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Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy

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Abstract

Since the "bird flu" incident in Hong Kong SAR in 1997, several studies have highlighted the substantial role of domestic birds, such as turkeys and chickens, in the ecology of influenza A viruses. Even if recent evidence suggests that chickens can maintain several influenza serotypes, avian influenza viruses (AIVs) circulating in domestic species are believed to be introduced each time from the wild bird reservoir. However, so far the direct precursor of influenza viruses from domestic birds has never been identified. In this report, we describe the antigenic and genetic characterization of the surface proteins of H7N3 viruses isolated from wild ducks in Italy in 2001 in comparison to H7N3 strains that circulated in Italian turkeys in 2002–2003. The wild and domestic avian strains appeared strictly related at both phenotypic and genetic level: homology percentages in seven of their genes were comprised between 99.8% (for PB2) and 99.1% (for M), and their NA genes differed mainly because of a 23-aminoacid deletion in the NA stalk. Outside this region of the molecule, the NAs of the two virus groups showed 99% similarity. These findings indicate that turkey H7N3 viruses were derived "in toto" from avian influenza strains circulating in wild waterfowl 1 year earlier, and represent an important step towards the comprehension of the mechanisms leading to interspecies transmission and emergence of potentially pandemic influenza viruses.

Keywords: Avian influenza; Interspecies transmission; Influenza ecology; Pandemics; Host-range determinants

Introduction

Wild waterfowl, gulls, and shorebirds are believed to be the natural hosts and reservoir of influenza A virus (Kawaoka et al., 1988; Slemons et al., 1974). Although stable lineages of several influenza A subtypes are present in mammals, phylogenetic evidence suggests that all influenza A viruses are derived from viruses circulating in aquatic bird species, in which they are considered avirulent and all the HA and NA subtypes are maintained (Webster et al., 1992). The 1957 and 1968 influenza pandemics imply the transfer of gene segments from the avian to the human virus gene pool (Kawaoka et al., 1989). However, avian influenza viruses (AIVs) do not appear to replicate efficiently in some mammalian species, such as nonhuman primates and humans (Beare and Webster, 1991; Murphy et al., 1982). One mechanism postulated to overcome this species barrier is the replication and reassortment of viruses in an intermediate mammalian host susceptible to infection by both human and avian influenza viruses, and a possible candidate for such a role was identified in the swine species (Scholtissek and Naylor, 1988).

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However, recent human infections caused by H5N1 and H9N2 viruses showed that the direct precursors of the human strains of avian origin circulated in domestic terrestrial poultry, for example, chickens, quail, and geese (Cameron et al., 2000; Guan et al., 2000; Shortridge et al., 1998). Although increasing evidence indicates that multiple subtypes of influenza viruses can establish stable lineages in domestic poultry (Liu et al., 2003; Suarez et al., 1999; Webby et al., 2002), the emergence of AIVs in chickens, turkeys, and other farm-raised birds (Alexander, 2000) is thought to be the result of primary virus introduction from the wild bird reservoir. In chickens and turkeys, viruses appear to undergo rapid evolution, a sign that they are adapting to a new host (Garcia et al., 1996; Zhou et al., 1999). Avian influenza in poultry may cause asymptomatic infections or a range of disease symptoms from mild respiratory illness to severe systemic infection with high mortality (highly pathogenic avian influenza, HPAI) (Alexander, 2000). In particular, AIV isolates of the H5 and H7 subtypes, which show the ability to mutate to the HPAI phenotype in domestic poultry, cause serious public health concern for at least two reasons. These AIV isolates can cause heavy economic losses in the poultry industry. In addition, these isolates might not only be transmissible to humans but also provoke severe (even fatal) outcomes, as seen in Hong Kong in 1997 when 6 of 18 infected people died, and in two more events that occurred early in 2003: (a) the infection of a man (who died) and a child in Hong Kong with an H5N1 virus of the same genotype that was concurrently circulating in several bird species in Hong Kong (Guan et al., 2003), and (b) the infection of more than 80 people (with one death) in The Netherlands; the causative agent was an H7N7 virus responsible for an outbreak of HPAI in commercial poultry (Osterhaus, 2003).

These findings have led to the hypothesis that domestic poultry, like swine, may act as an intermediate host for the transmission of viruses from aquatic birds to humans. This hypothesis is supported by recent studies in chickens, demonstrating that these birds possess both α2,3 and α2,6 sialic acid (SA) receptors (similar to those recognized by human viruses) on their epithelial cells, and the characteristics of the binding of chicken viruses to NeuAc-terminated ganglioside receptors are intermediate to those of human and duck viruses (Gambaryan et al., 2002). These attributes, in turn, may help overcome the restriction of chicken viruses (as compared with duck viruses) in humans.

Thus far, however, the direct virus progenitors of all reported cases of avian influenza in poultry have never been identified, although they presumably are circulating in wild waterfowl. During the past few years in Italy, there have been several epidemics of avian influenza in domestic poultry that were caused by the high-pathogenicity (HP) H5N2 virus (in 1997) and by both low-pathogenicity (LP) and HP H7N1 influenza viruses (in 1999 through 2001) (Capua et al., 2000, 2002a; Donatelli et al., 2001). For both events, virological and serological surveillance carried out since 1992 among wild waterfowl in wetlands in Central Italy has failed to identify closely related virus precursors in the aquatic avian influenza reservoir (De Marco et al., 2003a, 2003b). In autumn 2001, two influenza viruses belonging to the H7N3 subtype were isolated for the first time from wild ducks in those areas. Then, in October 2002, a new epidemic of LP H7N3 influenza virus started spreading among farms in Northern Italy that intensively rear turkeys and chickens. The H7N3 virus affected the same regions devastated by the 1999–2000 H7N1 epidemic (Capua et al., 2002b), and several viruses were obtained. The aims of our study were to determine whether and to what degree the Italian wild duck and poultry H7N3 strains were related and to investigate their evolutionary relationships with other Eurasian avian viruses.

Results

Background information

Viruses isolated and analyzed in this study are shown in Table 1. Wild avian strains were obtained from cloacal samples collected from mallard ducks (*Anas platyrhynchos*) between August 2000 and October 2001. During an ongoing virological and serological survey among resident and migratory wild waterfowl, the animals were caught in a wildlife refuge along the west coast of Central Italy (the Orbetello Lagoon). The Orbetello Lagoon is an important wintering site for several species of migratory aquatic birds whose main breeding sites are in Central and Northeastern Europe and whose migratory routes have been shown also to pass over the Northern Italian regions (De Marco et al., 2000).

Since the 1999–2000 H7N1 avian influenza epidemic, which led to the culling of more than 14 million birds in Northern Italy, local veterinary authorities adopted additional preventive measures, including systematic serological surveillance of each batch of poultry taken to the slaughterhouse. In October 2002 in the Lombardia region, one

Table 1
Viruses analyzed in this study

Virus	Subtype	Date of isolation	Location
A/Mallard/Italy/35/99	H2N3	December 1999	Tuscany
A/Mallard/Italy/36/99	H2N3	December 1999	Tuscany
A/Mallard/Italy/208/00	H5N3	August 2000	Tuscany
A/Mallard/Italy/33/01	H7N3	October 2001	Tuscany
A/Mallard/Italy/43/01	H7N3	October 2001	Tuscany
A/Turkey/Italy/214845/02	H7N3	October 2002	Lombardia
A/Turkey/Italy/220158/02	H7N3	October 2002	Lombardia

Note. All isolates were obtained from cloacal swabs. None of the animals from which the isolates were obtained showed any disease signs at the time of sample collection. However, 2 weeks before collection, the two birds that were the sources of A/Turkey/Italy/214845/02 and A/Turkey/Italy/220158/02 had shown mild respiratory symptoms, which were diagnosed and treated as mycoplasmosis.

turkey was found to be positive for influenza. As a result, 20 cloacal swabs were collected from other turkeys raised at the influenza-positive farm as well as from two others that were linked commercially and geographically to the first. Viruses A/Turkey/Italy/214845/02 and A/Turkey/Italy/220158/02 were isolated. Because the two regions affected (Lombardia and Veneto) are very densely populated with poultry, the epidemic spread very rapidly despite control measures immediately adopted in accordance with European Union Directive 92/40/EEC (CEC, 1992). As of 30 September 2003, when the last infected poultry farm was detected, 388 outbreaks had occurred.

Antigenic analysis of H7N3 influenza viruses isolated in Italy

The isolates were characterized antigenically by HI assay (Table 2). To test the viruses, horse red blood cells (HRBCs) were used, because horse erythrocytes (displaying almost exclusively α2,3-linked SA on their surface) have been shown to increase the sensitivity of the HI assays performed on avian influenza viruses, compared to turkey RBCs, which possess a mixture of α2,3- and α2,6-linked SA (Stephenson et al., 2003).

Because the current avian influenza epidemic in Italian poultry occurred in the same regions of Northern Italy in which the 1999–2000 H7N1 avian influenza epidemic took place, we wanted to compare the H7N3 viruses at antigenic level between themselves and with previously circulating H7 strains. Results showed that the two domestic and wild avian H7N3 virus groups shared the same HA antigenic profile (Table 2). In contrast, they all reacted at significantly lower titers against almost all of the sera and monoclonal antibody raised against the 1999-00 Italian H7N1 viruses compared to the reference H7N1 strain Ty/It/2676/99 (the only exception being the F6/02 ferret serum, against which the Italian H7N1 and H7N3 viruses had a similar antibody titer). When viruses were tested using either chicken or turkey RBCs, HI titers were generally lower or negative compared to those obtained with horse RBCs, and no significant antigenic differences between the H7N1 and H7N3 viruses could be detected (data not shown).

Analysis of the virus genome

To better understand the similarity between the wild and domestic H7N3 strains, we obtained full-length sequences for their HA and NA genes, and partial sequences of their internal genes.

HA genes and proteins

The HA genes of the two duck strains A/Mallard/Italy/33/01 and A/Mallard/Italy/43/01 were 100% homologous with each other and showed 99.6% similarity to the turkey strain Ty/It/214845/02 (the two turkey virus HAs were identical between themselves) at both the nucleotide and amino acid levels (Table 3). The highest homology of the duck viruses with published H7 sequences was to A/Turkey/Italy/1279/99 (H7N1), a virus strain isolated in April 1999 during the initial outbreaks in the 1999–2000 epidemic in Northern Italy and that showed 98% identity in the HA1 gene at the nucleotide level and 99.4% homology when the deduced amino acid sequence was considered (Table 3). It is noteworthy that homology values with some H7N1 viruses isolated several months later during the same epidemic (e.g., A/Turkey/Italy/4294/99, an LP strain collected in November 1999, Banks et al., 2001) decreased to 96.6% and 97.5% at the nucleotide and protein levels, respectively (data not shown).

Overall, the wild and domestic H7N3 avian strains differed by seven nucleotide substitutions throughout the HA molecule, six of which were in the HA1 subunit. Of these six, only two, R261S in HA1 and K161R in HA2 (corresponding to positions 271 and 161 on the H3 molecule), encoded amino acid changes in turkey strains (Table 4). Alignment of 248 published H7 sequences isolated from different hosts showed that neither of these amino acid positions could be considered specific for a given host species. In particular, S261, found in the H7N3 turkey

Table 2
Antigenic characterization of H7N3 influenza viruses by HI test performed using horse red blood cells

Viruses	Hemagglutination-inhibition titer						
	Hyperimmune antisera to			Postinfection ferret antisera Ck/It/13474/99 (H7N1)			Monoclonal antibody to
	RT/NJ (R)	Ty/2676 (Ck)	Ty/214845 (Ck)	F4/02	F5/02	F6/02	Ty/2676
A/RuddyTurnstone/NJ/65/85 (H7N3)	**5120**	320	320	40	<	40	160
A/England/268/96 (H7N7)	2560	320	320	20	<	40	160
A/Turkey/Italy/2676/99 (H7N1)	5120	**2560**	640	160	80	80	**1280**
A/Mallard/Italy/33/01 (H7N3)	5120	640	640	40	20	40	320
A/Mallard/Italy/43/01 (H7N3)	5120	640	640	40	<	40	320
A/Turkey/Italy/214845/02 (H7N3)	2560	640	**640**	40	20	40	160
A/Turkey/Italy/220158/02 (H7N3)	2560	640	640	40	<	40	160

<, <20; R, rabbit; Ck, chicken; titers in bold indicate virus reactivity with homologous antiserum.

Table 3
Homology of gene segments of A/Mallard/Italy/33/01 (H7N3) virus with A/Turkey/Italy/214845/02 strain and with other Eurasian avian viruses

Gene	Region analyzed	Percentage homology with Ty/It/214845/02 (H7N3)		Virus with highest identity within the Eurasian lineage (%)[a]			
		nt	aa		nt		aa
PB2	28–1029	99.8	99.7	Dk/Nanchang/1-100/2001	97.4	Dk/Nanchang/1-100/2001	100.0
PB1	24–922	99.3	99.0	Dk/Hong Kong/3461/99	96.7	Chicken/Shanghai/F/98	99.2
PA	25–1299	99.3	99.5	Dk/Nanchang/8-197/2000	95.8	Dk/Nanchang/8-197/2000	100.0
HA	22–1732	99.6[b]	99.6	Turkey/Italy/2676/99	98.0	Turkey/Italy/2676/99	99.4
NP	46–1005	99.2	99.4	Chicken/Italy/9097/97	97.9	Quail/Nanchang/1-026/00	99.4
NA[c]	247–1432	99.0	99.0	Mallard/Italy/208/00	97.7	Mallard/Italy/208/00	99.7
MP	44–781	99.1	99.2	Dk/Hong Kong/P54/97	98.7	Aquatic Bird/Hong Kong/399/99	100.0
NS	27–890	99.7	99.1[d]	Dk/Nanchang/2-045/2000	98.5	Chicken/Italy/9097/97	99.4

[a] Homology percentages were calculated on regions comprising nucleotides 41–631 for NP, 101–621 for NS, 65–603 for PB2, and 47–525 for PA to allow comparison with a greater number of partial sequences available in GenBank.
[b] No differences in HA glycosylation patterns were observed between the two viruses. No additional CHO sites in the globular region of the molecular were found.
[c] Homology was calculated excluding the first 250 bp that include the stalk region, as in the domestic poultry strain there is a 23-amino acid deletion.
[d] Amino acid homology was calculated on the NS1 coding region.

strains, was shared also by two duck strains, whereas R261 appeared unique to the two Italian duck strains. The percentage of coding-to-non-coding changes in the HA1 of the turkey isolates was 16.7% (one of six), a value comparable to that observed in wild ducks (Zhou et al., 1999). At amino acid residue 84 of the HA1, the Italian H7N1 and H7N3 strains both had asparagine instead of serine, which is found in almost all other H7 strains in GenBank.

No differences in glycosylation patterns were found between the duck and turkey strains. Overall, five potential glycosylation (CHO) sites were identified located at amino acid positions 12, 28, and 231 of the HA1 and 403 and 475 of the HA2 (H7 numbering). Thus, only the CHO site at position 231 (corresponding to residue 240 by H3 numbering) was found on the globular head of the HA1 (which includes positions 90 through 260, as defined for the H3 HA molecule).

Both virus groups possessed the same sequence (PEIPKGR*GLF) at the cleavage site, without the additional basic residues that are considered a marker of high virulence in domestic poultry. This motif is commonly found in LP H7 strains belonging to the Eurasian avian lineage. Similarly, no differences were observed concerning the amino acid residues at positions 138, 190, 194, 225, 226, and 228 (H3 numbering), which are part of the receptor binding site (RBS) and are homologous to the avian H7 consensus sequence (Nobusawa et al., 1991).

Table 4
Amino acid changes between duck and turkey H7N3 viruses in the surface glycoproteins

	Amino acid at position						
	HA[a]		NA				
	261	482 (161HA2)	37	83	140	266	355
Duck	R	K	E	T	L	Y	T
Turkey	S	R	G	P	V	H	K

[a] H7 numbering, with position 1 set at the first residue downstream of the signal peptide.

Because many nucleotide sequences in GenBank are only partial sequences, we performed a phylogenetic analysis of the H7 HA1 of all representative isolates (Fig. 1). As expected from homology data, the two H7N3 virus groups clustered together on the same branch, the six nucleotide differences accounting for their slight divergence. In addition, they showed a sister-group relationship with the H7N1 strains isolated in Italy in 1999, indicating that the HAs of the two Italian virus groups either shared a recent common ancestor or transmitted the HA from one to the other. The only human strain belonging to the H7 subtype whose sequence was available (A/England/268/96), although located in the same major sublineage as the Italian strains within the Eurasian avian branch, was not closely related to them, a finding consistent with the homology values (93.7% in the HA1).

NA genes and proteins

Because the NA protein has recently been involved in the adaptation of wild avian strains to land-based poultry (Banks et al., 2001; Matrosovich et al., 1999) and because almost no sequence data are available on the N3 genes, we sequenced the NA genes of the wild and domestic H7 avian strains as well as those of several strains isolated from aquatic and terrestrial birds in Europe and North America (Table 1). Mall/It/33/01 and Mall/It/43/01 NAs were 100% homologous to each other and shared highest nucleotide similarity (97.7%) with A/Mallard/Italy/208/00. This percentage decreased to 94.1–94.2% when these strains were compared with Ty/It/214845/02 and Ty/It/220158/02 (which were 99.9% homologous between themselves). Aligning all available N3 sequences revealed that whereas all other avian strains (including a turkey strain, A/Ty/Minnesota/916/80) coded for a polypeptide 470 amino acids long, the N3

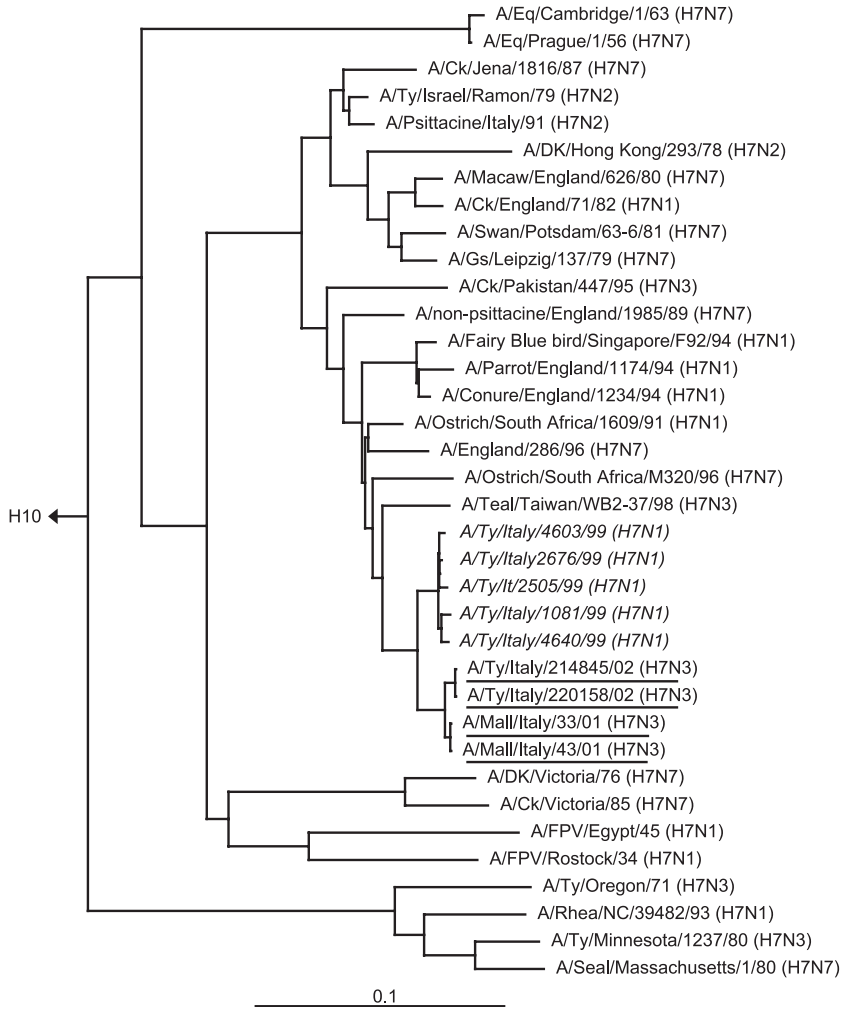

Fig. 1. Phylogenetic tree of the HA genes from four H7N3 Italian viruses and 33 avian, human, and equine strains. The cladogram was constructed by using the sequences coding for the entire HA1 subunit of the HA gene (nucleotides 76–1019). The tree was rooted to A/Chicken/Germany/N/49 (H10N7). Sequences were analyzed with the Neighbor program (Phylip, version 3.57). Accession numbers of the sequences used are listed in Banks et al., 2000. Branch lengths are proportional to genetic distances. The Italian H7N3 viruses are underlined; the Italian H7N1 strains are in italics. Abbreviations used: Dk, duck; Ty, turkey; Gs, goose; Ck, chicken; Eq, equine; Mall, mallard.

proteins of both Italian turkey strains lacked 23 amino acids (positions 56–78) in the stalk region (Fig. 2), thus accounting for the decreased homology. This NA stalk deletion occurs in N1, N2, and N3 NAs in avian viruses isolated from terrestrial or raised aquatic birds (including chickens, turkeys, quail, pheasants, teal, and chukar), but this deletion has never been identified in wild avian strains. When we calculated the homology percentages after excluding the first 250 bp (which corresponds to the stalk region), the duck H7N3 strains showed 99.0% similarity to the turkey isolates compared with a value of 97.7% with Mall/It/208/00 in the same region. Therefore, outside the stalk region, the N3 genes of the duck and turkey H7N3 strains appeared to be very closely related to each other, with a degree of homology comparable to that observed for the HA gene. Thus, this is the first report of an influenza virus from the wild bird reservoir whose NA gene appears to be the immediate precursor of the NAs of viruses circulating in domestic poultry that differs from their wild bird counterparts essentially because of the lack of a long stretch of amino acids in the stalk (a feature associated with early adaptation of wild avian viruses to turkeys and chickens).

Analysis of the glycosylation pattern showed that all the wild avian strains had six potential CHO sites: at positions 14, 57, 66, 72, 146, and 308. Because of the stalk deletion, the turkey N3s lacked three CHO sites (Fig. 2). Similar differences occur in the N1 of the Italian H7N1/99 poultry strains, in viruses of the H5N1 lineage from Hong Kong, in early human H1N1 isolates, and in the H5N2 viruses isolated during the Pennsylvania outbreak in 1983 (Banks et al., 2001; Matrosovich et al., 1999).

Apart from the deletion, the duck and turkey H7N3 strains had 11 nucleotide differences, five (45, 5%) of which were nonsynonymous and coded for the following amino acid changes (ducks versus turkeys): E37G (in the transmembrane region), T83P, L140V, Y266H, and T355K

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                                30        40        50        60        70        80
Tern/Astr/775/83          H13   AFNAVIHGKVENNKCETTPTTTPHPVYNCSDTVITKNHTTINNITTVVFQDPETHFRLPL
Mallard/It/208/00         H5    IFNTVIHEKIGDHQTVVYPTITAPVVPNCSDTIITYNNTVVNNITTTIITKAEKHFKSSL
Mallard/It/35/00          H2    IFNTVIHEKIGDHQTVVYPTIAAPVVSNCSDTIITYNNTVVNNITTTIITKAEKHFKSSL
Mallard/It/36/00          H2    IFNTVIHEKIGDHQTVVYPTIAAPVVSNCSDTIITYNNTVVNNITTTIITKAEKHFKSSL
Mallard/It/33/01          H7    IFNTVIHEKIGDHQTVVYPTVTAPVVPNCSDTIITYNNTVVNNITTTIVTKAETHFKSSL
Mallard/It/43/01          H7    IFNTVIHEKIGDHQTVVYPTVTAPVVPNCSDTIITYNNTVVNNITTTIVTKAETHFKSSL
Turkey/It/214845/02       H7    IFNTVIHGKIGDHQTVVYPTVTAPVV.....................TKAEPHFKSSL
Turkey/It/220158/02       H7    IFNTVIHGKIGDHQTVVYPTVTAPVV.....................TKAEPHFKSSL
Pigeon/Nan/9-366/00       H3    IFNAVIHEKIGDHQTVIYPTITPPVVPNCSDTIITYNNTVVNNITTTIITKAEKHFKSSL
Bantam/Nan/9-058/00       H3    IFNAVIHEKIGDHQTVIYPTITPPVVPNCSDTIITYNNTVVNNITTTIITKAEKHFKSSL
Duck/Ger/1215/73          H2    IFNTVIHEKIGDHSTVVYPTITTPVVPNCSDTIITYNNTVINNITTTIITEAERHFKPSL
Turkey /Min/916/80        H7    VFNTVIHEKIGDHQTVIHPTIMTPAVPNCSDTIITYNNTVINNITTTIITEAERLFKPPL
RuddyTurnstone/NJ/65/85   H7    IFNTVIHEKIGDHQTVIHPTITTPAVPNCSDTIITYNNTVINNITTTIITEAERPFKPPL
                                 ** *** *          **      *           *           *  *  *
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Fig. 2. Alignment of the NA stalk region of N3 viruses. The full names of virus strains are as indicated in Fig. 4. Potential glycosilation sites are underlined. Asterisks indicate conserved amino acid residues.

(Table 4). It is unclear whether one or more of these changes is related to adaptation of a wild aquatic bird virus to turkeys. However, it is worth noting that none of the listed substitutions occurred in any of the other wild bird N3 viruses we analyzed regardless of the site of isolation. Moreover, at amino acid level, the H7N3 duck strains appeared much more closely related to Mall/It/208/00 (99.7% similarity) than to the turkey strains (99.0% similarity).

Circulation of H9N2 viruses in chickens in Hong Kong has been associated with the accumulation of mutations in the amino acids coding for the NA hemadsorbing (HB) site,

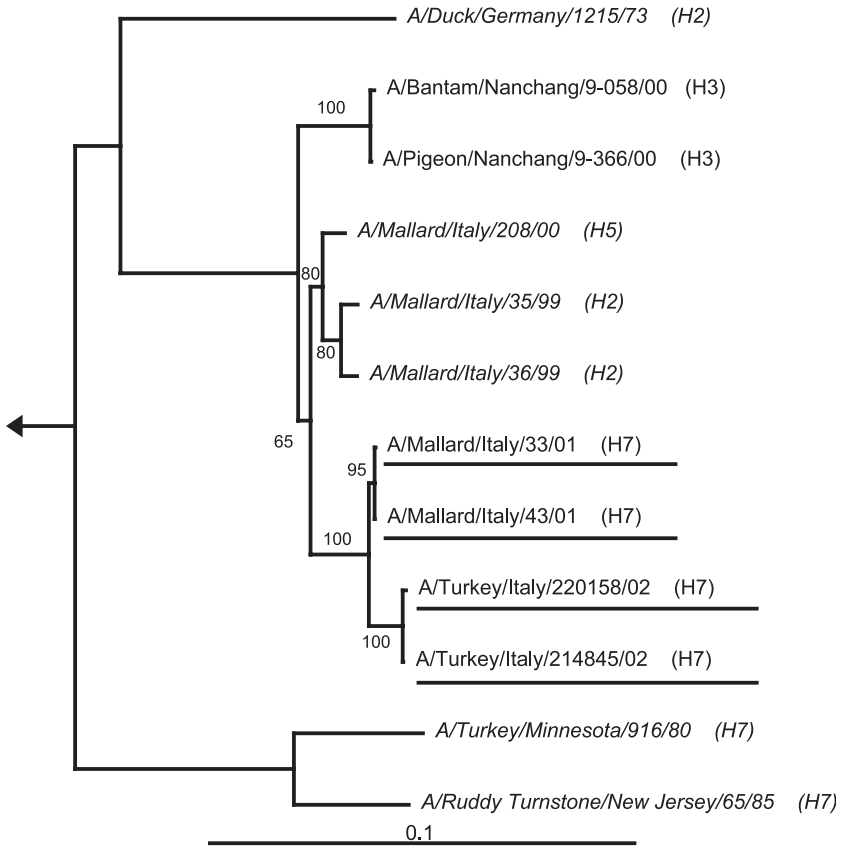

Fig. 3. Phylogenetic tree of 13 N3 NA genes, including the Italian H7N3 strains. The nucleotide region used to compute the tree comprised nucleotides 247–1432. A/Tem/Astrakhan/775/83 (H3N3) was used as root. Numbers at critical nodes represent bootstrap values obtained performing 1000 bootstrap replicates. Accession numbers of published sequences can be found in Liu et al. (2003). The Italian H7N3 strains are underlined, other strains sequenced in this study are in italics.

a second sialic acid-binding pocket on the NA surface (whose function is still unknown) that is typically highly conserved in aquatic bird strains (Matrosovich et al., 2001). Alignment of N3 HB and flanking sequences showed that the residues presumed involved in the hemadsorption activity of N3 strains (Kobasa et al., 1997) are all conserved in both duck and turkey H7N3 strains. Similarly, the 18 amino acids that define the enzymatic active site of the molecule, which have been highly conserved in all the NA subtypes analyzed so far (Colman et al., 1993), were retained in all the N3 strains we examined (data not shown).

A phylogenetic tree (generated with the limited number of sequences available) showed that the N3 genes can be grouped into two major lineages, the Eurasian and North American ones (Fig. 3). Within the Eurasian branch, all the Italian N3 genes clustered together and were clearly different from two H3N3 strains recently isolated in China, A/Pigeon/Nanchang/9-058/00 and A/Bantam/Nanchang/9-366/00. However, within the Italian virus group, the H7N3 duck and turkey strains were found on the same branch, whereas the other three duck strains formed two distinguishable groupings. Therefore, our homology and phylogenetic data indicate that (i) the H7N3 duck and turkey strains share a very recent precursor with regard to their NA genes; and (ii) some heterogeneity exists within the N3 gene pool circulating in wild ducks in Italy.

Homology and phylogenetic analysis of internal protein genes

Sequence analysis of the six internal protein genes of Mall/It/33/01 and Mall/It/43/01 showed that they were 99.9–100% identical in all segments, and the same values were found between the two turkey strains. Comparison of the two virus groups revealed a degree of similarity that ranged from 99.7% for the NS gene to 99.1% for the M gene, and from 99.7% for the PB2 protein to 99.1% for the NS1 protein, confirming the high level of similarity ob-

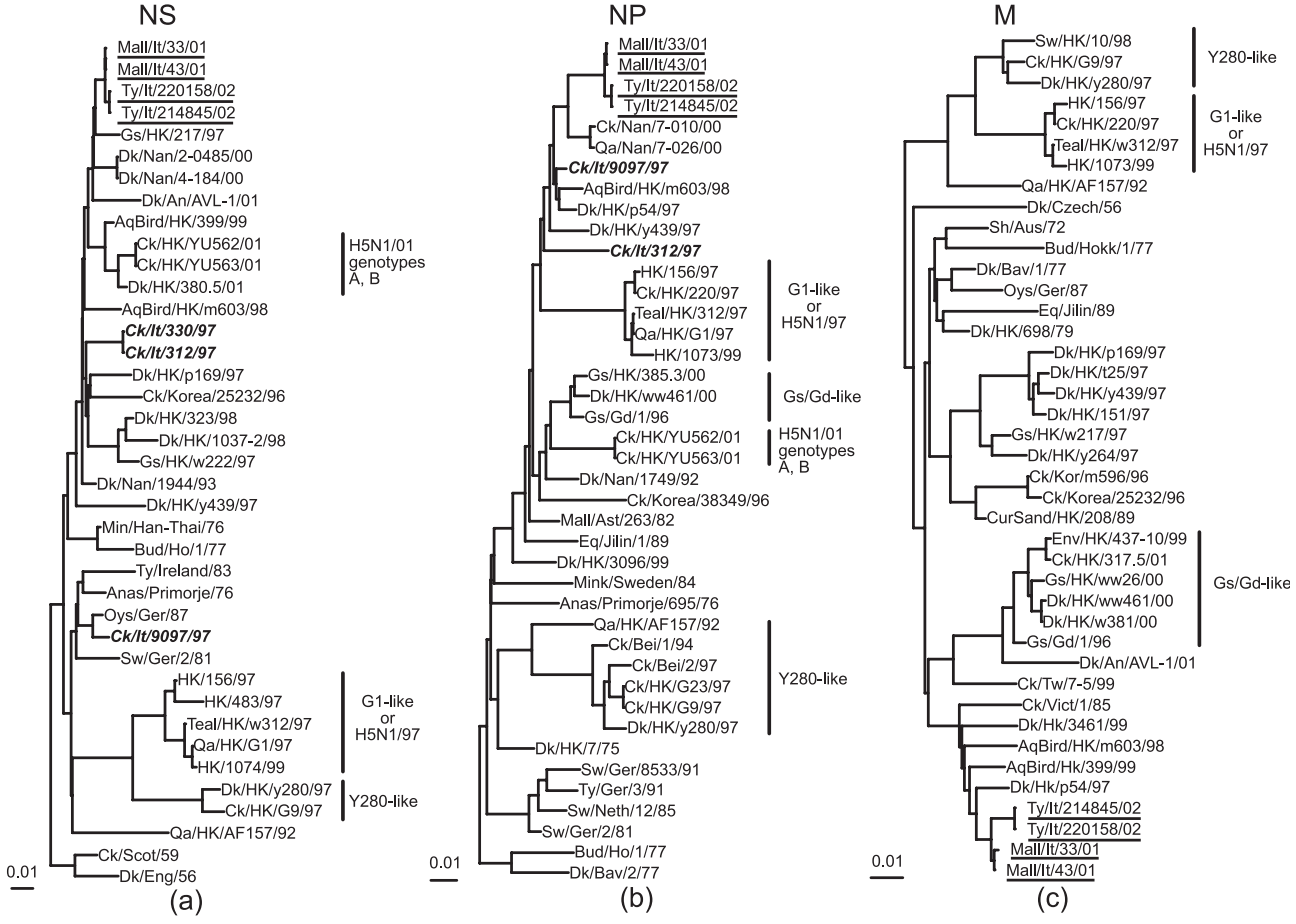

Fig. 4. Phylogenetic for the NS (a), NP (b), and M (c) genes of influenza A viruses. The nucleotide sequences were analyzed using the Fitch program (Phylip, version 3.57). Nucleotides 101–621 of the NS gene, 46–631 of the NP gene, and 44–781 of the M1 gene were used for the phylogenetic analysis. All gene trees were rooted to A/Equine/Prague/1/56 (H7N7). Branch lengths are proportional to genetic distances. Vertical lines are used to space branches and labels. The Italian H7N3 viruses are underlined, other viruses sequenced in this study are in italics, and the remaining sequences are available in GenBank. Abbreviations used: Ck, chicken; Dk, duck; Gs, goose; Sw, swine; AqBird, aquatic bird; Eq, Equine; Qa, Quail; Gu, gull; Env, Environment; FPV, fowl plague virus; Oys, Oystercatcher; Bud, Budgengar; Ty, turkey; Mal, Malard duck; Nan, Nanchang; Ho, Hokkaido; HK, Hong Kong; CurSand, Curfew sandpiper; Gd, Guangdong; Ast, Astrakhen; Bei, Beijing; Ger, Germany; Neth, Netherland; Bav, Bavaria; Tw, Taiwan; Vict, Victoria.

served in the surface genes (Table 3). Thus, the entire genome of the Italian turkey strains appeared to be derived from wild duck viruses circulating in Italy 1 year before.

All genes of both virus groups were most closely related to viruses circulating between 1997 and 2001 in Italy, South Central China, and Hong Kong (Table 3), with the highest values observed for M (98.7% homology to Duck/HK/P54/97-H11N6) and NS (98.5% homology to Dk/Nanchang/2-045/00-H2N9). As seen already with the NA gene, the percentage of similarity of the H7N3 duck strains was highest with the H7N3 turkey viruses at nucleotide level but not at amino acid level (Table 3).

Overall, phylogenetic analyses of the six internal genes confirmed the close clustering of the H7N3 duck and turkey viruses within the Eurasian lineage (Figs. 4 and 5). NP phylogeny showed that the Italian H7N3 virus genes are closely related to H5 viruses circulating in Italy in 1997, such as A/Chicken/Italy/9097/97 virus, located on a branch parallel to that of the 1997 H5N1 viruses from Hong Kong. On the contrary, NS genes clustered with viruses recently circulating in Southern China and were only distantly related to those of the H5 Italian strains (Fig. 4). An analogous pattern was observed with PB2, PB1, PA, and M genes that formed phylogenetic clusters with recent isolates from Southeast Asia (Fig. 5). However, none of the Italian virus genes appeared closely related to any of the genotypes previously involved in the transmission of avian influenza viruses to humans or other mammals, such as the H5N1/97 viruses or the Dk/HK/y280-like (H9N2) strains (Peiris et al., 2001).

Discussion

The results of our study demonstrate for the first time that domestic poultry viruses of the H7N3 subtype, which were responsible for the 2002–2003 LP avian influenza epidemic in Northern Italy, derived their entire genome directly from virus precursors circulating in wild ducks in Italy at least 1 year earlier.

Serological analysis of the HAs of duck and turkey H7N3 viruses showed an identical antigenic profile between the two virus groups, and comparison of the nucleotide and deduced amino acid HA sequences confirmed the serological data and revealed a very high degree of identity (99.6%) at both levels. As for the NA gene, the two virus groups showed a similarity of 99.0–99.1% at the nucleotide level and 99% similarity at the amino acid level throughout the molecule, except for the stalk region, where the turkey viruses differed by a 23-amino acid deletion. Therefore, ours is the first report in which a domestic poultry virus with a short-stalked NA and its direct wild bird counterpart, with

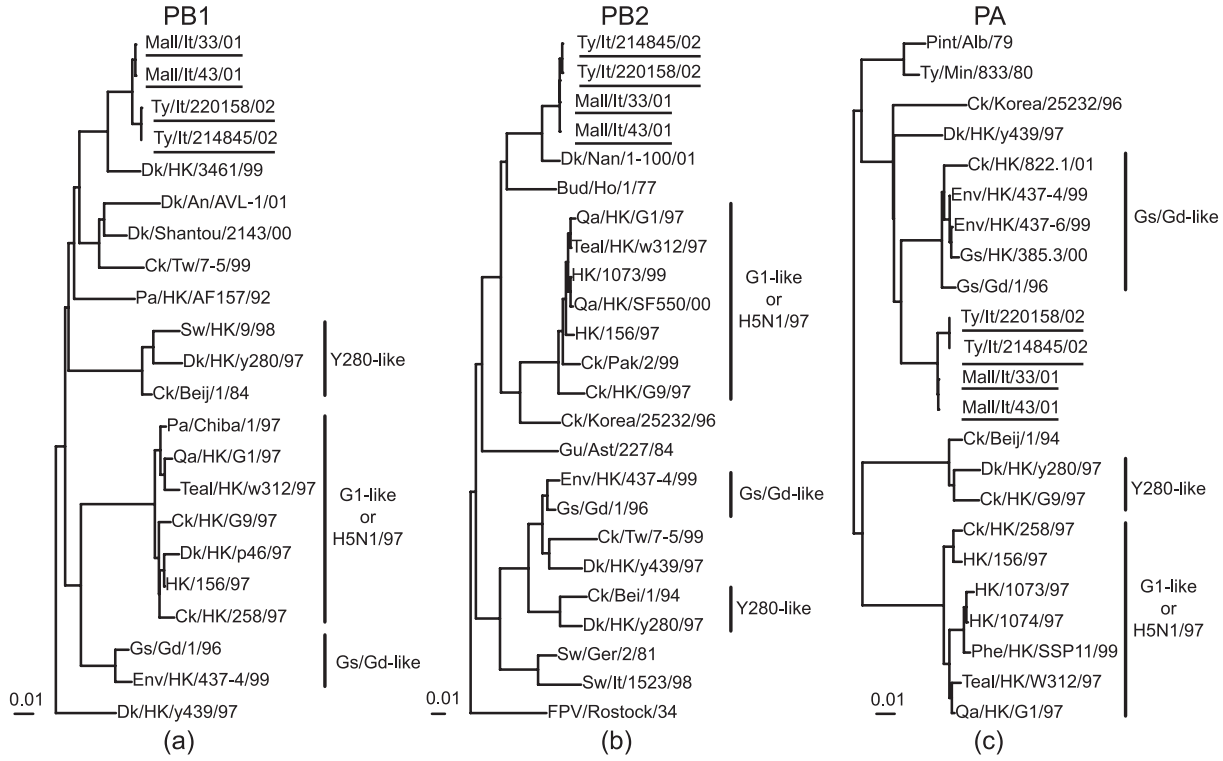

Fig. 5. Phylogenetic trees for the PB1 (a), PB2 (b), and PA (c) genes of influenza A viruses. Evolutionary analyses were performed as described in the legend to Fig. 4. Nucleotides 55–768 for PB1, 55–1283 for PA, and 65–603 for PB2 were used for the phylogenetic analysis. PB1 and PB2 gene trees were rooted to A/Equine/London/1416/73 (H7N7) and A/Equine/Prague/1/56 (H7N7), respectively. The PA gene tree was rooted to B/Singapore/222/79. The Italian H7N3 viruses are underlined, the remaining sequences are available in GenBank. Abbreviations used: Pa, Parakeet. Virus names and other abbreviations can be found in the legend of Fig. 4.

a homologous but long-stalked NA, are described, thus strengthening the hypothesis that a deletion in the NA stalk represents an adaptation of a wild bird virus to a new host, such as domestic poultry (Banks et al., 2001). Phylogenetic analysis of all genes confirms their very close relationship but apparently does not clarify whether the mallard strains were the progenitors of the turkey strains or the reverse. However, we must consider that: (a) the duck viruses were circulating during the fall of 2001, 1 year before the first isolation of the poultry viruses; (b) the regular serological monitoring of commercial poultry for influenza H7 and H5 antibodies, established as a mandatory preventive measure in Italy after the 1999–2000 epidemic, did not demonstrate any sign of H7 influenza circulation in the poultry farms until July 2002; (c) the NA stalk deletion, typical of both LP and HP poultry viruses, has never been found in association with influenza in feral birds. Therefore, the most plausible explanation for the presence of viruses with an almost identical genome in both bird groups is that an H7N3 influenza strain, circulating in populations of migratory wild waterfowl that congregate in early autumn in protected wetlands in Central Italy (used as wintering sites), was introduced into the domestic bird populations in Northern Italy during the migratory movements of wild ducks. However, the actual epidemiological link between feral birds and poultry farms has not yet been identified.

Analysis of the molecular differences in the surface genes of the duck and turkey strains revealed only two amino acid changes in the HA gene. When we aligned their HA genes with available sequences in GenBank, neither of the two amino acid positions at which a substitution was found between the duck and turkey H7N3 strains (271HA1 and 161HA2, according to H3 numbering) could be related to host specificity. Thus, apparently no species-specific adaptation of the duck virus H7 HA seemed necessary to allow infection of turkeys. On the other hand, host range-related changes of the H9 HA protein have been postulated for a duck virus to efficiently infect chickens (Perez et al., 2003). These observations are consistent with evidence from both field studies and experimental infections, which indicate that turkeys are more susceptible to infection by duck viruses than are chickens (Halvorson et al., 1983). Nonetheless, it is noteworthy that, although the majority of poultry farms affected by H7N3 LPAI reared turkeys, the epidemic also spread to chicken and hen farms, particularly in Lombardia, where the number of layers is about three times that of turkeys, indicating that this H7N3 virus is capable of effectively infecting these species, too. It is not known whether the H7N3 chicken viruses isolated in these outbreaks bear a different set of mutant residues on the HA than do the turkey viruses, and further studies will be important to elucidate this point. However, molecular changes in HA alone are insufficient for efficient replication and transmission of duck viruses in chickens, and mutations in the remaining genes may be necessary (Perez et al., 2003). In this regard, the possibility that wild bird viruses circulating in turkeys could undergo changes that favor their adaptation to growth in chickens cannot be ruled out.

Amino-acid changes observed in the N3 genes of duck and turkey strains up- and downstream of the stalk deletion did not affect the functionally active sites of the molecule, that is, the enzymatic active site (as defined by Colman et al., 1993) and the hemadsorbing (HB) site. Recently, accumulation of mutations in the HB site has been observed in H9N2 Hong Kong chicken viruses linked to those infecting humans, and has been correlated to the acquisition of a human-like HA binding specificity (Matrosovich et al., 2001). Therefore, both of these features—the mutations in the HA receptor binding site (RBS) and those in the HB—may represent markers predictive of the potential of avian viruses to infect humans. The conservation of the avian consensus sequence in both the HB site and the RBS of all the Italian H7N3 strains suggests that these viruses may lack some requirement in the HA gene that facilitates transmission to humans, although the experience with the 1997 H5N1 viruses indicates that a human-like receptor specificity is not strictly necessary to overcome the species barrier, and the contribution of the internal genes in the adaptation to a new host also has to be considered.

A deletion of variable length in the NA stalk is a molecular feature frequently found in viruses isolated from domestic poultry, and this change is thought to be the result of early adaptation of wild avian influenza viruses to land-based poultry. Previous studies have demonstrated that viruses with NA stalk deletions tend to compensate for their low NA activity (that affects negatively the release of progeny virions from infected cells) by two mechanisms: restoration of the NA stalk by RNA–RNA recombination or a decrease in HA binding affinity to sialic acid, for example, by increased glycosylation of the HA globular head (Baigent and McCauley, 2001; Mitnaul et al., 2000). The latter mechanism seems to be operating in nature, too, because several chicken and turkey viruses with NA stalk deletions possess additional potential CHO sites on their HA1 subunits. The H7N3 turkey and duck viruses analyzed in this study do not contain any extra CHO signals in the globular head of the HA as they share the same pattern of glycosylation that is found among all duck H7 viruses. The two turkey strains were isolated during the initial outbreaks in October 2002, presumably shortly after the initial virus introduction into poultry (the first serological positivity for H7 influenza from the same turkey farm at which the index case virus was isolated was in July 2002). Therefore, the positive selection pressure on viruses in the poultry host to acquire glycosylation motifs around the RBS had not had enough time to exert its effect. Similarly, in the 1999–2000 H7N1 epidemic, the initial isolates, which already had a short-stalked NA, did not have additional CHO sites, but variants soon emerged with extra CHO motifs at either position 123 or 149 (133 and 158 in H3 numbering) (Banks et al., 2001). Although HA sequencing of a few turkey strains isolated later during the epidemic in Northern Italy

showed an unchanged glycosylation pattern (L. Campitelli, unpublished results), some other isolates may have acquired this feature. These data indicate that the NA stalk deletion is likely to be essential for effective virus growth in the poultry host, although its functional relevance remains unknown, and mutations in the affinity of HA for virus receptors may arise afterwards to compensate the reduced NA activity.

Previous studies showed that the HA1 region of the genome undergoes the greatest number of amino acid changes when an influenza virus is first introduced into a new host (Ludwig et al., 1995; Schafer et al., 1993). During the 1994–1995 H5N2 epidemic in Mexican poultry, more than 50% of nucleotide substitutions were non-silent (Garcia et al., 1996). Instead, we observed a much higher proportion of coding to non-coding changes in the NA gene than in the HA gene (45% versus 16.7%) between the two Italian H7N3 virus groups. Moreover, none of the amino acid substitutions found in the Italian turkey N3 NAs were observed in any of the N3 subtype strains from wild ducks and shorebirds. Despite the fairly brief circulation of the H7N3 strains in poultry flocks, this difference suggests that the HA protein of the duck virus is already fairly well adapted to the turkey host, whereas the NA seems to be under higher selection pressure upon introduction into the new poultry host, as has been observed among the chicken H5N1 viruses isolated in Hong Kong (Zhou et al., 1999). Amino acid homology indicates that nonsynonymous substitutions occur also in the internal protein genes of the H7N3 turkey strains, in contrast with the high amino acid conservation between the mallard strains and other wild avian viruses (Table 3), suggesting that the adaptation process may involve also the rest of the genome.

According to phylogenetic analysis of the four Italian H7N3 viruses, none of the six internal protein genes is found in association with any of the evolutionary clusters that include avian viruses involved in avian-to-human transmission in Hong Kong (such as A/Hong Kong/156/97 (H5N1)-like or A/Hong Kong/1073/99 (H9N2)-like strains). Nonetheless, they are all closely related to several avian viruses recently isolated in Southeast Asia, particularly in China (Liu et al., 2003). This evidence confirms the continuous flow of avian influenza viruses between Europe and Asia due to bird migrations and reinforces the need for a regular influenza monitoring in wild birds to identify viruses with pandemic potential.

An important question was whether the HA gene of the H7N3 strains derived from the H7N1 virus responsible for the avian influenza epidemic that occurred in Italian poultry between March 1999 and April 2000 and was followed by two further waves of H7N1 LPAI between August 2000 and March 2001 (Capua et al., 2002a). Serologic analysis shows that the H7N1 and H7N3 viruses can be clearly distinguished by antisera raised against the H7N1 strains, and phylogenetic analysis (with the exception of viruses from the two most recent H7N1 outbreaks, as no sequence data are available) confirms these data, indicating a sister-group relationship between the two virus groups that mirrors the high nucleotide and amino acid homology between the H7N3 and H7N1 strains. Since there was no serological evidence of H7 circulation until July 2002 in domestic poultry, whereas a H7N3 virus lacking the NA deletion circulated in wild waterfowl late in 2001, the most plausible explanation remains that the precursor of the H7N1 viruses in the wild bird reservoir was maintained in aquatic birds, although virological monitoring in these species did not detect any H7 virus before 2001 (De Marco et al., 2003b and personal communication). In this reservoir, very likely this precursor virus reassorted with a N3 subtype virus (H2N3 and H5N3 viruses had been isolated in wild birds in previous years) and was introduced again into domestic poultry in 2002.

One of the most serious concerns of the LP H7N3 influenza epidemic in Italian poultry was related to its prolonged duration, about 1 year (Report to the European Commission, 5 November 2003), as it is well known that the risk of emergence of a H7 or a H5 strain bearing a multibasic cleavage site in the HA (a primary molecular determinant of the HP phenotype) increases with time: an occurrence of this type took place in Mexico in 1994–1995 (Horimoto et al., 1995), and in Italy in 1999–2000 led to the culling of more than 14 million birds (Capua et al., 2000). However, during this period, no HP H7N3 domestic poultry viruses arose.

A second reason for concern was related to the zoonotic potential of avian influenza viruses circulating in terrestrial poultry. The most recent cases of avian-to-human transmission were associated with an outbreak of HPAI in poultry in The Netherlands that started in February 2003 and was caused by an H7N7 virus (Osterhaus, 2003). More than 80 people in contact with infected poultry were found positive for H7N7 virus. Most of them developed conjunctivitis, but in a few cases, flu-like symptoms were observed, and in one case, a veterinarian died of pneumonia, and the H7N7 virus was found in his lungs. Moreover, there appeared to be at least three cases of secondary transmission to family members. We do not know the relationship between the Italian and Dutch H7 strains. The HP Dutch viruses are strongly suspected to have originated from the wild bird reservoir because H7 strains had been isolated from wild ducks in The Netherlands in previous years (Fouchier et al., 2003) and because migratory movement of feral birds between The Netherlands and Italy cannot be ruled out (De Marco et al., 2000). Therefore, to better understand differences and similarities between the H7N3 and H7N7 European viruses, particularly in regard to the ability to transmit to people, it will be essential to compare and analyze their surface and internal gene proteins.

A final consideration can be drawn from these data. China has long been considered a potential epicenter for the emergence of both epidemic and pandemic influenza viruses (Webster et al., 1992). Our findings, with those from the recent Dutch outbreaks, suggest that the chain of events

leading to the emergence of a potentially pandemic virus may occur in other regions as well, and reinforce the importance of continuing surveillance of influenza virus in multiple animal reservoirs worldwide. In particular, this continued surveillance may enable us to characterize the influenza gene pool in the avian reservoir in Europe and help to identify the gene combinations and mutations that can be involved in the generation of a potentially pandemic virus.

Materials and methods

Viruses

For influenza A virus detection and isolation from wild waterfowl, cloacal swabs were processed as follows: pools of five to six fecal specimens were prepared, viral RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), and RT-PCR with primers M52C and M253R, which are specific for a conserved region of the influenza matrix protein, was performed as previously described (Fouchier et al., 2000). Samples from PCR-positive pools were inoculated into 10-day-old embryonated SPF hen's eggs, and influenza isolates were identified by both the hemagglutination test (according to standard procedures) and a double-antibody "sandwich ELISA" for the detection of influenza A virus nucleoprotein (Foni et al., 1995).

Turkey viruses were obtained during the initial outbreaks of the LP H7N3 avian influenza that started in Northern Italy in October 2002 (Capua et al., 2002b). For virus isolation, samples were inoculated in embryonated hens' eggs, as described earlier. A/Ruddy Turnstone/New Jersey/65/85 (H7N3), A/Duck/Germany/1215/73 (H2N3), A/Turkey/Minnesota/916/80 (H7N3), and A/Tern/Astrakhan/775/83 (H13N3) were obtained from the virus repository at St. Jude Children's Research Hospital, Memphis, TN.

Antigenic characterization

Subtype identification of influenza viruses was performed by using hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays with a panel of reference antigens and antisera, as previously described (WHO, 2002). A more detailed analysis of HA antigenic reactivity was carried out by HI test using a panel of hyperimmune chicken sera to A/Ruddy Turnstone/New Jersey/65/85 (H7N3), A/Turkey/Italy/2676/99 (H7N1), the index case strain isolated during the 1999-00 H7N1 Italian poultry outbreak, and A/Turkey/Italy/214845/02 (H7N3) virus. In addiction, a panel of postinfection ferret sera against A/Chicken/Italy/13474/99 (H7N1) virus, also isolated during the 1999-00 H7N1 outbreak, and a monoclonal antibody to Ty/It/2676/99 were used. The HI test was performed using horse red blood cells (HRBCs) according to standard procedures with minor modifications (Stephenson et al., 2003). Basically, a 1% suspension of HRBCs in 0.5% BSA–PBS was used throughout the procedure and HI titers were read after 60 min.

Gene sequencing and analysis

Viral RNA was extracted from infected allantoic fluids as described earlier and reverse-transcribed using a 12-bp oligodeoxynucleotide primer (5′ AGCAAAAGCAGG) and Superscript II reverse transcriptase (Invitrogen, Paisley, UK), as described previously (Campitelli et al., 2002). We PCR-amplified the coding region of the viral gene segments with gene-specific primers using the Expand High-Fidelity PCR system (Roche, Mannheim, Germany) according to the manufacturer's protocol. Amplified products of the expected size were purified with the Qiaquick PCR purification kit (Qiagen), sequenced using the BigDye Terminator Cycle-Sequencing Ready Reaction (Applied Biosystems, Foster City, CA), and analyzed on ABI PRISM 310 or 377 DNA sequencers (Applied Biosystems).

Sequence and phylogenetic analysis

Editing, analysis, and alignment of sequence data were performed with the Lasergene package (version 4.0; DNASTAR, Madison, WI). Phylogenetic analysis was carried out using the Fitch program (PHYLIP, version 3.57; Felsenstein, 1981). Transition/transversion ratios were calculated for the eight data sets using Tree-Puzzle version 5.0 (Schmidt et al., 2002).

Nucleotide sequence accession numbers

Sequences obtained in this report can be found under the following accession numbers: AY586408 though AY586446.

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