

## H3N2 influenza viruses from domestic chickens in Italy: an increasing role for chickens in the ecology of influenza?

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In Italy, multiple H3N2 influenza viruses were isolated from chickens with mild respiratory disease and were shown to replicate in the respiratory tracts of experimentally infected chickens; this finding is the first to show that H3N2 influenza viruses can replicate and cause disease in chickens. H3N2 influenza viruses in pigs on nearby farms seemed a likely source of the virus; however, antigenic and molecular analyses revealed that the gene segments of the viruses in chickens were mainly of Eurasian avian origin and were distinguishable from those isolated from pigs and wild aquatic birds in Italy. Thus, several different H3 influenza viruses were circulating in Italy, but we failed to identify the source of the chicken H3N2 influenza viruses that have disappeared subsequently from Italian poultry. Until recently, the transmission of influenza viruses (other than the H5 and H7 subtypes) from their reservoir in aquatic birds to chickens was rarely detected and highly pathogenic and non-pathogenic viruses were considered to be restricted to poultry species. However, the recent reports of the transmission of H9N2 and H5N1 influenza viruses to chickens in Hong Kong and, subsequently, to humans and our findings of the transmission of H3N2 influenza viruses to domestic chickens in Italy suggest an increased role for chickens as an intermediate host in the ecology of influenza.

### Introduction

Avian species, particularly wild waterfowl, have been recognized as the main reservoir of influenza A viruses in nature (Hinshaw & Webster, 1982). Avian influenza viruses that belong to the 15 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified. Moreover, all influenza viruses isolated in mammals, including those that cause epidemics and pandemics in humans, have been derived, either directly or indirectly, from avian influenza viruses. One of the ecological characteristics of influenza A viruses include interspecies transfer and infection of new hosts. Although the virus genetic factors that control such events remain unknown,

previous studies have suggested that the introduction of avian influenza viruses into other species is unidirectional and the transmission of mammalian viruses back to birds is unlikely (Lin *et al.*, 1994). However, classic swine H1N1 influenza virus and avian-like swine H1N1 influenza virus have been detected in domestic turkeys (Hinshaw *et al.*, 1983; Wright *et al.*, 1992; Ludwig *et al.*, 1994). At least in the case of the avian-like swine H1N1 influenza viruses circulating in European pigs, the viruses may have retained avian host-range determinants, which allowed their transfer back to birds.

In European pigs, H3N2 influenza viruses possess genes encoding non-surface proteins of avian origin (Campitelli *et al.*, 1997). This phenomenon was caused by a reassortment event between human-like H3N2 swine influenza viruses and avian-like H1N1 swine influenza viruses (Castrucci *et al.*, 1993). These viruses, which have been circulating during the past 15 years, can be transmitted to and cause disease in humans (Claas *et al.*, 1994).

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The GenBank accession numbers of the sequences reported in this paper are AF213900–AF213916.

During the winter of 1994–1995, we monitored several chicken farms in northern Italy for the presence of respiratory pathogens in commercial poultry showing signs of mild respiratory disease and a temporary reduction in egg production. Three H3N2 influenza virus strains were isolated from broiler chickens and battery hens located in three distinct farms. Because the farms containing chickens from which H3N2 influenza viruses were isolated were located in an area with many pig farms and because H3N2 swine influenza virus had been detected in those pigs during the autumn of 1994, we questioned whether the pigs were the source of the H3N2 influenza virus that infected the chickens.

Here we report the ability of H3N2 influenza viruses from Italian poultry to replicate in the respiratory tract of experimentally infected chickens and the antigenic and molecular relationships between H3N2 influenza viruses of chickens and swine in Italy.

## Methods

■ **Virus strains.** During the winter of 1994–1995, tracheal swabs and ovary tissue samples were collected from broiler chickens showing signs of mild respiratory disease and battery hens with a temporary reduction in egg production. Specimens were sent to the National Reference Centre for Avian Influenza, Naples, Italy, for virus isolation and characterization. After specific-pathogen-free (SPF), embryonated chickens' eggs were inoculated with the samples, the following three influenza viruses were obtained: Ck/It/5783/95, Ck/It/5954/95 and Ck/It/5945/95. On the basis of their antigenic characteristics, they were classified as viruses of the H3N2 subtype. The identification of the viruses was confirmed by the International Reference Centre for Avian Influenza, Weybridge, UK. During the fall of 1994, respiratory disease was observed among pigs on a nearby farm and one H3N2 influenza virus strain (Sw/It/25823/94; H3N2) was isolated from the lung of one pig.

■ **Antigenic characterization.** Haemagglutination inhibition (HI) assays were performed according to standard procedures (US Department of Health and Human Services, 1982). Viruses were tested with a panel of hyperimmune polyclonal antisera to the following viruses: Dk/Ukr/1/63 (H3N8), Aichi/1/68 (H3N2), PChal/1/73 (H3N2) and Coot/It/153/94 (H3N8), a recent wild duck isolate. Monoclonal antibodies (mAbs) to the early human H3N2 influenza virus strains England/42/72 and Tex/1/77 were also used.

■ **Experimental infections.** Four groups of 3-week-old SPF White Leghorn chickens ( $n = 4$  birds per group) were inoculated intranasally, orally and intratracheally with one of the four avian influenza viruses. Tracheal and cloacal swabs were collected 3 days after infection. Swabs and faeces were suspended in PBS with antibiotics and the suspensions were injected into 11-day-old embryonated chickens' eggs for isolation and titration of virus.

To evaluate the extent of replication and shedding of the H3N2 influenza virus chicken isolates, five 3-week-old SPF White Leghorn chickens were inoculated intranasally, orally and intratracheally with allantoic fluid containing Ck/It/5945/95 (H3N2) influenza virus. Tracheal swabs were collected on days 1–5 after infection for isolation and titration of virus. Two birds were dissected on day 3 to determine the organs in which the virus was replicating. Tissue samples of brain, lung, trachea, colon, spleen, heart, blood, gall bladder, pancreas and intestine were collected aseptically for virus titration. Suspensions (10% v/v) were

prepared in a Stomacher blender and the virus was assayed as described previously (Webster *et al.*, 1981).

To evaluate the degree of pathogenicity of the viruses in chickens, we infected chickens intravenously with one of the three swine influenza virus strains isolated during different years (Sw/It/526/85, Sw/It/25823/94 or Sw/It/1452/96) or with the human variant PChal/1/73. Except for Sw/It/25823/94, which was tested in four birds, each virus was tested in two birds.

■ **DNA sequence analysis.** We used the RNeasy kit (Qiagen) to extract viral RNA (vRNA) from infected allantoic fluid and RT-PCR to amplify gene segments, as described previously (Lin *et al.*, 1994). Briefly, cDNA was synthesized using reverse transcriptase and a 12 bp oligodeoxynucleotide primer complementary to a common sequence in the 3' terminus of all influenza vRNA genes. Regions of gene segments encoding internal proteins were amplified using gene-specific primers. PCR products were sequenced using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI 373A automated sequencer (Applied Biosystems).

■ **Phylogenetic analysis.** We used the GCG program package, version 8.0, to assemble a map of contiguous nucleotides and to perform multiple sequence alignments. Evolutionary analysis of the gene segments was performed by using the neighbour-joining method of the PHYLIP software package, version 3.5 (Felsenstein, 1993). Gene sequences available from GenBank were used in all sequence comparisons and phylogenetic analyses.

## Results

### Isolation of viruses

During the winter of 1994–1995, mild respiratory disease among broiler chickens and a slight reduction in egg production among battery hens were observed at several chicken farms in northern Italy. Although none of the ill chickens died, tracheal specimens from birds with respiratory symptoms and ovary specimens from hens with reduced egg-laying frequency were collected and examined to determine whether pathogenic microbes or viruses, or both, were present.

Three H3N2 influenza viruses were isolated from samples taken at three different farms, which raised a total of 103 000 birds. Ck/It/5954/95 and Ck/It/5945/95 influenza viruses were present in tracheal samples, whereas Ck/It/5783/95 influenza virus was found in ovary samples.

During the weeks before the influenza outbreak in chickens, the H3N2 swine influenza virus Sw/It/25823/94 had been isolated from a nearby swine herd that belonged to the same commercial network. Farm personnel had worked at both the swine farm and the poultry farms.

### Pathogenicity of H3N2 chicken and swine influenza virus isolates in domestic chickens

Because the chicken and swine H3N2 influenza virus strains were isolated in temporal and geographical proximity in Italy, we hypothesized that the avian strains may have been derived from a virus infecting a swine host. To test this hypothesis, we compared first the pathogenicity of the two virus groups in

**Table 1.** Replication of Italian chicken and swine H3N2 influenza viruses in experimentally infected chickens

SPF 3-week-old White Leghorn chickens were infected intranasally (0.1 ml), intratracheally (0.4 ml) and orally (0.5 ml) with allantoic fluid containing one of four viruses: Ck/It/5783/95, Ck/It/5954/95, Ck/It/5945/95 or Sw/25823/94. In a separate experiment, chickens were infected intravenously with 1.0 ml of allantoic fluid containing one of three viruses: Sw/It/1452/96, Sw/It/526/85 or PChal/1/73. Ck, chicken; Sw, swine; It, Italy; PChal, Port Chalmers.

H3N2 influenza virus strain	Virus replication (no. from which virus was isolated/total no. infected)	
	Trachea	Cloaca
Ck/It/5783/95	4/4	0/4
Ck/It/5954/95	2/4	0/4
Ck/It/5945/95	4/4	0/4
Sw/It/25823/94	0/4	0/4
Sw/It/1452/96	0/2	0/2
Sw/It/526/85	0/2	0/2
PChal/1/73	0/2	0/2

**Table 2.** Pattern of shedding of Ck/It/5945/95 virus by chickens

Five SPF 3-week-old White Leghorn chickens were infected intranasally (0.1 ml), intratracheally (0.4 ml) and orally (0.5 ml) with allantoic fluid containing Ck/It/5945/95.

Source of sample	No. days after infection	Ck/It/5945/95 titre ( $\log_{10}$ EID <sub>50</sub> /ml)
Trachea*	1	—
	2	3.10
	3	3.21
	4	1.90
	5	1.25
Spleen†	3	2.75
Other organs‡	3	—

\* Swabs were taken from the trachea on days 1–5 after infection and virus was titrated in chickens' eggs.

† Two chickens were dissected on day 3 after infection to determine the spread of infection. Tissue suspensions were prepared from samples of spleen and virus was titrated in chickens' eggs.

‡ Other organs included tissue suspensions prepared from samples of brain, blood, pancreas, heart, intestine, lung, colon and gall bladder and virus was titrated in chickens' eggs.

experimentally infected chickens. When chickens were inoculated intranasally, orally and intratracheally, the three chicken strains (Ck/It/5783/95, Ck/It/5954/95 and Ck/It/5945/95) replicated in the oropharyngeal tracts of the infected birds but no virus was recovered from the cloacae (Table 1).

The birds showed no signs of disease and their consumption of food did not decrease during the study. The three H3N2 swine influenza virus strains (Sw/It/25823/94, Sw/It/1452/96 and Sw/It/526/85) and the antigenically related early human influenza virus PChal/1/73 did not replicate in either the respiratory tract or the intestinal tract of chickens. Antibody levels in sera of chickens that were experimentally infected with the avian strains were consistently elevated 10 days after infection, whereas antibody levels in sera of chickens infected with the human and swine strains were comparable to the low non-specific antibody titres in sera before infection (data not shown).

To evaluate virus growth and dissemination to other organs, we inoculated chickens with Ck/It/5945/95 and titrated virus from the tracheae and other organs in chickens' eggs. Low levels of virus were detected in the tracheae on days 2–5 (maximum titre 3.21  $\log_{10}$  EID<sub>50</sub>/ml) and the birds showed neither signs of disease nor evidence of gross pathological change (Table 2). Virus was also detected in the spleen of one of the two birds dissected on day 3 (2.75  $\log_{10}$  EID<sub>50</sub>/ml) but not in the other organs of either bird.

#### Characterization of HA antigenicity

To investigate the antigenic relationships among chicken and swine H3 influenza virus HAs, we tested the viruses in HI assays. We used a panel of polyclonal antisera specific for representative H3 influenza virus strains from different hosts and a panel of mAbs to two early H3N2 human influenza virus strains (England/42/72 and Tex/1/77) to compare the viruses. The H3N2 human influenza virus strains are related to human variants, such as PChal/1/73, shown previously to be antigenically similar to the swine H3N2 influenza viruses circulating in pigs in Italy (Castrucci *et al.*, 1994).

Analysis with polyclonal antisera showed antigenic cross-reactivity among the swine and chicken H3N2 influenza virus isolates from Italy, with highest titres to Aichi/1/68 and PChal/1/73 (Table 3). The antisera for Aichi/1/68 reacted weakly with Coot/It/153/94 and the Dk/Ukr/63 and PChal/1/73 antisera did not react with Coot/It/153/94, an H3N8 influenza virus strain isolated from wild waterfowl in central Italy in 1994. In contrast, the Coot/It/153/94-specific antiserum, which reacted with two early human influenza virus variants (PChal/1/73 and Tex/1/77), also reacted with Sw/It/526/85. This finding illustrates the reduced sensitivity of the HI antibody to avian viruses, as reported previously (Lu *et al.*, 1982).

Analysis with mAbs to early human H3N2 influenza viruses showed that both Italian strains of swine influenza virus, although isolated 9 years apart, reacted with the mAbs specific for Tex/1/77. On the other hand, none of the chicken viruses was recognized by any of the mAbs to early human H3N2 influenza virus strains. These results indicate that the swine and chicken influenza viruses represent two antigenically distinguishable groups. The different reactivity patterns of the

**Table 3.** Antigenic characterization of chicken and swine H3N2 influenza viruses isolated in Italy

HI titres in bold indicate the titre of antibodies against the virus for which they are specific. Titres less than 10 (—) and 100 (<) are also indicated. Dk, duck; Sw, swine; Ck, chicken; Tex, Texas; Ukr, Ukraine; PChal, Port Chalmers; It, Italy.

Virus	HI titres										
	Polyclonal antisera specific for				mAbs to early human H3N2 influenza virus						
	Dk/Ukr/ 63	Aichi/1/ 68	PChal/1/ 73	Coot/It/ 153/94	England/42/72				Tex/1/77		
				11/1	28/1	31/4	37/4	10/1	29/1	26/1	
Dk/Ukr/63 (H3N8)	<b>240</b>	320	640	—	<	<	<	<	<	<	<
Aichi/1/68 (H3N2)	960	<b>2560</b>	1280	—	2400	200	6400	800	<	<	<
PChal/1/73 (H3N2)	1920	1280	<b>10 240</b>	80	<	800	200	800	200	<	100
England/42/72 (H3N2)	1280	2560	2560	30	<b>25 600</b>	<b>12 800</b>	<b>12 800</b>	<b>4800</b>	<	<	<
Tex/1/77 (H3N2)	120	320	480	80	<	<	<	<	<b>9600</b>	<b>6400</b>	<b>12 800</b>
Coot/It/153/94 (H3N8)	—	20	—	<b>160</b>	<	<	<	<	<	<	<
Sw/It/526/85 (H3N2)	80	480	1280	160	<	4800	400	400	3200	600	3200
Sw/It/25823/94 (H3N2)	30	320	640	—	<	<	<	<	4800	3200	4800
Ck/It/5783/95 (H3N2)	80	240	160	—	<	<	<	<	<	<	<
Ck/It/5954/95 (H3N2)	40	160	640	—	<	<	<	<	<	<	<
Ck/It/5945/95 (H3N2)	60	240	640	—	<	<	<	<	<	<	<

**Table 4.** Genetic similarity between the CK/It/5945/95 influenza virus isolated from chickens in Italy and other influenza viruses

PB2, PB1 and PA, polymerase proteins; HA, haemagglutinin; NP, nucleoprotein; M, matrix; NS, non-structural protein.

Ck/It/5945/95 gene	Nucleotides sequenced	Similarity (%) to Sw/It/25823/94 (H3N2)	Eurasian avian virus with highest genetic similarity to Ck/It/5945/95	
			Virus (strain)	Similarity (%)
PB2	967–1309	88·3	A/Chicken/Korea/25232-96006/96 (H9N2)	91·0
PB1	388–586	89·9	A/Duck/HongKong/62/76 (H11N2)	91·5
PA	52–398	84·8	A/Duck/HongKong/y439/97 (H9N2)	90·0
HA	439–1066	82·1	A/Duck/Hokkaido/5/77 (H3N8)	88·2
NP	1076–1398	90·0	A/Mallard/Astrakhan/244/82 (H14N6)	93·2
M	177–970	91·8	A/Oystercatcher/Germany/87 (H1N1)	96·6
NS	580–873	91·5	A/Anas acuta/Primorje/695/76 (H3N2)	96·3

two swine influenza virus strains with respect to mAbs to England/42/72 and antiserum to Coot/It/153/94 revealed that the more recent H3 swine influenza virus strain has antigenically drifted away from the earlier one.

#### Sequence and phylogenetic analysis of the genes encoding HA and internal proteins

To evaluate the genetic relationships among the swine and chicken H3N2 influenza viruses and to elucidate the origin of the chicken H3N2 influenza virus strains, we sequenced portions of genes encoding HA1 and the six internal proteins of the chicken H3N2 influenza viruses in Italy and compared

these nucleotide sequences with corresponding sequences from other avian and swine influenza virus strains.

**Genes encoding HA1.** Sequence similarity among the genes of the three chicken viruses ranged from 99·5 to 100% (data not shown). Of the HA1 genes of H3 influenza viruses from different animal hosts, the HA1 genes of duck influenza viruses of Eurasian origin were most similar to those of the representative chicken influenza virus Ck/It/5945/95 (88·2%) (Table 4). On the other hand, similarity between the HA1 gene of Ck/It/5945/95 and Sw/It/25823/94 was much lower (82·1%). The swine strain Sw/It/25823/94 was related most closely to H3N2 swine influenza viruses isolated recently from

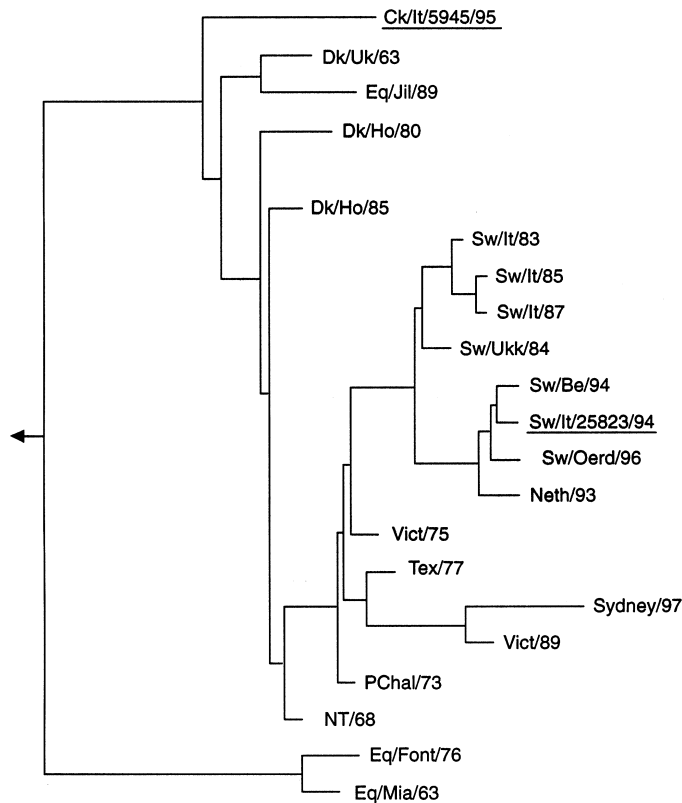


Fig. 1.

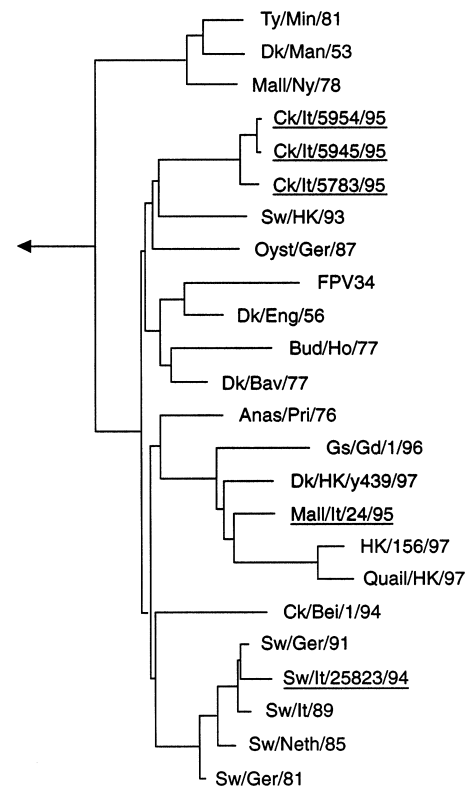


Fig. 2.

**Fig. 1.** Phylogenetic tree of the H3 HA gene of influenza A viruses. The tree is rooted to the H2 HA gene of Pintail/Praimoric/625/76 (H2N2) and is based on 628 bp (residues 439–1066). Horizontal lines are proportional to the numbers of nucleotide substitutions between branch points. Underlined viruses indicate those that were sequenced in this study. Abbreviations of virus sequences are as follows: Ck/It/5945/95, A/Chicken/Italy/5945/95 (H3N2); Dk/Uk/63, A/Duck/Ukraine/1/63 (H3N8); Eq/Jil/89, A/Equine/Jilin/1/89 (H3N8); Dk/Ho/80, A/Duck/Hokkaido/8/80 (H3N8); Dk/Ho/85, A/Duck/Hokkaido/10/85 (H3N8); Sw/It/83, A/Swine/Italy/309/83 (H3N2); Sw/It/85, A/Swine/Italy/520/85 (H3N2); Sw/It/87, A/Swine/Italy/635/87 (H3N2); Sw/Ukk/84, A/Swine/Ukkel/1/84 (H3N2); Sw/Be/94, A/Swine/Belgium/231/94 (H3N2); Sw/It/25823/94, A/Swine/Italy/25823/94 (H3N2); Sw/Oerd/96, A/Swine/Oerdenrode/70/96 (H3N2); Neth/93, A/Netherlands/5/93 (H3N2); Vict/75, A/Victoria/3/75 (H3N2); Tex/77, A/Texas/1/77 (H3N2); Sydney/97, A/Sydney/5/97 (H3N2); Vict/89, A/Victoria/5/89 (H3N2); PChal/73, A/PortChalmers/1/73 (H3N2); NT/68, A/Northern Territory/60/68 (H3N2); Eq/Font/76, A/Equine/Fontainebleau/76 (H3N8); Eq/Mia/63, A/Equine/Miami/1/63 (H3N8). The sequence for influenza virus Tex/1/77 (accession no. ISDNTX77) is taken from C. Bender and N. Cox, Centers for Disease Control and Prevention, Atlanta, GA, USA (unpublished data).

**Fig. 2.** Phylogenetic tree of the NP gene of influenza A viruses. The tree is rooted to the NP gene of B/Lee/40 and is based on 323 bp (residues 1076–1398). Horizontal lines are proportional to the numbers of nucleotide substitutions between branch points. To preserve space, only the terminal branches of the avian lineage are presented and underlined viruses indicate those that were sequenced in this study. Abbreviations of virus sequences are as follows: Ty/Min/81, A/Turkey/Minnesota/1661/81 (H1N1); Dk/Man/53, A/Duck/Manitoba/1/53 (H10N7); Mall/Ny/78, A/Mallard/New York/6750/78 (H2N2); Sw/HK/93, A/Swine/Hong Kong/168/93 (H1N1); Oyst/Ger/87, A/Oystercatcher/Germany/87 (H1N1); FPV34, A/FPV/Rostock/34 (H7N1); Dk/Eng/56, A/Duck/England/1/56 (H11N6); Bud/Ho/77, A/Budgerigar/Hokkaido/1/77 (H4N6); Dk/Bav/77, A/Duck/Bavaria/2/77 (H1N1); Anas/Pri/76, A/Anas Acuta/Primorje/695/76 (H2N3); Gs/Gd/1/96, A/Goose/Guangdong/1/96 (H5N1); Dk/HK/y439/97, A/Duck/Hong Kong/y439/97 (H9N2); Mall/It/24/95, A/Mallard/Italy/24/95 (H1N1); HK/156/97, A/Hong Kong/156/97 (H5N1); Quail/HK/97, A/Quail/Hong Kong/61/97 (H9N2); Ck/Bei/1/94, A/Chicken/Beijing/1/94 (H9N2); Sw/Ger/91, A/Swine/Germany/8533/91 (H1N1); Sw/It/89, A/Swine/Italy/839/89 (H1N1); Sw/Neth/85, A/Swine/Netherlands/12/85 (H1N1); Sw/Ger/81, A/Swine/Germany/2/81 (H1N1).

northern Europe (97.9% similarity). Sw/It/25823/94 showed a marked genetic drift from the earlier Italian H3N2 influenza virus isolates Sw/It/309/83, Sw/It/520/85 and Sw/It/635/87 (between 92.4 and 92.6% similarity; data not shown).

Within the phylogenetic tree that was based on our sequence data, the HA gene of the Ck/It/5945/95 H3 influenza virus clustered consistently with those of the avian influenza virus group within the Eurasian avian lineage (Fig. 1)

and it was clearly distinct from those of the human and swine branch. Therefore, the genes of the chicken H3 influenza virus are of avian origin.

**Genes encoding internal proteins.** The genes encoding internal proteins from the three chicken influenza virus strains shared a high degree of similarity, which ranged from 98.2% for the nucleoprotein (NP) gene to 100% for the genes encoding the polymerase (PA) and non-structural (NS) proteins (data not shown). A comparison of the nucleotide sequences of the representative strains Ck/It/5945/95 (H3N2) and Sw/It/25823/94 revealed that the similarity between the internal protein genes of the two ranged from as high as 91.8% for the matrix (M) gene to as low as 84.8% for the PA gene (Table 4). Thus, the avian and swine H3N2 influenza viruses isolated in Italy are not genetically closely related. To determine the closest relatives of the internal protein genes of the avian H3N2 influenza strain, we compared their sequences with the available sequences in GenBank. Each of the internal protein genes of the Italian avian H3N2 influenza virus was most similar to the corresponding gene from an Eurasian avian strain (Table 4). Overall, the extent of similarity between the P genes of Ck/It/5945/95 and those of corresponding avian influenza viruses in GenBank was low (between 90.0 and 91.5%); however, this finding may reflect the limited amount of information that was available for comparison. The closest relationship was between the M genes of the avian H3N2 Italian influenza virus and A/Oystercatcher/Germany/87 (H1N1); these genes shared 96.6% identity.

Phylogenetic analysis of the NP gene (Fig. 2) identified distinct subgroups within the Eurasian avian influenza virus lineage. The NP gene of the Italian avian H3N2 influenza viruses clustered with that of Sw/HK/168/93, an H1N1 influenza virus of avian origin that was detected recently in pigs in China (Guan *et al.*, 1996). In contrast, the NP gene of the H3N2 swine influenza virus clustered with those of the European swine influenza viruses of avian descent. Both branches were distinguishable from the group represented by the NP gene of HK/156/97. This group also included a strain isolated from wild ducks in Italy (Mal/It/24/95; H1N1) (L. Campitelli, unpublished data). Thus, on the basis of the antigenic and phylogenetic data, the chicken influenza viruses do not seem to be derived from contemporary strains circulating in wild ducks in Italy, such as Coot/It/153/94 and Mal/It/24/95. Instead, the antigenic heterogeneity among the avian H3 proteins and the genetic diversity among the NP genes indicate that multiple sublineages exist within the gene pool of avian influenza viruses that are circulating in Italy.

## Discussion

The sporadic isolation of H3N2 influenza viruses in chickens that show no evidence of disease has been reported previously (Siebinga & de Boer, 1988; Lin *et al.*, 1994). The

present study shows that H3N2 influenza viruses isolated from Italian chickens with mild respiratory disease replicate in the respiratory tract of experimentally infected chickens but are not shed in the faeces. Relatively low titres of these viruses were found in chickens after experimental infection and no signs of disease were observed. In the poultry houses, chickens that were naturally infected showed mild respiratory symptoms. However, the antigenic and genetic identity among the isolates suggests that the virus had spread from farm to farm as a result of the fact that the three chicken houses belonged to the same commercial network: the practice of sharing personnel and equipment among farms is rather common in this area (Capua & Marangon, 2000). The isolation of Ck/It/5783/95 from ovarian tissue and Ck/It/5945/95 from spleen indicates that these H3N2 influenza viruses infect tissues beyond the respiratory tract. In contrast to the chicken H3N2 influenza viruses, the Italian swine and the human H3N2 influenza viruses failed to replicate in chickens. This failure is consistent with differences in the host ranges among viruses of the same subtype (Rogers & Paulson, 1983).

Because human H3N2 influenza viruses that infected pigs in Italy before 1980 continue to circulate (Castrucci *et al.*, 1994; Campitelli *et al.*, 1997) and were found shortly before the avian virus isolations in pigs on farms near those where the outbreak in chickens occurred, we hypothesized that the Italian H3N2 chicken influenza virus arose from strains that were present in the nearby pigs (Ludwig *et al.*, 1994). However, results of our antigenic analysis with mAbs and results of our sequencing analysis of the genomes of the avian H3N2 influenza viruses do not support this hypothesis. Instead, the chicken H3N2 influenza viruses in Italy were most similar to the Eurasian avian influenza viruses. HA sequence and evolutionary analyses suggest an introduction of the virus from wild aquatic birds, although the relatively low percentage of similarity (88.2%) between the HA1 gene of Ck/It/5945/95 and that of Dk/Ho/5/77 indicates that the Italian chicken influenza viruses are only distantly related to other known avian H3 influenza virus strains. In the NP phylogenetic tree, the viruses under study were grouped with an avian-like swine influenza virus from Hong Kong (Sw/HK/168/93; H1N1).

Several different subtypes of influenza viruses have been isolated from domestic chickens. The two highly pathogenic subtypes H5 and H7 have been isolated from chickens for many years (Stubbs, 1965; Bean *et al.*, 1985). Other influenza viruses isolated in chickens include the following subtypes: H1 (Siebinga & de Boer, 1988), H2 (Schäfer *et al.*, 1993), H4 (Donis *et al.*, 1989), H6 (Lin *et al.*, 1994) and H10 (Feldmann *et al.*, 1988). Unlike the highly pathogenic strains, these less pathogenic viruses have been detected sporadically and appear to be confined to the poultry species from which they had been isolated.

The transmission of H9N2 influenza viruses to domestic chickens in Asia, the establishment of stable lineages of influenza virus strains in chickens (Guan *et al.*, 1999; Guo *et al.*,

2000) and the transmission of these viruses to humans (Peiris *et al.*, 1999) suggest that the role of domestic chickens in the ecology of influenza viruses may be expanding. Alternatively, these three observations may result from increased surveillance, which is more frequently detecting viruses that are transmitted to other species but do not necessarily establish themselves in that species. As domestic chickens become an important source of protein for humans, their increasing population raises the possibility of an increased role for them in the ecology of influenza.

Our studies suggest that the circulation of non-pathogenic influenza viruses in commercial poultry may be significantly underestimated because these infections are asymptomatic and are often not detected. This theory raises some concern because of the episodes of transmission of highly pathogenic H5N1 chicken influenza viruses to humans in Hong Kong (de Jong *et al.*, 1997; Claas *et al.*, 1998; Subbarao *et al.*, 1998; Suarez *et al.*, 1998). Although the genetic determinants that favour interspecies transmission of avian influenza viruses have not been identified, the role of the host cell receptor does not appear to be crucial. Receptor specificity for avian viruses, as defined by oligosaccharides that contain the Sia( $\alpha$ 2,3)Gal-linkage (Rogers & Paulson, 1983), does not necessarily restrict avian-to-human transmission (Matrosovich *et al.*, 1999). This finding suggests that chickens may also serve as an intermediate host either instead of or in addition to the pig. The presence of Sia( $\alpha$ 2,3)-containing (avian influenza virus-specific) receptors and Sia( $\alpha$ 2,6)-containing (human influenza virus-specific) receptors (Ito *et al.*, 1998) in pigs makes this an attractive model for transmission of avian influenza viruses to man, but the direct transmission of H5N1, H9N2 and H7N7 (Kurtz *et al.*, 1996) avian influenza viruses to humans indicates that the interspecies transmission of influenza virus does not necessarily require the pig.

The antigenic differences among the H3N2 influenza viruses isolated from chickens in Italy and the H3N8 influenza viruses from wild aquatic birds of the same region suggest that multiple types of H3 influenza viruses are circulating in Italy. This finding also indicates that an extensive gene pool for influenza viruses continues to circulate in nature; however, the actual source of the H3N2 influenza virus that infected chickens in Italy is unknown. Therefore, our studies emphasize the continued need to monitor domestic and wild avian populations to better understand interspecies transmission and the importance of avian hosts in the ecology of influenza.

This work was supported in part by Public Health Research grants AI-29680 and AI-95357 from the National Institute of Allergy and Infectious Diseases by Cancer Center Support (CORE) grant CA-21765 and by the American Lebanese Syrian Associated Charities (ALSAC). This research was also funded by grant 1% D.Lgs 502/92 to Dr Isabella Donatelli. We thank Dr Lijuan Zhang for technical support, Alice Herren and Romina Tomasetto for preparation of this manuscript and Angela J. McArthur for scientific editing.

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Received 2 August 2001; Accepted 2 November 2001  
 Published ahead of print (15 November 2001) in JGV Direct as  
 DOI 10.1099/vir.O.18036-0