Characterization of H5N2 influenza viruses from Italian poultry

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From October 1997 to January 1998, highly pathogenic H5N2 avian influenza viruses caused eight outbreaks of avian influenza in northern Italy. A nonpathogenic H5N9 influenza virus was also isolated during the outbreaks as a result of virological and epidemiological surveillance to control the spread of avian influenza to neighbouring regions. Antigenic analysis showed that the Italian H5N2 isolates were antigenically similar to, although distinguishable from, A/HK/156/97, a human influenza H5N1 virus isolated in Hong Kong in 1997. Phylogenetic analysis of the haemagglutinin (HA) genes showed that the highly pathogenic Italian viruses clustered with the Hong Kong strains, whereas the nonpathogenic H5N9 virus, despite its epidemiological association with the highly pathogenic Italian isolates, was most closely related to the highly pathogenic A/Turkey/England/91 (H5N1) strain. Like the HA phylogenetic tree, the nonstructural (NS) phylogenetic tree showed that the H5N2 Italian virus genes are clearly separate from those of the H5N9 strain. In contrast, results of the phylogenetic analysis of nucleoprotein (NP) genes indicated a closer genetic relationship between the two Italian virus groups, a finding suggesting a common progenitor. Comparison of the HA, NS and NP genes of the Italian H5 strains with those of the H5N1 viruses simultaneously circulating in Hong Kong revealed that the two groups of viruses do not share a recent common ancestor. No virological and serological evidence of bird-to-human transmission of the Italian H5N2 influenza viruses was found.

Introduction

All 15 haemagglutinin (HA) and nine neuraminidase (NA) subtypes of influenza viruses have been isolated from feral aquatic birds and from a wide range of domestic avian species, such as chickens, turkeys, pheasants, geese and ducks (Webster et al., 1992). Most influenza virus infections tend to be subclinical in poultry, producing only mild to moderate signs of disease that include respiratory distress, decreased egg production and renal deficiency. However, in chickens, geese and ducks, some avian H5 and H7 viruses cause severe systemic disease that is associated with high rates of morbidity and mortality. Such highly virulent strains have been isolated in widespread locations: Pennsylvania in 1983 (Bean et al., 1985), Australia in 1987 (Murphy, 1986), Mexico in 1994 and 1995 (Horimoto et al., 1995; García et al., 1996), and continental Europe and Great Britain (reviewed in Alexander, 2000). Classification of an avian influenza virus as highly pathogenic is based on the isolate’s intravenous pathogenicity index (IVPI) and the amino acid sequence of the HA cleavage site (Wood et al., 1993). Further studies have demonstrated that highly pathogenic strains do not constitute distinct influenza virus lineages but are derived from nonpathogenic avian strains that have acquired mutations that render the HA cleavable by intracellular proteases (Röhm et al., 1995; Perdue et al., 1997). Avian influenza viruses are rarely transmitted directly to humans. A notable exception is the avian virus that caused the
1997 influenza outbreak in Hong Kong (Yuen et al., 1998; de Jong et al., 1997). All of the virus isolates were closely related to avian H5N1 strains that had previously caused outbreaks of influenza-like illness among poultry flocks in the new territories of Hong Kong, SAR, China (Shortridge et al., 1998; Suarez et al., 1998; Subbarao et al., 1998). The transmission of an avian virus directly to humans raised serious concern over the emergence of an influenza pandemic.

From October 1997 to January 1998, eight outbreaks of highly pathogenic avian influenza (HPAI) occurred in poultry in northeastern Italy (Capua et al., 1999). An epidemiological survey was conducted to control the spread of avian influenza to neighbouring regions and to identify the origin and mechanism of transmission of infection among flocks. Here we present the results of antigenic and molecular characterizations of the isolated highly pathogenic and non-pathogenic H5 influenza viruses and the results of our comparison of these viruses with other avian and avian-like influenza viruses, including those isolated in Hong Kong.

Methods

■ Origin and serological characterization of virus isolates. The highly pathogenic H5N2 strains analysed in this study were isolated as previously described (Capua et al., 1999). The non-pathogenic strain, Ck/lt/9097/97, was isolated after an initial passage of chicken organ homogenates into 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Five pharyngeal swabs were also collected from persons in close contact with infected birds during the last outbreak, and these samples were passaged in SPF eggs as described (Bean et al., 1985) and in SPF primary chick embryo fibroblast cells. All samples were handled in a biosafety level 3 containment facility.

Viral HAs were characterized by haemagglutination inhibition (HI) assays with antisera to influenza virus (US Department of Health and Human Services, 1982). A more detailed antigenic analysis was performed with polyclonal antisera to representative H5 strains and a panel of monoclonal antibodies (MAbs) to the HA of Ck/PA/1370/83 (H5N2). These MAbs and antisera were prepared as previously described (Kawaoka et al., 1987).

Ferret antisera to HK/156/97 (H5N1) was kindly provided by the Centers for Disease Control (Atlanta, GA, USA). Viral NAs were characterized by using the NA inhibition test (US Department of Health and Human Services, 1982).

■ Pathogenicity testing in chickens. The pathogenicity of the Ck/lt/9097/97 virus was determined in 6-week-old chickens at the European Union (EU) Reference Laboratory according to the IVPI test described in the EU Directive (CEC, 1992). In this test, an IVPI of 3–00 indicates that all birds died within 24 h of infection, whereas an index of 0–00 indicates the absence of any clinical signs of illness in any birds during the 10 day observation period. The pathogenic potential of the isolate was also evaluated by sequencing the amino acids at the HA cleavage site (Senne et al., 1996).

■ Serological analysis of human sera. Sera obtained from people who had been in close contact with sick or dead birds were collected at the end of the outbreaks. Since the HI test is not considered a sensitive method for detecting antibodies against avian influenza viruses in humans (Lu et al., 1982; Zhou et al., 1996), we analysed human sera by the new microneutralization test previously described (Rowe et al., 1999), using the first avian H5N2 (Ck/lt/312/97) virus isolate as reference antigen. Neutralizing antibody titres below 80 were considered negative by Rowe et al. (1999).

The human sera were also tested by the single radial haemolysis (SRH) assay, using RT/DE/253/91 (H5N2) as a reference virus. This assay was performed by standard methods (Mancini et al., 1984) using chronic chloride-treated turkey erythrocytes, with minor modifications including 15% turkey erythrocyte suspension and adsorption of sera with both human H1N1 and H3N2 strains prior to testing with H5 antigens (I. Wood, personal communications).

■ Sequence and phylogenetic analyses. Four influenza virus strains (Ck/lt/312/97, Gf/lt/330/97, Ck/lt/365/97, Ck/lt/367/97) isolated in different areas (Fig. 1) and at different times were selected as representatives of the highly pathogenic virus isolates, and their HA.
genes were characterized by sequence analysis. The nonpathogenic CK/It/9097/97 strain was also included in the molecular study. Viral RNA was extracted and amplified by RT–PCR, as described previously (Campitelli et al., 1997). The PCR products were subjected to cycle sequencing with an ABI dye terminator sequencing system.

The nucleotide sequences from this study were compared with published sequences in GenBank by using the GCG software package (Madison, WI, USA). Phylogenetic trees were constructed with the Fitch–Margoliash algorithm (PHYLIP package, version 3.57c).

Accession numbers of the genes sequenced in this report are AF194169, AF194990–92 and AF319644–51.

Results

Epidemiological, pathogenic and antigenic properties of the avian influenza virus isolates

The eight outbreaks of HPAI occurred in poultry on rural farms in two regions of Italy, Veneto and Friuli-Venezia Giulia (Fig. 1; Capua et al., 1999). The Istituto Zooprofilattico Sperimentale of Padua (the peripheral veterinary centre responsible for animal disease surveillance in the northeastern regions of Italy) conducted an epidemiological survey to identify the origin and mechanism of transmission of infection among flocks. The exception, CK/It/9097/97, was isolated from healthy birds on a weaning farm with commercial links to two of the farms where HPAI outbreaks occurred. This virus, antigenically characterized as an H5N9 influenza virus strain, did not cause any sign of disease in experimentally infected birds (IVPI = 0.0), and sequence analysis of the HA-connecting peptide region showed that it did not possess multiple basic amino acids at the cleavage site (KETR*GLF). Therefore, the virus was classified as a nonpathogenic chicken influenza virus.

To evaluate the antigenic characteristics of the isolates and to determine the diversity of their HAs, we expanded the analysis by using a panel of MAbs and goat, chicken and ferret polyclonal antisera to the H5 HA (Table 1). Because MAb CP46 can detect the presence of a carbohydrate at residue 158 near the receptor binding site on HA, this MAb has been used to distinguish two groups of Hong Kong H5N1 influenza virus isolates: one group has a carbohydrate at residue 158, and the other group lacks the carbohydrate (Shortridge et al., 1998). Therefore, the HK/156/97 and CK/HK/W162/97 (H5N1) viruses were included in the analysis as representatives of the antigenic group containing the carbohydrate, whereas CK/HK/258/97 and HK/483/97 viruses were used as serological prototypes of the group lacking the carbohydrate. We also included two nonpathogenic H5 viruses, CK/Chia/15224/97 (H5N2) and Dk/Sing-Q/F119-3/97 (H5N3).

Each of the Italian strains reacted with polyclonal reference antisera, a result that confirmed their identity as H5 viruses (Table 1). The Italian strains also reacted similarly with the panel of MAbs, but the nonpathogenic CK/It/9097/97 virus

### Table 1. HI reactivity of representative H5 virus isolates from Italian poultry

<table>
<thead>
<tr>
<th>Virus</th>
<th>CP24</th>
<th>CP25</th>
<th>CP46</th>
<th>CP58</th>
<th>364/</th>
<th>Te/SA/G</th>
<th>CK/Chia/Ct</th>
<th>HK/156/F</th>
<th>HK/156/C</th>
</tr>
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<tbody>
<tr>
<td>CK/It/312/97</td>
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<td>200</td>
<td>1600</td>
<td>3200</td>
<td>6400</td>
<td>320</td>
<td>640</td>
<td>160</td>
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</tr>
<tr>
<td>CK/It/326/97</td>
<td>100</td>
<td>200</td>
<td>800</td>
<td>6400</td>
<td>12800</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>Gf/lt/330/97</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>3200</td>
<td>6400</td>
<td>320</td>
<td>640</td>
<td>80</td>
<td>320</td>
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<tr>
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<td>100</td>
<td>400</td>
<td>6400</td>
<td>6400</td>
<td>160</td>
<td>640</td>
<td>80</td>
<td>320</td>
</tr>
<tr>
<td>CK/lt/9097/97</td>
<td>&lt;</td>
<td>100</td>
<td>100</td>
<td>1600</td>
<td>3200</td>
<td>40</td>
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<td>20</td>
<td>80</td>
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<td>640</td>
<td>640</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>CK/HK/258/97</td>
<td>800</td>
<td>800</td>
<td>&lt;</td>
<td>3200</td>
<td>6400</td>
<td>40</td>
<td>320</td>
<td>20</td>
<td>320</td>
</tr>
<tr>
<td>HK/483/97</td>
<td>&lt;</td>
<td>100</td>
<td>&lt;</td>
<td>3200</td>
<td>3200</td>
<td>40</td>
<td>160</td>
<td>&lt;</td>
<td>160</td>
</tr>
<tr>
<td>Dk/Sing-Q/F119-3/97</td>
<td>&lt;</td>
<td>100</td>
<td>1600</td>
<td>6400</td>
<td>3200</td>
<td>160</td>
<td>320</td>
<td>80</td>
<td>320</td>
</tr>
<tr>
<td>CK/PA/1370/83</td>
<td>200</td>
<td>800</td>
<td>3200</td>
<td>1600</td>
<td>6400</td>
<td>320</td>
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<td>&lt;</td>
<td>1600</td>
<td>3200</td>
<td>&lt;</td>
<td>320</td>
<td>10</td>
<td>80</td>
</tr>
</tbody>
</table>

* Goat antiserum to isolated HA of Te/SA/61 virus.
† Chicken antiserum to CK/Chia/15224/97 virus.
‡ Ferret antiserum to HK/156/97 virus.
§ Chicken antiserum to HK/156/97 virus.

Abbreviations: chicken, Ck; guinea fowl, Gf; duck, Dk; tern, Te. Italy, It; Chiapis, Chia; Hong Kong, HK; Singapore, Sing; Pennsylvania, PA; South Africa, SA. <, Titre that is less than 10; <, titre that is less than 10. nt, Not tested.
Table 2. Nucleotide sequence homology of the HA gene among Italian H5 chicken influenza viruses and between these strains and closely related H5 influenza viruses of the Eurasian avian lineage
Abbreviations of virus strains are given in the legends to Table 1 and Fig. 2. A dash (–) means that the corresponding homology value is already shown elsewhere in the table.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence analysed</th>
<th>% Identity of the HA genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ty/En/91</td>
</tr>
<tr>
<td>Ck/lt/9097/97</td>
<td>51–1729</td>
<td>97:1</td>
</tr>
<tr>
<td>(HSN9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ck/lt/312/97</td>
<td>13–1746</td>
<td>95:4</td>
</tr>
<tr>
<td>(HSN2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HSN2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HSN2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HSN2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Homology to DK/HK/78 was limited to the HA1 portion of the gene.
† A/HK/156/97 (HSN1).
failed to react with CP24. Although distinguishable from the Hong Kong H5 reference strains, the Italian isolates showed appreciable cross-reactivity with the HK/156/97 virus, as indicated by their binding to the CP46 MAb. As predicted, sequencing of the viral HAs revealed the absence of a carbohydrate at residue 158, which would account for reactivity with CP46.

One of the nonpathogenic Asian viruses (Dk/Sing-Q/F119-3/97) reacted with all but one of the MAbs and with the reference polyclonal antisera (Table 1). The Italian Ck/It/9097/97 strain reacted with all of the antisera, but the titres of the reacting antibodies were low. This nonpathogenic strain did not react at all with CP24 MAb; thus, the antigenic pattern of the strain was distinguishable from that of the H5N2 group. Sequence analysis showed that HA1 of the Ck/It/9097/97 (H5N9) strain lacked the potential glycosylation site at amino acid residue 158; this characteristic is identical to that of the Italian H5N2 strains.

**Sequence and evolutionary analysis of the H5 HAs**

We sequenced the Italian H5 HA genes to investigate their relationships with the Hong Kong H5 HA genes and to test the hypothesis that the HAs of the highly pathogenic H5N2 viruses were derived from the HA of the nonpathogenic H5N9 virus after the acquisition of basic amino acids at the connecting peptide. This mechanism has already been observed in previous HPAI outbreaks (Horimoto et al., 1995). Table 2 reports the HA1 and HA2 nucleotide sequence identities among four highly pathogenic Italian isolates, the nonpathogenic strain and a group of antigenically related H5 viruses of the Eurasian avian lineage. The HA genes of the highly pathogenic Italian viruses were almost identical to each other (99-4% to 99-9% identity). The percentages of identities between the HA genes of the highly pathogenic H5N2 viruses and that of Ck/It/9097/97 (H5N9) were appreciably lower (94-3% to 94-6%). Comparison of the HA sequences of the Italian strains with the H5 HA sequences available in GenBank revealed that the Italian strains were most closely related to Ty/Eng/50-92/91, although the degree of identity differed for the highly pathogenic (95-4% to 95-6%) and nonpathogenic Italian strains (97-1%). Similar results were obtained in comparisons with HK/156/97 HA: the identities with HA genes of the highly pathogenic strains ranged from 93-7% to 93-9%, whereas the identity with the HA genes of the nonpathogenic strain was 92-2%.

These findings were supported by phylogenetic analysis of H5 HA1 sequences (Fig. 2). Despite their epidemiological association, the highly pathogenic and nonpathogenic Italian viruses were positioned on discrete branches of the H5 HA phylogenetic tree; this pattern indicates that the highly pathogenic viruses probably did not originate from Ck/It/9097/97. Thus, two distinct H5 sublineages were identified within the Italian chicken H5 strains. Moreover, the highly pathogenic strains formed an evolutionary cluster with the Hong Kong viruses and their putative progenitor Goose/Guangdong/1/96 (Xu et al., 1999). Similar groupings were obtained in the evolutionary analysis of the corresponding amino acid sequences (data not shown).

Regardless of their pathogenicity, the sequenced viruses showed an identical pattern of six potential glycosylation sites at positions 11, 23, 165, 286, 482 and 539. Only one glycosylation site (residue 165) appears to be located on the HA1 globular head of the highly pathogenic and nonpathogenic viruses isolated in Italy. This finding is similar to that for nonpathogenic H5 viruses isolated from aquatic birds (Matrosovich et al., 1999). Ty/Eng/50-92/91 virus, the most closely related, highly pathogenic strain in the Eurasian avian lineage, lacks a glycosylation site at the same position.

**Evolutionary analysis of NP and NS genes**

We analysed the nucleotide sequences of the genes encoding the nucleoprotein (NP) and the nonstructural (NS) protein to evaluate the phylogenetic relationships of the Italian
H5 viruses as indicated by genes encoding internal proteins (Fig. 3). The Eurasian avian terminal branches are shown. Results of the phylogenetic analysis of the NS genes showed that the H5N2 Italian virus genes are clearly separated from those of the H5N9 strain; this separation is similar to that seen in the tree created from the HA gene data. The NS genes of the Italian isolates form a subgroup with that of Dk/HK/y439/97 (an H9N2 avian virus representing one of the three H9N2 lineages cocirculating in southern China), distinguishable from the Hong Kong H5N1 influenza viruses and from other H9N2 strains considered as possible donors of the internal protein genes to the HK H5N1 virus group (Guan et al., 1999). In contrast, the NP phylogenetic tree indicated a closer genetic relationship between the highly pathogenic and the nonpathogenic Italian viruses, which clustered on the same branch, again together with Dk/HK/y439/97 (Fig. 3).

Although the analysis of the genes encoding internal proteins was limited to only two, these results suggest that at least the NP genes of the Italian H5N9 and H5N2 chicken strains share a recent common precursor but that both the NP and NS genes (except for the NP gene of the H5N9 virus) appear to be related to those of currently circulating Asian viruses.

Lack of evidence for transmission of Italian avian H5N2 viruses to humans

During the outbreak of highly pathogenic H5N2 avian influenza in Italy, there was no evidence of clinical respiratory infection of poultry workers or other humans working with the viruses. Despite the lack of even mild infection, analysis of a small number of human samples was done. Throat samples collected from intensively exposed poultry workers from the last outbreak failed to yield influenza viruses after three blind passages in chicken embryos and primary chick embryo fibroblast cells. Detailed serological analysis of 32 human sera from poultry workers and laboratory technologists (data not shown) by microneutralization and SRH gave negative results in both tests. Therefore, no evidence of anti-H5 antibodies in human sera was observed.
Discussion

Our investigations of the possible origin of the highly pathogenic Italian H5N2 influenza viruses showed that all of the viruses analysed in this study reacted with a panel of MAbs to Ck/PA/1370/83 (H5N2), the virus responsible for a devastating influenza outbreak in the northeastern United States (Bean et al., 1985), and with four polyclonal antisera prepared in goats, chickens and ferrets. Thus, the Italian viruses (except for Ck/It/9097/97) can be considered as representatives of the same antigenic group. This finding is in contrast to that seen with the H5N1 viruses from Hong Kong, which could be separated into distinct subgroups on the basis of the presence or absence of a carbohydrate at residue 158 of the HA (Shortridge et al., 1998). Analysis of the HA sequences confirmed that each of the Italian strains lacked a carbohydrate residue at position 158.

Because of the epidemiological features of the Italian avian influenza outbreaks, it is tempting to speculate that the pathogenic Italian viruses derived their HAs from the non-pathogenic Ck/It/9097/97 strain after polymerase stuttering (García et al., 1996) or acquisition of a mutation at the cleavage site (Rohm et al., 1995). However, neither the nucleotide homology nor the phylogenetic relationship between these viruses supports such conjecture. Rather, the viruses in Italian poultry represent a distinct group that has a sister-group relationship to the 1997 H5N1 chicken and human isolates from Hong Kong. The nonpathogenic strain is more closely related to Ty/Eng/50-92/91 (97.1% homology) than to the pathogenic Italian strains (maximum identity 94.6%). These findings indicate the cocirculation of at least two separate H5 sublineages in Italian poultry.

Both the pathogenic and nonpathogenic Italian viruses had only one glycosylation site (residue 165) within the globular head region of the HA1 subunit. Recently, it has been postulated that the presence of more than one glycosylation site in this region and of deletions in the NA stalk is characteristic of several chicken H5 and H7 influenza viruses; these features are absent in viruses from aquatic birds (Matrosovich et al., 1999). However, the pathogenic relevance of this finding is still unclear, because several other highly virulent strains, including those in the present study, do not possess more glycosylation sites than do nonpathogenic viruses from wild aquatic birds. Future sequence analysis of the NA gene of the Italian viruses may provide additional clues about the interrelationships between the NA and the HA surface antigens.

Phylogenetic analysis of the NP genes of the Italian H5 viruses revealed that unlike the NS and HA genes, the NP genes of the two H5 sublineages are highly similar. Therefore, genetic exchange between the NP genes of viruses from the two sublineages (or their precursor virus) appears to have been possible. Also, all of the Italian NP genes are very similar to the NP gene of Dk/HK/y439/97 virus, a Hong Kong H9N2 virus isolated in 1997. Because migratory bird flyways cross northeastern Italy and because contact between poultry on the farms and migratory waterfowl may have occurred, the results of our NP genetic analysis suggest that a precursor virus with a similar NP gene was introduced by common waterfowl into Italian poultry. NP is part of the replicase complex of influenza viruses; therefore, it will be important to elucidate the origin of the rest of the genome, especially the polymerase genes.

Investigations of people who had been highly exposed to infected poultry showed no evidence of virus transmission to humans; the absence of even mild clinical signs of disease in humans was supported by the results of serological studies performed with a limited number of serum samples from persons directly involved in the Italian poultry industry in this region. Although the molecular determinants responsible for zoonotic transfer of H5N1 influenza viruses to humans in Hong Kong are largely unresolved, evidence suggests that the PB2, PA, NP and M genes are involved (Zhou et al., 1999).

Recent studies have shown that the Hong Kong H5N1 influenza viruses were reassortants that derived their HA gene from Goose/Guangdong/1/96 (H5N1) virus, whereas all of their genes encoding internal proteins were closely related to those of an H9N2 strain, Quail/HK/G1/97 (Xu et al., 1999; Guan et al., 1999). These findings emphasize the role played by poultry species in the generation of new influenza virus strains with altered virulence and host range. Our results underscore the importance of increased surveillance of domestic birds to detect circulating nonpathogenic avian influenza viruses that could contribute to the emergence of influenza viruses potentially pathogenic in both birds and mammals.

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