1983 and 1985, genetic reassortment took place between avian- and human-like viruses in Italian pigs. The reassortant generated by this event circulated in the swine population until at least 1989.

It has long been suggested that pigs could serve as a "mixing vessel" for the generation of pandemic strains. The current study presents the first evidence that genetic reassortment occurs between human and avian viruses in pigs in a natural setting, although the genotype of the reassortment (human HA and NA and avian internal protein genes) differs from that of the 1957 and 1968 pandemic strains (avian HA and NA and human internal protein genes). Certain requirements would have to be met before a human pandemic strain of influenza A virus could emerge from pigs. First, both human and avian influenza A viruses would have to infect pigs and be maintained in this population long enough for reassortment to occur. Second, there would have to be a reassortment event. And, third, after reassortment, the new virus would have to be transmitted from pigs to humans. Evidence to support the possible occurrence of the first step (1–8, 15) and the third step (16–19) was available at the outset of our study; however, there was no indication that human and avian influenza virus genes could reassort in a natural porcine milieu. The phylogenetic relationships revealed by this analysis support genetic reassortment in pigs as a possible mechanism for generation of the 1957 and 1968 pandemic strains, but do not exclude the possibility of such an event occurring in birds and humans.

The reassortant H3N2 viruses we identified arose between 1983 and 1985. Whether they superseded the original H3N2 viruses is not clear. In humans, influenza A viruses of two different subtypes have been cocirculating since the H1N1 virus reappeared in 1977 (27). Reassortants derived from these two viruses were isolated soon after the appearance of the H1N1 strain,

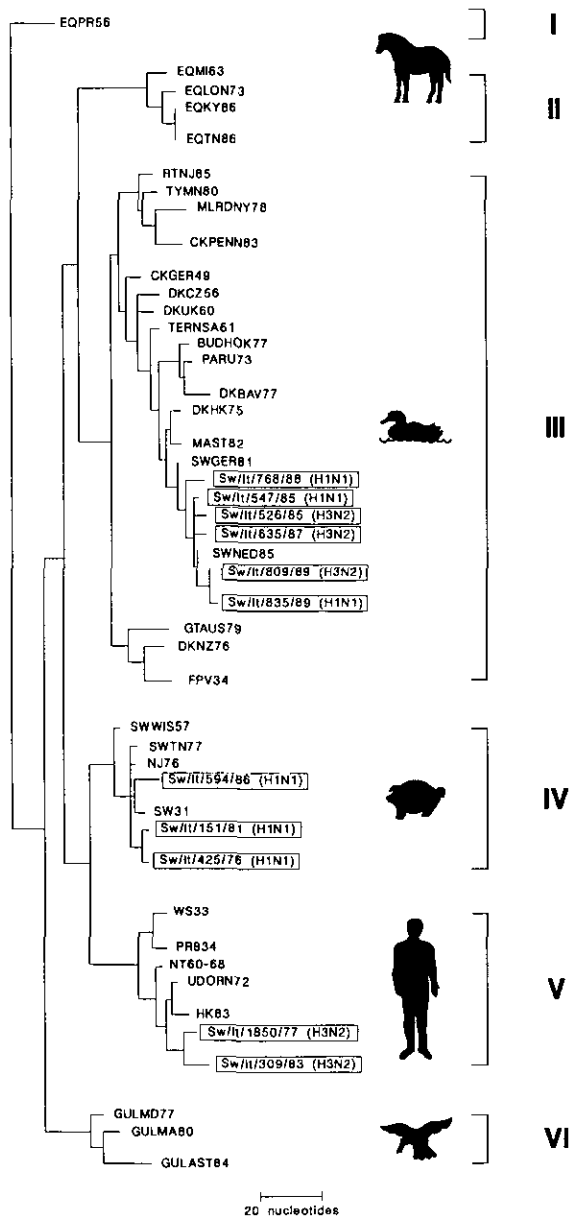


Fig. 1. Phylogenetic tree for influenza A virus NP genes. Nucleotide residues 274–476 of all the NP genes were analyzed by the maximum parsimony method (30) using PAUP. Specific methods of analysis are given in the text and in the footnote to Table 1. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and NP sequences. Vertical lines are for spacing branches and labels. Viruses isolated from Italian pigs are in boxes. Roman numerals indicate species-specific lineages: I, equine/Prague/56; II, recent equine; III, avian; IV, classic swine; V, human; VI, gull. Abbreviations for viruses may be found in Gorman *et al.* (21).

but they circulated for only several years (28). Similarly, reassortant viruses with the H1N2 subtypes were isolated in China in 1990 but did not continue to circulate or spread globally (29). By contrast, equine H7N7 viruses isolated after 1973 were reassortants whose internal protein genes were derived from the equine H3N8 virus, and they superseded the original H7N7

viruses (21, 23). Thus, given the available information, it would be difficult to predict the longevity of human–avian reassortants circulating in pigs.

Each of the three types of influenza A viruses with different origins of the HAs and NAs has been isolated from Italian pigs for over 10 years. This diversity is unique, considering that in the United States only the classic H1N1 viruses have been continuously isolated from pigs. Closer examination of Italian agricultural practices may provide a clue to the differences in epidemiology between swine influenza A viruses in Italy and the United States. Because we analyzed partial sequences in the present study, it is not clear whether each type of influenza virus was introduced only once and then maintained or was introduced many times. The answer will depend on the results of phylogenetic analysis of complete nucleotide sequences.

Previous findings underscore the pivotal role of pigs in the interspecies transmission of influenza A viruses (21, 26). Thus, consideration should be given to eradication of influenza A viruses from pigs. Inactivated vaccines to prevent swine influenza are available, although they have not been widely used to date. This might be a reasonable strategy to lessen the odds for emergence of new pandemic strains.

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