

## Continued Evolution of H1N1 and H3N2 Influenza Viruses in Pigs in Italy

Laura Campitelli,<sup>\*1</sup> Isabella Donatelli,<sup>\*</sup> Emanuela Foni,<sup>†</sup> Maria Rita Castrucci,<sup>\*</sup> Concetta Fabiani,<sup>\*</sup> Yoshihiro Kawaoka,<sup>‡</sup> Scott Krauss,<sup>‡</sup> and Robert G. Webster<sup>‡</sup>

<sup>\*</sup>Department of Virology and WHO National Influenza Centre, Istituto Superiore di Sanita, Rome 00161, Italy; <sup>†</sup>Istituto Zooprofilattico Sperimentale, Parma 41300, Italy; and <sup>‡</sup>Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105-0318

Received November 26, 1996; returned to author for revision January 17, 1997; accepted February 24, 1997

Swine influenza viruses possessing avian genes were first detected in Europe in 1979 (Scholtissek *et al.*, 1983, *Virology*, 129, 521–523) and continue to circulate in pigs in that region of the world. To characterize the molecular epidemiology of swine influenza viruses currently circulating in Europe, we used dot-blot hybridization and sequence analysis to determine the origin of the genes encoding the nonsurface proteins ("internal" genes) of 10 H1N1 and 11 H3N2 swine influenza viruses isolated in Italy between 1992 and 1995. All of the 126 genes examined were of avian origin; thus the currently circulating H3N2 strains which possess A/Port Chalmers/1/73-like surface glycoproteins appear to be descendants of the reassortant human–avian viruses that emerged between 1983 and 1985 in Italy. Sequence analysis of matrix (M), nonstructural, and nucleoprotein genes, as well as phylogenetic analysis of M gene showed that the H1N1 and H3N2 viruses from the pigs were closely related to recent isolates of the avian-like swine H1N1 influenza strain currently circulating in northern Europe and were distinguishable from the genes of viruses isolated from European swine in 1979. To evaluate the frequency of transmission of swine H1N1 and H3N2 viruses to man, we tested 123 human sera for hemagglutination-inhibiting antibodies against avian and mammalian H1N1 and H3N2 virus strains. Our findings indicate that swine influenza viruses possessing A/Port Chalmers/1/73-like hemagglutinin may have transmitted to approximately 20% of young persons under 20 years of age who had contact with pigs. Thus, H3N2 swine viruses, possibly possessing avian-derived internal genes, may be entering humans more often than was previously thought. We strongly recommend that pigs be regularly monitored as a potential early warning system for detection of future pandemic strains. © 1997 Academic Press

### INTRODUCTION

Influenza virus strains responsible for human pandemics during this century appear to have arisen either by genetic reassortment between human and avian influenza viruses or by introduction of an avian virus *in toto* into a human host. The viruses that caused the 1957 and 1968 influenza pandemics resulted from reassortment events (Webster and Laver, 1972; Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989). In contrast, according to phylogenetic data, the causative agent of the 1918 "Spanish" flu pandemic apparently derived all of its eight genes (without reassortment) from an avian influenza strain (Scholtissek *et al.*, 1993; Webster *et al.*, 1992).

Evidence indicates that the avian influenza genes introduced in the 1957 and 1968 pandemic strains were of Eurasian origin (Webster *et al.*, 1992). However, avian influenza viruses replicate poorly, if at all, in humans (Beare and Webster, 1991), possibly because humans do not possess the NeuAc 2,3Gal receptors required for virus attachment to epithelial cells. For this reason, an intermediate host has been postulated. Pigs are the best

candidate for this role because they possess both NeuAc 2,3Gal and 2,6Gal receptors (Y. Kawaoka, unpublished data) and are susceptible to infection by both avian and human influenza viruses (Kida *et al.*, 1994; Schultz *et al.*, 1991; Kundin, 1970). Furthermore, swine influenza viruses can directly infect humans (Rota *et al.*, 1989; Wells *et al.*, 1991).

There was no evidence of classical H1N1 swine influenza in Europe until 1976–1977 (Nardelli *et al.*, 1978; Donatelli *et al.*, 1991). In the mid-1970s, human H3N2 influenza viruses (A/Port Chalmers/1/73-like) were first transmitted to pigs in Italy (Castrucci *et al.*, 1994). In the late 1970s and early 1980s, an avian H1N1 influenza virus was transmitted to swine in Europe, and this virus became established in pigs in Europe as a stable lineage (Scholtissek *et al.*, 1983, 1993). In 1983–1985, swine influenza strains were isolated from pigs in Italy that appeared to result from reassortment between the genes encoding the surface proteins of the human-like swine H3N2 virus and the internal proteins of the swine H1N1 viruses of avian origin (Castrucci *et al.*, 1993). These H3N2 avian–human reassortant swine viruses were capable of transmitting and causing disease in man (Claas *et al.*, 1994).

Thus, the ecoepizootic picture of swine influenza in Europe strongly supports the role of pigs as an intermedi-

<sup>1</sup> To whom correspondence and reprint requests should be addressed at the Department of Virology, Istituto Superiore di Sanita, Viale Regina Elena, 299, Rome 00161, Italy. Fax: (039 6) 4453369. E-mail: epsilon@virus1.net.iss.it.

ate host for the generation of new human influenza viruses and prompts the following questions:

1. Have the H1N1 and H3N2 avian gene-containing swine viruses replaced human-like and classic swine viruses in pigs in Italy? How are the internal protein genes of the Italian swine viruses related to those of swine and avian strains from northern Europe?

2. How frequently are swine H3N2 viruses that express human-like surface antigens transmitted to susceptible human populations (i.e., young persons who lack immunity to early human H3N2 variants and have contact with pigs)?

To provide answers to these questions, we characterized the genes of H1N1 and H3N2 swine influenza viruses isolated in northern Italy from 1992 through 1995. We also examined human sera collected in 1993 from residents of northern Italy for evidence of antibody levels against the influenza variants circulating in swine and humans.

## MATERIALS AND METHODS

### Virus strains

During 1992–1995, 405 samples (nasal swabs or tracheal exudates) were collected from sick or dead pigs from 40 herds located in northern Italy. All the animals had shown clinical symptoms of acute respiratory disease. At the Istituto Zooprofilattico di Parma, a total of 84 influenza viruses were isolated using embryonated chicken eggs. The virus strains were characterized antigenically in hemagglutination-inhibition (HI) and neuraminidase-inhibition tests using polyclonal antisera against reference strains of influenza A viruses. Additional antigenic analysis using monoclonal antibodies to reference influenza A viruses was carried out at the WHO Reference Influenza Surveillance Center (The National Institute for Medical Research, Mill Hill, London). Eleven H3N2 and 10 H1N1 viruses, isolated throughout 1992–1995, were "randomly" selected for the genetic investigations described in the present study.

### Human sera

In May 1993, 123 sera were collected from residents of northern Italy; 97 were from people younger than 20 years of age who were unlikely to have been exposed to early H3N2 human variants antigenically related to A/Port Chalmers/1/73 and A/Victoria/3/75. The remaining 26 sera were from persons older than 20 years (adult group). The younger group was subdivided according to their exposure to pigs. Because they lived in rural areas or due to their profession, 57 of the 97 donors were considered to have had high or moderate exposure to pigs. The remaining 40 people constituted the low-exposure group, because they lived in urban areas and had

no known contact with pigs. Everyone in the adult group had had high exposure to pigs.

### Serologic evaluation by HI tests

HI tests were performed according to standard procedures (Donatelli *et al.*, 1991). Each serum sample was tested against a panel of avian, swine, and human H1N1 and H3N2 influenza strains for the presence of HI antibodies. All tests included antisera to the homologous influenza virus strains.

### Hybridization assay

To characterize the six genes encoding the internal proteins, we performed dot-blot hybridization assays as described previously (Wright, 1992). Briefly, virus RNA was extracted, and a cDNA of each internal protein gene was synthesized by using reverse transcriptase and a 12-base oligodeoxynucleotide primer (5' AGCAAAAGCAGG) that is complementary to a sequence common to the 3' terminus of all influenza vRNA genes. After polymerase chain reaction (PCR) amplification of these cDNAs, the products were cross-linked to a nylon membrane for dot-blot hybridization. Sequences and nucleotide locations of primers and probes have been previously reported (Wright *et al.*, 1992; Lin *et al.*, 1994). All probe synthesis and hybridization reagents were obtained from Boehringer Mannheim (Indianapolis, IN) and used according to the manufacturer's recommendations. Four probes were prepared for each gene: (i) a positive control probe that bound a region common to all influenza viruses, (ii) a probe specific for human influenza sequences, (iii) a probe specific for avian influenza sequences, and (iv) a probe specific for classic swine influenza sequences. In order to assess the degree, if any, of nonspecific probe–virus binding, a genetically unrelated strain (e.g., A/Eq/London/73 [H7N7] or A/Eq/Prague/56 [H7N7]) was included in each assay as a negative control. Probe binding was detected by a colorimetric reaction with x-phosphate and nitroblue tetrazolium salt. The specificity of probe binding was evaluated as previously described (Lin *et al.*, 1994).

### Sequence analysis

Because dot-blot hybridization occasionally led to non-definitive results, some genes were partially sequenced. Purified PCR products corresponding to the PB1, matrix (M), nonstructural (NS), and nucleoprotein (NP) genes were sequenced by *Taq* Dye Terminator chemistry according to the manufacturer's instructions (Applied Biosystems, Inc.), then analyzed on an ABI 373 DNA sequencer. The PB2 and PA genes were analyzed from PCR products by using the fmol sequencing method (Krisnan *et al.*, 1991) with the DNA Cycle Sequencing System (Promega). The sequences obtained were examined with the FastDB sequence analysis program (Intelli-

TABLE 1

## Swine Influenza Viruses Isolated in Northern Italy, 1992–1995

Year of isolation	No. of samples collected	Isolation rates, by subtype			
		H3N2 viruses <sup>a</sup>		H1N1 viruses <sup>b</sup>	
		No.	% of total	No.	% of total
1992	132	14	10.6	19	14.4
1993	94	3	3.2	11	11.0
1994	89	1	1.0	11	12.4
1995	90	19	21.0	6	6.6
Total	405	37	9.1	47	11.6

<sup>a</sup> Antigenically most closely related to A/Sw/OMS/3633/84, A/Sw/OMS/4955/87, and Sw/Belgium/220/92, which are antigenically similar to the early human H3N2 strain (A/Port Chalmers/1/73 [H3N2]).

<sup>b</sup> Antigenically most closely related to avian-like H1N1 strains (A/Sw/OMS/2899/82, A/Sw/OMS/3614/84, and A/Sw/Belgium/303/93).

genetics, Inc.) for homology with other influenza viruses in GenBank.

### Evolutionary analysis

Phylogenetic analysis of the sequencing data was performed with PAUP (phylogenetic analysis using parsimony) software package, Version 2.4, from David Swofford of the Natural History Survey (Champaign, IL), which relies on maximum parsimony to generate a phylogenetic tree.

## RESULTS

### Antigenic properties of viruses isolated from pigs in northern Italy

In 1992–1995, 405 clinical samples were collected from sick or dead pigs during swine influenza outbreaks in northern Italy. The influenza episodes in swine occurred year-round in contrast to the typical influenza circulation pattern in humans in Europe, which is restricted to the winter season. A total of 84 viruses (Table 1) was isolated comprising 37 H3N2 and 47 H1N1 viruses (44 and 56% of the isolates, respectively). Both subtypes circulated simultaneously as had been observed previously (Donatelli *et al.*, 1991). However, the prevalence of the two subtypes varied considerably from year to year. In particular, H1N1 viruses predominated in 1993–1994, and H3N2 strains were detected most frequently in 1995. Antigenic characterization with polyclonal antisera and monoclonal antibodies showed a limited degree of heterogeneity among the strains of each subtype (data not shown). The H1N1 isolates were most closely related to A/Sw/OMS/2899/82, A/Sw/OMS/3614/84, and A/Sw/Belgium/303/93, which are representative of the avian-like H1N1 lineage established in European pigs since 1979. In contrast, the reactivity pattern of the H3N2 vi-

ruses demonstrated close homology with the prototype strains A/Sw/OMS/3633/84, A/Sw/OMS/4955/87, and A/Sw/Belgium/220/92, which are antigenically similar to each other and related to the early human variant A/Port Chalmers/1/73 (H3N2).

### Genotypic characterization of swine influenza isolates

**Dot-blot hybridization.** Since we wanted to determine the host of origin of each of the gene segments of the influenza viruses currently circulating in swine in Italy, we characterized the genes encoding the “internal” proteins of 10 H1N1 and 11 H3N2 isolates from these animals. These represented 13% of the viruses isolated over 4 years and selection for inclusion in the study was “random.” Overall, 126 genes were tested: 94 of them were of avian origin and bound only to avian probes (Table 2). All of the 21 NP and NS gene segments reacted strongly with the avian probe, and most of the PB1, PA, and M gene segments also reacted strongly with the avian probe. A group of 32 genes could not be characterized by the hybridization assay: 20 PB2 genes reacted weakly with only the human probe, 3 M genes cross-reacted with both avian and swine probes while 1 PB1 and 1 PB2 gene reacted weakly with both avian and human probes, and 7 PA genes reacted only with the control probe. Among the 20 PB1 genes, 6 reacted strongly with the avian probe (same signal intensity as the positive control), but 14 bound only weakly. Because they failed to react with any other probe, these 20 PB1 genes were tentatively classified as being of avian origin. This “assignment” was later confirmed by partial sequencing of these genes.

**Sequence analysis.** To identify the host of origin of the RNA segments that could not be clearly defined by dot-blot hybridization and to confirm the dot-blot results, we sequenced the genes listed in Table 3. Base pair regions

TABLE 2

### Genotype Analysis of the “Internal” Protein Genes of H1N1 and H3N2 Swine Influenza Viruses from Northern Italy by Dot-Blot Hybridization

Gene	No. of genes binding to					
	Avian probe only	Human probe only	Swine probe only	Avian and human probes	Avian and swine probes	Control probe only
PB2	0	20 <sup>a</sup>	0	1	0	0
PB1	20	0	0	1	0	0
PA	14	0	0	0	0	7
NP	21	0	0	0	0	0
M	18	0	0	0	3	0
NS	21	0	0	0	0	0
Total	94	20	0	2	3	7

<sup>a</sup> Weak reaction, 10% of the intensity of the positive control.

TABLE 3

## Homology Analysis of the Internal Protein Genes of Recent Italian Swine Influenza Viruses by Partial Sequencing

Virus strain	Gene	Subtype	Nucleotides analyzed	Virus with highest homology	(%)
A/Sw/It/1273/93	PA	H1N1	177–406	A/FPV/Rostock/34	87.2
A/Sw/It/1158/92	PA	H3N2	147–590	A/Dk/OK/8/80	86.0
A/Sw/It/1142/92	PA	H3N2	176–400	A/FPV/Rostock/34	86.2
A/Sw/It/1247-6/92	PA	H3N2	176–400	A/FPV/Rostock/34	87.1
A/Sw/It/1385-1/95	PA	H3N2	144–479	A/Dk/OK/8/80	87.7
A/Sw/It/1259/93	PA	H3N2	177–420	A/Pintail/Alb/119/79	86.4
A/Sw/It/1393-2/95	PA	H3N2	162–627	A/FPV/Rostock/34	88.3
A/Sw/It/1151/92	M	H1N1	496–866	A/Sw/S-H/1/93	96.6
A/Sw/It/1273/93	M	H1N1	420–861	A/Sw/Ger/8533/91	98.0
A/Sw/It/1390-2/95	M	H1N1	526–865	A/Sw/Ger/8533/91	97.9
A/Sw/It/1367-2/94	M	H3N2	623–893	A/Sw/S-H/1/93	98.1
A/Sw/It/1385-1/95	M	H3N2	544–893	A/Sw/S-H/1/93	97.1
A/Sw/It/1394-2/95	M	H3N2	540–886	A/Sw/S-H/1/93	98.5
A/Sw/It/1273/93	NP	H1N1	1080–1455	A/Sw/Ger/8533/91	96.7
A/Sw/It/1385-1/95	NP	H3N2	1054–1406	A/Sw/S-H/1/93	97.4
A/Sw/It/1151/92	NS	H1N1	251–550	A/Sw/Ger/8533/91	96.6
A/Sw/It/1149/92	NS	H3N2	133–599	A/Sw/Ger/8533/91	98.4
A/Sw/It/1385-1/95	NS	H3N2	308–599	A/Sw/Ger/8533/91	96.5
A/Sw/It/1149/92	PB1	H3N2	836–1420	A/Singapore/1/57	88.6
A/Sw/It/1407-4/95	PB1	H3N2	980–1491	A/Singapore/1/57	89.1
A/Sw/It/1145/92	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	95.8
A/Sw/It/1151/92	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	96.6
A/Sw/It/1198/92	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	97.0
A/Sw/It/1247-8/92	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	97.0
A/Sw/It/1273/93	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	98.3
A/Sw/It/1376-3/94	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	97.4
A/Sw/It/1353/94	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	96.6
A/Sw/It/1369-7/94	PB2	H1N1	1353–1588	A/Sw/Ger/2/81	96.6
A/Sw/It/1158/92	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	98.3
A/Sw/It/1149/92	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	97.0
A/Sw/It/1247-6/92	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	98.3
A/Sw/It/1259/93	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	97.9
A/Sw/It/1278/93	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	97.0
A/Sw/It/1367-2/94	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	96.6
A/Sw/It/1393-2/95	PB2	H3N2	1353–1588	A/Sw/Ger/2/81	96.6

*Note.* For reasons of space, only 15 of the 21 PB2 genes sequenced are included. The data on the remaining 6 genes are comparable to those shown. DK, duck; Sw, swine; FPV, fowl plague virus; S-H, Schleswig–Holstein; Alb, Alberta; Ger, Germany; OK, Oklahoma.

of approximately 200–500 nucleotides were compared among these Italian swine isolates and to sequences available in GenBank. Homology between the Italian swine isolates was very high; the maximum difference within each gene ranged between 6.4 (for PB2) and 3.8% (for NP). Comparison with sequences in GenBank showed that the “internal” protein genes from the pig influenza viruses were most closely related to genes from avian influenza viruses or to genes derived from avian influenza virus sources (e.g., A/Singapore/1/57 PB1; A/Sw/Ger/8533/91, M, NP, and NS). Overall, the homology for the PA and PB1 genes with avian genes ranged from 86.0 to 89.1%. This reflects the lack of sequence information available from recent European avian PA and PB1 genes in GenBank and will be considered in the discussion. The NP, NS, M, and PB2 genes demonstrated much higher homology (95.8–98.6%). In the case of NP, the

homologies of A/Sw/Italy/1273/93 (H1N1) and A/Sw/1385-1/95 (H3N2) with the 1991 and 1993 swine isolates from Germany were 96.7 and 97.1%, respectively. When aligned with NP, M, and NS sequences from virus groups of different host origin (Table 4), the genes of the representative H1N1 and H3N2 swine influenza strains were most homologous to European avian-like swine viruses (96.1–98.4%). No classic swine or human-derived “internal genes” were detected, either by dot-blot hybridization or by sequencing, among the viruses we analyzed. All of the H3N2 strains contained a full constellation of avian-derived internal genes, as did the first human–avian H3N2 reassortant viruses which emerged during 1983–1985 in pigs in Italy. All of the sequenced genes from the H3N2 viruses were highly homologous (in some cases identical) to the corresponding genes from the H1N1 isolates.

TABLE 4

Homology of Nucleotide Sequences of Italian Swine Influenza Isolates (1992–1995)  
with Representative Viruses of Avian, Swine, and Human Origin

Virus strain	Gene	Subtype	Virus groups (% homology to test strain)				
			Avian-like swine	Recent European avian	Avian	Classic swine	Human
A/Sw/It/1273/93	NP	H1N1	96.7	93.2 <sup>a</sup>	88.6	83.7	84.2 <sup>b</sup>
A/Sw/It/1385-1/95	NP	H3N2	97.1	92.8 <sup>a</sup>	88.7	83.2	84.1 <sup>b</sup>
A/Sw/It/1151/92	NS	H1N1	96.6	93.6	86.9	84.3	83.9
A/Sw/It/1385-1/95	NS	H3N2	96.1	94.0	88.7	85.2	85.6
A/Sw/It/1151/92	M	H1N1	98.4	93.2	91.6	87.3	89.5
A/Sw/It/1385-1/95	M	H3N2	97.1	93.4	92.3	86.8	89.3
A/Sw/It/1273/93	PB2	H1N1	98.3 <sup>c</sup>	Not done	87.7	81.4	80.1
A/Sw/It/1247-6/94	PB2	H3N2	98.3 <sup>c</sup>	Not done	87.7	81.4	80.1

Note. The nucleotide positions analyzed are those described in Table 3. Representative virus strains used for comparison were A/Sw/Germany/8533/91 (avian-like swine), A/Oystercatcher/Germany/87 (recent European avian), A/Fowl Plague Virus/Rostock/34 (avian), A/Sw/Iowa/15/30 (classic swine), and A/WSN/33 (human).

<sup>a,b,c</sup> Comparison with A/Dk/Bavaria/2/77 (H1N1), A/WS/33 (H1N1), and A/Sw/Ger/2/81 (H1N1), respectively.

### Phylogenetic analysis

To assess the evolutionary relationships of these H1N1 and H3N2 swine strains, a phylogenetic tree of the M gene was constructed, which included three representative viruses from pigs in Italy (one H1N1 and two H3N2 isolates). The viruses examined clustered on the European avian-like swine branch, which forms a clearly distinct sublineage within the avian branch (Fig. 1). Both subtypes were grouped with recent North European avian-like H1N1 isolates, A/Swine/Germany/8533/91 and A/Turkey/Germany/3/91 (which was transmitted to turkeys from pigs), giving further support to the indication emerging from dot-blot and homology data that the swine H3N2 viruses, containing avian-like "internal protein" genes, established a stable lineage in Italian pigs.

### Detection of antibodies in human sera

To determine if H3N2 swine influenza viruses currently circulating in Italian pigs have a propensity to spread to humans, we examined 123 human sera collected in northern Italy during May 1993 using HI tests. We detected HI antibodies against swine H1N1 strains (either classic swine or avian-like) at HI titers of 40 in only 4 samples (Table 5). In contrast, 77 (63%) of the sera had HI antibodies to the human-avian reassortant influenza virus A/Sw/Italy/1394-2/95 (H3N2) (antigenically similar to the older human H3N2 variant A/Port Chalmers/1/73), with titers  $\geq 10$ , and 47 (38%) had titers  $\geq 40$ . When assayed in HI tests against the currently circulating human strain A/Beijing/32/92 (H3N2), 68 (55%) of the sera had titers  $\geq 10$  and 31 (25%) had titers  $\geq 40$ . Thus, more sera were reactive with the swine A/Port Chalmers/1/73-like virus than with the currently circulating human H3N2 strain.

From a subset of 97 persons under the age of 20 years, 13 sera either reacted exclusively with the A/Sw/It/1394-2/95 (Port Chalmers-like, H3N2) virus (9/13) or had concomitant HI titers to the swine H3N2 strain fourfold above those to A/Beijing/32/92 (H3N2) virus (4/13) (Table 6). When the exposure of this group of subjects to swine was examined, 19.3% of the young persons with moderate to high exposure to pigs had HI titers of  $\geq 10$  to the H3N2 swine virus (73% of them being  $\geq 40$ , data not shown). In contrast, only 5% of young people who lacked exposure to pigs had antibodies to this strain. The difference between the two frequencies was significant ( $\chi^2 = 3.0$ ,  $0.05 < P < 0.10$ ) and the odds ratio value, 4.54, indicated that the subjects with antibodies had a more than fourfold higher risk of having been exposed to infected pigs when compared to the subjects without antibodies. Overall, our findings suggest that H3N2 swine viruses circulating in Italian pigs are infecting humans who have contact with swine. Since the HI test does not discriminate between human-like and human-avian reassortant swine virus, from our test results we could not establish which of the two virus types was transmitted to the positive subjects. However, 3 of the young people whose sera reacted exclusively with A/Port Chalmers-like antigens had been exposed to live and dead pigs only after 1989. Since no Port Chalmers-like viruses have been isolated in Italy since 1985, we can conclude that at least in some cases the antibodies detected were elicited by infection with human-avian reassortant viruses.

## DISCUSSION

### Analysis of the gene pool of swine influenza viruses isolated in Italy

Analysis by dot-blot hybridization and nucleotide sequencing of influenza viruses isolated during 1992–1995

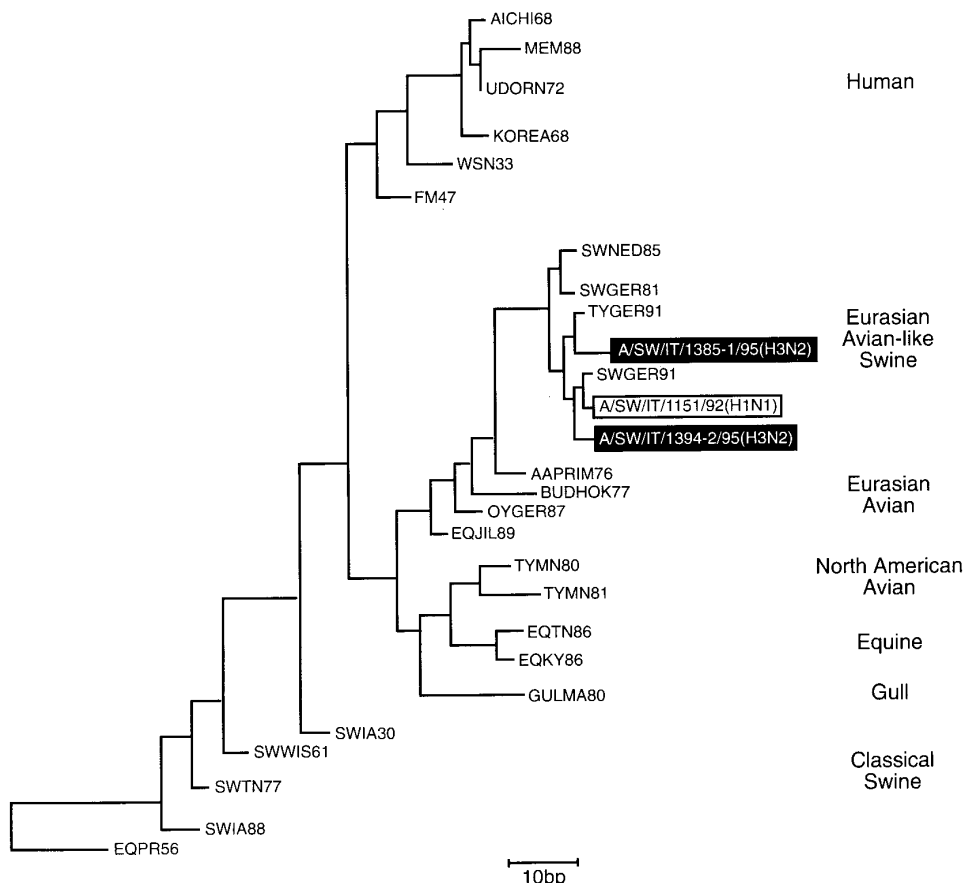


FIG. 1. Phylogenetic tree for the M genes of influenza A viruses. The tree is rooted to the M gene of A/Equine/Prague/56 (H7N7) and is derived from 306 bp from residues 540 to 845. The M genes of three Italian swine influenza viruses (one H1N1 and two H3N2 isolates) were analyzed in combination with the nucleotide sequences of M genes available in GenBank. Analysis was performed with the PAUP program, Version 2.4 (David L. Swofford, Illinois Natural History Survey), which utilizes a maximum parsimony algorithm. The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes. Vertical lines are for spacing between branches only, and do not represent any evolutionary distance. Virus names can be identified in Ito *et al.* (1991) or Gorman *et al.* (1991) except for the following: TYGER91, A/Turkey/Germany/3/91 (H1N1); SWGER91, A/Swine/Germany/8533/91 (H1N1); AAPRIM76, A/*Anas acuta*/Primorje/695/76 (H3N2); OYGER87, A/Oystercatcher/Germany/87 (H1N1); and EQJIL89, A/Equine/Jilin/1/89 (H3N8).

from pigs in Italy revealed that the "internal" genes of both H1N1 and H3N2 Italian swine viruses were most similar to genes of avian origin. Strong evidence (95.8–98.6% homology) was obtained with the M, NP, NS, and

PB2 genes when compared to avian-like swine strains, such as A/Swine/Germany/8533/91.

Despite the absence of any data on the PA and PB1 genes of Eurasian avian origin, the Italian swine PA and

TABLE 5

Prevalence of Antibody to Swine and Human Influenza Viruses in 123 Human Sera Collected in May 1993

Antigens	Host of origin	Titer $\geq 10$		Titer $\geq 40$	
		No.	Percentage of sera samples	No.	Percentage of sera samples
A/Swine/Italy/1145/92 (H1N1)	Avian-like	8	6.5	2	1.6
A/Swine/Italy/594/86 (H1N1)	Classic swine	13	11	1	0.8
A/Swine/Beijing/47/91 (H1N1)	Classic swine	8	6.5	1	0.8
A/Texas/31/91 (H1N1)	Human	66	54	38	31
A/Swine/Italy/1394-2/95 (H3N2)	Human-avian	77	63	47	38
A/Beijing/32/92 (H3N2)	Human	68	55	31	25

TABLE 6

Antibody to A/Swine/Italy/1394-2/95 among People Younger Than 20 Years of Age

Degree of exposure to pigs	No. of people	HI antibody titers to A/Sw/It/1394-2/95 (H3N2) Titer $\geq 10$	
		No.	%
High or moderate	57	11	19.3***
Low or none	40	2	5.0
Total	97	13	13.4

Note. Sera taken into account in this evaluation had titers for A/Beijing/32/92 (human H3N2 strain) which were either  $< 10$  (9/13) or fourfold lower than the titers to A/Sw/It/1394-2/95 (4/13).

\*  $\chi^2 = 3.0$  (Yates corrected),  $0.05 < P < 0.10$ .

\*\* Odds ratio = 4.54.

PB1 sequences were most closely related to avian influenza genes, although homology was only 86.0–89.1%. However, similar values (86.9–88.7%) were observed with NS, NP, and PB2 genes when they were compared with the old European avian variant A/Fowl Plague Virus/Rostock/34 (Table 4), indicating conservation of these genes.

Phylogenetic analysis of the M gene indicated that the Italian swine isolates were most closely related to recent northern European H1N1 swine viruses (1991 and 1993 strains) rather than to earlier strains, confirming the homology data. It also showed that the avian-like European swine virus group forms a lineage with a clearly distinct evolutionary pattern within the avian branch, even when compared to the most closely related avian strains, like A/Oystercatcher/Germany/87. The evolutionary analysis of NP and PB2 showed a similar pattern (data not shown). Previous reports demonstrated that the hemagglutinin (HA), NP, M, and NS genes of north European avian-like H1N1 swine viruses have the highest rate of evolution ever measured for influenza viruses (Scholtissek *et al.*, 1993; Ludwig *et al.*, 1994). A mutated polymerase complex (mutator mutation) capable of rapidly creating changes in all genes may account for this observation and such a mutation could enable an avian virus to cross the species barrier, adapting more easily to a new mammalian host (Suàrez *et al.*, 1992; Ludwig *et al.*, 1995). Overall, our observations suggest that both H3N2 and H1N1 Italian swine isolates underwent variation comparable to that of northern European swine viruses.

Of the four swine influenza types circulating in pigs in Italy in the early 1980s, two have disappeared (classic swine H1N1 and human-like A/Port Chalmers/1/73 viruses). The H3N2 reassortant virus possessing avian "internal" genes, which first emerged in 1983–1985 in Italian pigs, has apparently superseded the antigenically related human-like variant. Introduction of later H3N2 hu-

man variants into pigs appears not to have occurred despite the multiple transmissions of early H3N2 human variants to pigs, which took place several times in different geographic areas (Castrucci *et al.*, 1994; Kundin, 1970; Hinshaw *et al.*, 1978; Shu *et al.*, 1994). In addition, our data show that since their introduction into pigs in Italy, avian-like H1N1 strains appear to have replaced the classic swine viruses. In contrast, classic swine viruses still predominate in the United States and China (Noble *et al.*, 1993; Shu *et al.*, 1994).

The cocirculation of multiple influenza strains of different host origins among swine populations raises concern about a possible new major epidemic in humans. Factors prompting this concern include the observations that swine viruses infect humans relatively often (Goldfield *et al.*, 1977; Rota *et al.*, 1989; Claas *et al.*, 1994) and that all influenza pandemics during this century were caused by viruses that contained (*in toto* or in part) avian genes (Webster and Laver, 1972; Scholtissek *et al.*, 1978, 1993; Kawaoka *et al.*, 1989). The successful adaptation in pigs in Italy of avian-derived H1N1 and H3N2 virus lineages suggests that these viruses might have a growth advantage in pigs compared to classic swine and human-derived viruses (which disappeared after a short period of cocirculation). Based on our data, we are unable to say whether the growth advantage depends on a higher mutation rate (possibly due to a mutator mutation), on the high susceptibility of pigs to be infected with avian viruses (Kida *et al.*, 1994), or on some other still unknown mechanism.

### Serologic evidence of human infection with swine influenza viruses

The HI test is useful for detecting influenza viruses with limited circulation in man, like swine viruses, even though its strain specificity is not absolute, and the confounding effect of low-level cross-reactivity must be addressed (Monto and Maassab, 1981). We detected significant HI antibody titers against avian-like swine H1N1 viruses among a limited number of people who had been in close contact with pigs. Since avian-like swine H1N1 viruses have very low cross-reactivity with human H1N1 viruses, humans are unlikely to be protected against European swine viruses containing the avian H1N1 hemagglutinin molecule. In addition, HAs from both human and avian-like swine H1 viruses have the same receptor-binding specificity (Rogers and D'Souza, 1989).

The study in humans suggests that young people in contact with swine may become infected with H3N2 swine influenza viruses at a higher frequency than one would expect on the basis of the sporadic swine virus isolations from man. It can be argued that some of the antibodies to A/Port Chalmers/1/73 (H3N2) may have been due to cross-reactivity with later variants that these persons would have experienced as children, but the

statistically significant correlation with pig exposure and the possibility of person-to-person transmission (Claas *et al.*, 1994) argue in favor of passage of these viruses to humans.

Although H3N2 human-like and human-avian reassortant strains are indistinguishable by HI test, at least in three cases, it is highly probable that the antibodies detected were elicited by H3N2 reassortant strains. This indication reinforces the previous evidence that avian influenza viruses, when reassorted with suitable surface glycoproteins, can infect humans (Claas *et al.*, 1994). Therefore these viruses recently isolated from pigs in Italy may represent a potential threat for the emergence of new human viruses. The H2N2 subtype, which disappeared from man in 1968, or one of the other HA subtypes (H4 through H13), may be introduced into humans by reassortment with avian viruses in pigs, as these animals can be experimentally infected with at least 13 avian virus subtypes (Kida *et al.*, 1994) and the human population under the age of 27 years lacks immunity to these influenza surface antigens. Alternatively, human virus genes encoding proteins involved in host-range specificity (NP, M, and NS) could enter the pig population and generate reassortants more capable of spreading to humans (Scholtissek *et al.*, 1985; Tian *et al.*, 1985; Treanor *et al.*, 1989). Regardless of which mechanism proves to be true, we speculate that the precursor of the next human pandemic could currently be circulating in pigs in Europe.

The above studies provide additional evidence for the possible role of swine in the evolution of human influenza viruses. There is no doubt that influenza viruses in pigs can transmit to humans, and the frequency of this event appears to be higher than the transmission of avian viruses to humans. As the world prepares for the next pandemic, we need as much early warning of this event as possible to facilitate preparation of vaccines and an adequate supply of antivirals. Therefore we strongly recommend that pigs be surveyed on a continuing basis for evidence of novel influenza A virus subtypes or reassortants.

## ACKNOWLEDGMENTS

We thank Dr. Michele Grandolfo for providing statistical analysis of the serological data, Amy L. B. Frazier for editing, and Giulia Pacetto and Dayna Baker for typing the manuscript. This work was supported in part by Public Health Service Research Grants AI-29680 and AI-08831 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) Grant CA21765, CNR-ISS Grant 95.01666.04, and American Lebanese Syrian Associated Charities.

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