Influenza surveillance in birds in Italian wetlands (1992–1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots?

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Abstract

We report the results of a 6-year serological and virological monitoring performed in ducks and coots in Italy, in order to assess the degree of influenza A virus circulation in these birds during wintering. A total of 1039 sera collected from 1992 to 1998 was screened by a double antibody sandwich blocking ELISA (NP-ELISA): seroprevalence of antibodies to influenza A viruses was significantly higher in ducks compared to coots (52.2% vs. 7.1%, respectively). The hemagglutination-inhibition (HI) assay, performed on NP-ELISA positive sera, showed that 16.9% of these duck sera and 33.3% of these coot sera had antibodies to at least one influenza virus HA subtype: ducks showed HI antibodies against most of the HA subtypes, except for the H3, H4, H7, and H12; coots were seropositive to the H3 and H10 subtypes, only. From 1993 to 1998, 22 virus strains were obtained from 802 cloacal swabs, with an overall virus isolation frequency of 2.7%. Viruses belonging to the H1N1 subtype were by far the most commonly circulating strains (18/22) and were isolated mainly from ducks (17/18). The remaining viruses were representative of the H10N8, H5N2 and H3N8 subtypes. Our data indicate some differences between influenza A virus circulation in sympatric ducks and coots and a significant antigenic diversity between some reference strains and viruses recently isolated in Italy.

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Keywords: Avian influenza; HA subtype circulation; Serological survey; Virological survey; Wild aquatic birds; Sympatric waterfowl species; Italy

1. Introduction

Aquatic birds are considered the main reservoir of influenza A viruses in nature, harbouring in their populations viruses belonging to all the 15...
hemagglutinin (HA) and the 9 neuraminidase (NA) subtypes, in almost every possible antigenic combination. In particular, among wild waterfowl, ducks are of paramount importance for the ecology of this infection since they provide most of the gene pool from which all influenza A viruses in both human and animal populations originate (Horimoto and Kawaoka, 2001).

Since 1996 several reports have provided first evidences of the direct transmission of influenza viruses from birds to humans (Kurtz et al., 1996; Subbarao et al., 1998; Peiris et al., 1999). In particular, the high lethality of the avian H5N1 virus that in 1997 infected 18 people in Hong Kong, six of whom died (Subbarao et al., 1998), emphasises the risk of a new pandemic arising from an avian source (Subbarao, 2001). Moreover, it has often been suspected that influenza viruses infecting domestic avian species originate from free-living birds (Alexander, 2000). Almost all of the isolates circulating in wild avian populations, in particular in aquatic birds, are low pathogenicity avian influenza (LPAI) viruses which cause little or no disease. Nevertheless, when LPAI viruses belonging to the H5 or H7 subtypes are transmitted to intensively reared Galliformes species, their pathogenicity can increase; the effects of the disease can be catastrophic with heavy economic and social implications, as occurred during the 1999–2000 Italian HPAI epidemic associated with a virulent H7N1 subtype (Capua and Marangon, 2000).

Because of the central epidemiological role played by wild waterfowl in the emergence of influenza viruses potentially dangerous for both humans and other animal species, it is important to understand the dynamics of influenza infection in wildlife populations, and long-term surveillance studies represent an effective means in this regard, as has already been shown by other authors (Süss et al., 1994). For this reason, in the present paper we report the results of a 6-year monitoring of avian influenza viruses in wild ducks and coots caught in Italian wetlands during the wintering periods.

Aims of this epidemiological study were: (1) to assess the presence of antibodies against 14 influenza A virus subtypes and (2) to evaluate the prevalence of circulating virus subtypes by virological exams of cloacal swabs collected from birds.

In a previous epidemiological study we observed a different trend of influenza A infection in ducks and coots (De Marco et al., 2000). Thus, a further objective of the present study was to evaluate differences in virus subtypes circulating during the wintering period in these two bird groups, belonging to different taxonomic orders (Anseriformes and Gruiformes, respectively) but living in the same environments (sympatric species).

2. Materials and methods

2.1. Sample collection

From 1992 through 1998 a total of 1039 serum samples and 802 cloacal swabs were collected from wild ducks and coots caught for ringing purposes. The waterfowl species and the number of sampled birds were: Mallard (Anas platyrhynchos) n. 407; Shelduck (Tadorna tadorna) n. 37; Wigeon (Anas penelope) n. 13; Teal (Anas crecca) n. 13; Pintail (Anas acuta) n. 6; Pochard (Aythya ferina) n. 94; Ferruginous duck (Aythya nyroca) n. 16; Tufted duck (Aythya fuligula) n. 4; Coot (Fulica atra) n. 449. Sampling intensity is shown in Table 1. Since all duck species belong to the Anseriformes order, they were treated as a single group to allow comparison with birds belonging to the Gruiformes order (coots) as regards the evaluation of influenza virus circulation. Bird age was identified whenever possible; in this report the term juveniles refers to birds hatched during the last breeding season whereas the term adults refers to individuals hatched any year before the last breeding season. Serum samples were stored at −20 °C until tested. Cloacal swabs were stored at −20 °C up to 6 days, otherwise at −80 °C, in PBS/glycerol (1:1) with antibiotics.

2.2. Area description

The study areas were two neighbouring wildlife protected areas (“Laguna di Orbetello” and “Lago di Burano” WWF Oases) located on the West coast of Italy, about 140km north of Rome. They represent important wintering sites for migratory and resident waterfowl; the main breeding sites of these migrant populations are wetlands in Central and North Eastern Europe.
Table 1
Serological and virological results showing influenza A virus circulation in wild waterfowl wintering in Italya

<table>
<thead>
<tr>
<th>Wintering period</th>
<th>Ducks</th>
<th>Coots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Seroprevalence % to NP-ELISA (no. of positive/examined sera)</td>
<td>Virus isolation prevalence % (no. of positive/examined cloacal swabs)</td>
</tr>
<tr>
<td>November 1993/January 1994</td>
<td>73.7 (84/114)</td>
<td>1.1 (1/93)</td>
</tr>
<tr>
<td>January 1995/February 1995</td>
<td>42.3 (69/165)</td>
<td>0 (0/148)</td>
</tr>
<tr>
<td>November 1995/January 1996</td>
<td>39.9 (58/146)</td>
<td>10.4 (10/96)</td>
</tr>
<tr>
<td>December 1996/January 1997</td>
<td>61.7 (50/81)</td>
<td>8.6 (7/81)</td>
</tr>
<tr>
<td>January 1998/March 1998</td>
<td>39.2 (20/51)</td>
<td>0 (0/51)</td>
</tr>
<tr>
<td>Total (November 1992/March 1998)</td>
<td>52.3 (308/590)</td>
<td>3.8 (18/486)</td>
</tr>
</tbody>
</table>

| GMT [min–max titre]       | [27.9] (2^2–2^13)                          | [21.1] (2^0–2^5)                           |

a Serological data include seroprevalence, overall geometric mean titres (GMT), and overall minimum (min) and maximum (max) antibody titres observed in ducks and coots.

b Test not done.

2.3. Serological tests

2.3.1. ELISA test
A double antibody sandwich blocking ELISA, using a monoclonal antibody against influenza A virus nucleoprotein (anti-NP MAb) was developed in order to detect antibodies against influenza A virus (NP-ELISA). The assay was performed using a standard technique (De Boer et al., 1990) with some modifications (De Marco et al., 2000). Twofold dilutions of the serum samples were tested and NP-ELISA titres of ≥8 were considered positive.

2.3.2. Hemagglutination-inhibition (HI) test
The HI assay was used to test available NP-ELISA positive serum samples (De Boer et al., 1990). In total, 19 avian influenza strains were employed in the HI test (Table 2). Of these, 14 were reference strains representative of HA virus subtypes from H1 to H14 (seven belonging to the Eurasian lineage and seven to the North American lineage). Four more strains were isolated from waterfowl wintering in the study area [A/Mallard/Italy/23/95 (H1N1), A/Mallard/Italy/80/93 (H5N2), A/Coot/Italy/153/94 (H3N8), A/Coot/Italy/114/95 (H10N8)], and finally one low pathogenicity avian influenza virus (AIV) A/Turkey/Italy/6423-1/99 (H7N1) was obtained from commercial poultry in Emilia Romagna Region (Northern Italy). Therefore H1, H3, H5, H7 and H10 antigens were represented by both a reference and a local strain. The HI assay was performed as described (Alexander, 1989). HI titres ≥16, detected against an antigen dose of 4 HAU, were considered positive. In order to reduce false-positive or false-negative reactions serum samples were treated both with RDE, as described (Anonymous, 1982), and at 37°C for 1 h with 10% (v/v) chicken red blood cells, respectively.

2.4. Virus isolation
Cloacal swabs were tested for influenza viruses by inoculation into embryonated SPF hens eggs according to standard procedures. Each sample underwent at least two passages, and influenza isolates were identified by both hemagglutination (HA) assay (Anonymous, 1982) and a double antibody sandwich ELISA for detection of influenza A viral nucleoprotein (Siebinga and de Boer, 1988). Influenza viruses were characterised by HI assay and neuraminidase-inhibition (NI) test (Anonymous, 1982) using reference virus strains and antisera, kindly provided by Dr. R.G. Webster (St. Jude Children’s Research Hospital, Memphis, TN, USA).

2.5. Statistical analysis
2.5.1. Statistical tests
Significant differences in both the overall NP-ELISA seroprevalences and virus isolation frequencies between ducks and coots were analysed by the
Table 2

Number of NP-ELISA seropositive ducks and coots showing hemagglutination-inhibition (HI) antibodies to several strains of influenza A viruses subtypes, overall geometric mean titres (GMT), and minimum (min) and maximum (max) antibody titres observed in ducks and coots

| Sampling period          | No. of birds with antibodies to at least one HA subtype/no. of NP-ELISA seropositive birds tested by HI (% | No. of serum samples showing HI antibodies to the following influenza subtypes | H1a | H1b | H2 | H3 | H5a | H5b | H6 | H8 | H9 | H10a | H10b | H11 | H13 | H14 |
|-------------------------|---------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----|-----|----|----|-----|-----|----|----|------|------|-----|-----|-----|
| November 1992/December 1992 | Ducks 9/39 (23.1)                                                                                           | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 5/59 (8.6)                                                                                             | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| November 1993/January 1994 | Ducks 4/44 (9.1)                                                                                             | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 1/9 (11.1)                                                                                             | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| January 1995/February 1995 | Ducks 3/59 (5.1)                                                                                             | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 0/9 (0)                                                                                                 | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| January 1996/February 1996 | Ducks 5/45 (11.1)                                                                                             | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 0/9 (0)                                                                                                 | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| December 1996/January 1997 | Ducks 8/49 (16.3)                                                                                             | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 0/9 (0)                                                                                                 | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| January 1998/March 1998  | Ducks 8/20 (40)                                                                                               | Ducks 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | Coots 0/1 (0)                                                                                                 | Coots 0 (0)                                                                 | 0 (0) | 0 (0) | 0 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 8 (24.6) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Total (November 1992/March 1998) | Ducks (GMT) 47/278 (16.9)                                      | Ducks (GMT) 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | (non-max titres)                                                                                              | (non-max titres)                                                                 | 2^4 | 2^3 | 2^2 | 2^5 | 2^3 | 2^2 | 2^2 | 2^2 | 2^3 | 2^5 | 2^3 | 2^2 | 2^2 | 2^2 |
|                          | Coots (GMT) 8/24 (33.3)                                                                                      | Coots (GMT) 16 (27.9)                                                                 | 6 (18.7) | 3 (18) | 0 | 14 (24.3) | 13 (22.6) | 7 (24.3) | 1 (16) | 5 (18) | 6 (18.7) | 3 (32) | 7 (17.1) | 1 (16) | 2 (22) | 2 (22) |
|                          | (non-max titres)                                                                                              | (non-max titres)                                                                 | 2^4 | 2^3 | 2^2 | 2^5 | 2^3 | 2^2 | 2^2 | 2^2 | 2^3 | 2^5 | 2^3 | 2^2 | 2^2 | 2^2 |

* H1a: A/DuckAlberta/3576/76 (H1N1); H1b: A/MallardItaly/23/95 (H1N1); H2: A/DuckGermany/525/73 (H2N3); H3: A/Chicken/England/31369/88 (H3N2); H5a: A/Ruddy Turnstone/Delaware/253/91 (H5N2); H5b: A/Mallard/Italy/80/93 (H5N2); H6: A/Shearwater/Australia/72 (H6N5); H7a: A/Ruddy Turnstone/New Jersey/65/85 (H7N3); H7b: A/Turkey/Italy/6423-1/99 (H7N1); H8: A/Duck/Maryland/504/77 (H8N4); H9: A/Turkey/Italy/6423-1/99 (H9N1); H10a: A/Chicken/Austria/72/86; H10b: A/Coot/Italy/114695 (H10N8); H11: A/Duck/England/58 (H11N9); H13: A/Gull/Maryland/504/77 (H13N9); H14: A/Mallard/Italy/82 (H14N7).

** The NP-ELISA seropositive samples were also tested and were negative to the following influenza viruses: H1a: A/Duck/Ukraine/1/63 (H1N8); H1b: A/Duck/Czechoslovakia/90 (H1N6); H2: A/Duck Alberta/3576 (H2N6); H3: A/Chicken/New Jersey/95/86 (H3N2); H5a: A/Turkey/Ontario/6118/68 (H5N4); H5b: A/Duck/Alaska/59/76 (H5N2).
Chi-square test. Differences between ducks and coots in the percentages of sera which resulted positive for at least one HA subtype were evaluated using the Fisher’s exact test. This test was also performed to test non-random associations between the number of ducks and coots showing HI antibodies against one or more HA subtype as correlated to bird age. Significance was set at $\alpha = 0.05$.

2.5.2. Antibody mean titres

Geometric mean titres (GMT) were calculated on the overall seroprevalences against influenza A viruses (Table 1) and on the sera showing antibodies against each of the HA subtypes (Table 2). Moreover, GMT were calculated in order to compare serological reactivities against virus pairs belonging to H1, H3, H5, H7 and H10 subtypes (Table 3).

3. Results

3.1. Serological tests

The overall antibody frequencies to type A influenza viruses, detected by NP-ELISA assay, were 52.2% in ducks (308/590) and 7.1% in coots (32/449), with a seroprevalence value significantly higher in the first bird group. Moreover, during the 6-year study period, the observed values were always higher in ducks than in coots (Table 1), ranging between 39.2 and 73.7% in the first group of birds and between 1.3 and 17.7% in the second one.

HI antibodies against at least one influenza virus HA subtype (Table 2) were detected in 16.9% of NP-positive ducks (47/278) and 33.3% of NP-positive coots (8/24), with a positivity percentage significantly higher in the latter group. Tested sera were found positive to 11 of the 14 influenza virus HA subtypes used in the assay.

From 1992 through 1998, the sampled ducks showed HI antibodies against most of the HA subtypes, except for the H3, H4, H7 and H12. On the other hand, coots were seropositive to the H3 and H10 subtypes, only. The overall percentages of HI seropositive birds, by strain, are shown in Fig. 1. As shown in Tables 2 and 3 the HI antibody frequency detected among the 47 ducks showing antibodies against at least one HA subtype ranged between 2.1% (against H8 and H13 subtypes) and 40.4% (against both the H5 strains). The HI antibody percentages observed...
Table 3
Comparison of serological reactivity of ducks and coots to virus pairs belonging to the H1, H3, H5 and H10 subtypes (one reference strain and one local strain per pair), which were used in the HI test (see Table 2 for explanation of antigen abbreviations).

<table>
<thead>
<tr>
<th>Serum samples resulting positive for the following virus strains a</th>
<th>H1a only</th>
<th>H1a + H1b b</th>
<th>H3a only</th>
<th>H3a + H3b b</th>
<th>H5a only</th>
<th>H5a + H5b b</th>
<th>H10a only</th>
<th>H10a + H10b b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ducks No. of seropositive birds</td>
<td>10</td>
<td>6</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GMT</td>
<td>18.4</td>
<td>52</td>
<td>19.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Coots No. of seropositive birds</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>GMT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>32</td>
<td>–</td>
<td>–</td>
<td>16</td>
<td>45.3</td>
</tr>
</tbody>
</table>

a H1a, H3a, H5a, H10a: reference strains. H1b, H3b, H5b, H10b: Italian strains.
b In the case of sera positive to both strains in a pair, GMT values against each one of the viruses (a and b columns) are given.

Among the eight coots with antibodies against at least one HA subtype, were 50% (against an H3 strain isolated from coot), 25 and 100% (against two H10 strains isolated from chicken and coot, respectively).

When we considered the HI serological data in ducks during each winter (Fig. 2), it appeared that the H1 and H5 subtypes were detected every winter; the H6 and H11 subtypes during five and four seasons, respectively; H9 and H10 subtypes, in three winters; H2 and H14 subtypes, twice; H8 and H13 subtypes, once.

As indicated in Fig. 3, HI seropositive coots were detected during the first three sampling periods, only. While in the winter of 1992 only the H10 subtype was found, during the two following seasons antibodies to both H3 and H10 viruses were detected in this bird group. HI seropositive coots were detected during the last three sampling periods, only. When we considered the HI serological data in ducks during each winter (Fig. 2), it appeared that the H1 and H5 subtypes were detected every winter; the H6 and H11 subtypes during five and four seasons, respectively; H9 and H10 subtypes, in three winters; H2 and H14 subtypes, twice; H8 and H13 subtypes, once.

Table 4 shows the number of ducks and coots seropositive to more than one subtype. Again, the most frequently detected antibody combinations in ducks involved the H1, H5 and H6 subtypes. As for coots, H3 and H10 antibodies were found in the same sera in 50% of cases (4/8 birds). Statistical analysis showed that seropositivity to more than one HA subtype was significantly higher in adult ducks than in juveniles. No age-related differences were observed in coots.

Comparison of serological reactivity against the virus pairs belonging to both reference and local strains of the H1, H3, H5, and H10 subtypes (including the geometric mean titre) are shown in Table 3. Of 19 H5-positive duck sera, six (31.6%) reacted exclusively with the North American strain, five (26.3%) with the Italian virus only, while eight birds (42.1%) were positive to both antigens, with comparable geometric mean titres (32 vs. 24.3, respectively). A similar picture was seen with the H10-positive duck sera.

In contrast, among the 16 duck sera with antibodies to the H1 subtype, the majority (62.5%, 10/16) reacted with the reference strain A/Duck/Alberta/35/76 (American lineage), and the remaining ones, although positive to both strains, had much higher titres to the American strain than to the Italian virus A/Mallard/Italy/23/95 (GMT: 52 and 19.7, respectively).

Coot sera with antibodies against the H10 subtype virus were clearly more reactive towards the coot strain A/Coot/Italy/42/95 (American lineage) than to the remaining ones (Table 3).
strains A/Coot/Italy/114/95, since 75% of them (6/8) were positive to this strain only. As regards the H3 subtype, the four H3-positive coot sera reacted to A/Coot/Italy/153/94 virus only. As already shown in Table 2, no birds were found positive to either H7 strain used in the HI test.

3.2. Virus isolation

From 1993 to 1998 a total of 22 influenza A viruses were isolated in the study area. Overall, the level of virus isolation was 2.7%, with isolation frequencies significantly higher in ducks than in coots.
(3.8 and 1.2%, respectively) (Table 1). Sampling periods, virus subtypes and bird species are shown in Table 5. In contrast with the continuous detection of influenza antibodies in ducks throughout the period considered, viruses were isolated during three wintering seasons, only. The virological results (Table 5) indicate that the most commonly isolated viruses belonged to the H1N1 subtype, with a total isolation frequency of 2.2% (18/802) compared to the overall 0.5% (4/802) of the other three subtypes (two H10N8, one H3N8, one H5N2, respectively).
Table 5: Avian influenza viruses isolated from wild waterfowl trapped in Italy

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>Virus subtype</th>
<th>Distribution of virus isolates by bird group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ducks</td>
<td>Coots</td>
</tr>
<tr>
<td>November 1993/January 1994</td>
<td>H3N8, H5N2, H10N8</td>
<td>1, 1</td>
</tr>
<tr>
<td>November 1995/January 1996</td>
<td>H1N1, H10N8</td>
<td>10, 1</td>
</tr>
<tr>
<td>December 1996/January 1997</td>
<td>H3N1</td>
<td>7, 1</td>
</tr>
</tbody>
</table>

4. Discussion

NP-ELISA assay, used in the present study, enabled a serological screening for influenza A antibodies in waterfowl, which harbour several influenza virus subtypes but rarely produce precipitating antibodies following infection (Alexander, 1989).

The influenza antibody frequency detected in ducks by this method during the seasons examined was 52.2%, indicating a high degree of positivity among these birds even in periods of apparently low virus circulation such as the winter months (Webster and Bean, 1998), as also confirmed by the small number of strains isolated at the same time (3.8%). These seroprevalence rates are in accordance with those observed in other Mediterranean areas such as Spain (Arenas et al., 1990), although they appear higher than those reported by other authors (De Boer et al., 1990; Astorga et al., 1994).

On the other hand, the percentage of ELISA-positive coots was significantly lower (7.1%) in comparison with that observed in ducks and was almost exclusively concentrated during the first three seasons examined. The difference observed could be due to either a higher susceptibility to influenza infections of the Anseriformes species or to a slower diffusion of influenza viruses in coots, or both, as previously described (De Marco et al., 2000).

As shown in Table 2, and in contrast with the above evidence, HI antibodies were detected much more frequently in coots seropositive to influenza NP (82/24, 33.3%) than in NP seropositive ducks (47/278, 16.9%). This result could be accounted for by a lower HI antibody activity in ducks compared to other bird species, as already observed by other authors. In one report, when gulls and ducks were infected with an influenza A virus, a good HI antibody response was obtained in the former species, whereas HI antibody levels were low and erratic in the latter (Bahl and Pomeroy, 1977). Moreover, several authors have shown a scarcely detectable serum HI antibody response in ducks, following experimental infections with influenza virus (Slemons and Easterday, 1972; Hinshaw et al., 1980; Kida et al., 1980; Lu et al., 1982). The discrepancy with the data reported by De Boer et al. (1990), showing that 93.3% of sera positive to type A influenza were also positive for at least one HA subtype, could be partly explained by the higher HI titre positivity threshold used in the present study compared to that employed by De Boer et al. (1990).

We have considered the possibility that the presence of antibodies against NA could give false positive results in the HI test performed with viruses with the same NA but different HA antigens. In our study, four coot sera were HI positive to both H3N8 and H10N8 coot strains (Table 4). However, in the same test these sera were all negative to another H3N8 virus, A/Duck/Ukraine/18/93 (Table 2). Similarly, one duck serum was positive to both H5N2 and H9N2 virus subtypes but gave negative results with a second H5N2 strain, A/Ruddy Turnstone/Delaware/253/91. Thus we can conclude that in the present study the HI test identified specifically anti-HA antibodies.

In general, ducks showed antibodies against ten different HA subtypes, spanning from H1 to H13, whereas coots were seropositive to two subtypes only, H3 and H10 (Fig. 1). HI seropositive coots were detected only during the first three seasons (Fig. 3), whereas in the duck populations serological data indicated a constant circulation of several influenza virus subtypes throughout the 6 years considered (Fig. 2). In particular, H1, H5, H6 and H11 antibodies were
found every, or almost every, year, whereas the detection of other subtypes occurred more sporadically. No positivity to the H3 subtype was found in ducks, in contrast with evidence from coots. Considering also the virus isolation results (Table 5), it appears that influenza circulation in coots, as compared to ducks, occurred at low levels, was limited to few subtypes and was not persistent, even though these two bird groups share the same habitat. The single H1N1 isolation in coots suggests that this species, although susceptible to infection with this virus, is not able to maintain in the wild an influenza subtype which, in contrast, commonly circulates among ducks. Moreover, the circulation of the H3 subtype seemed to be restricted to coots, suggesting the existence of distinct influenza cycles in these two groups of waterfowl.

Epidemiological data obtained in ducks by both serological HI results and isolate characterisation, showed an apparently contrasting situation: antibodies against 10 different HA subtypes were detected by the HI assay (Fig. 1) whereas only two different subtypes (H1N1 and H5N2) were isolated in the study area during five consecutive wintering periods (Table 5). However, anti-H5 and anti-H1 antibodies were by far those most frequently represented in these birds (40.4 and 34% of HI positive ducks, respectively). Moreover, although only one H5 virus was obtained during the period under study, in previous years two more H5N2 strains had been isolated in the same area (Fioretti et al., 1991). On the other hand, the presence of antibodies to several other HA subtypes could be explained by various factors: (i) at least a part of the wild ducks sampled in the present study was represented by contingents migrating from and to breeding sites in north-eastern Europe (Prigioni and Boano, 1992), where different HA subtypes could be harboured and perpetuated (Süss et al., 1994); (2) some of the sampled ducks could be domestic ducks introduced into the wild as a part of restocking operations for hunting purposes (De Marco et al., 1996, 1999), and previous studies performed in Germany have already demonstrated that wild and domestic ducks differ with regard to the influenza HA subtypes most frequently circulating in both groups (Süss et al., 1994). The above-mentioned factors could account for those sera showing antibodies against multiple HA subtypes, too (Table 4). As expected, this phenomenon was observed more frequently in adult birds with a longer history of contacts with influenza viruses than in the young ones.

In the case of the H1, H3, H5, H7 and H10 subtypes, sera were HI tested using additional strains isolated in Italy in the time lapse considered. Since in some instances the two strains representative of each subtype belonged to two different avian lineages, the North American and the Eurasian ones, this enabled us to evaluate the effects of a possible antigenic heterogeneity on the frequency and the level of HI antibody detection in birds. As shown in Tables 2 and 3, in all cases (except for the H7, against which no antibodies were found), most of the sera positive for these subtypes showed a differential reactivity against the two representative strains: (i) Coots reacted to the virus A/Coot/Italy/153/94 (H3N8), but not to the older duck strain A/Duck/Ukraine/1/63 (H3N8) (Table 3). In a previous report, it had already been shown that the same coot strain reacted poorly, if at all, with a panel of antisera representative of avian, human and swine H3 strains (Campitelli et al., 2002); as for the H10 subtype, all of the eight coot sera appeared specific for A/Coot/Italy/114/95 (H10N8); and only in two cases cross-reactivity with an older chicken strain, at lower titres, was observed (Table 3). Thus, viruses circulating in coots appear to be antigenically different from those isolated in ducks. (ii) In ducks, with regard to the H1 subtype, sera reacted much better to the North American strain A/Duck/Alberta/35/76 (H1N1) than to a duck strain isolated in the study area during the period considered. This phenomenon could be explained by the circulation of antigenically distinguishable H1 influenza strains. The genetic characterisation of these viruses will clarify this aspect, as well as their relationship to the H1N1 influenza regularly occurring in swine and humans in Italy. The existence of distinct antigenic sublineages seems evident when considering those sera positive to the H5 and H10 subtypes. In both cases, two groups were identified, each one reacting with only one of the two subtype-representative strains. These results suggest a significant antigenic diversity within at least some subtypes and raise the problem of which strains to choose as reference viruses for animal influenza surveillance.

Data on the HA subtypes of the viruses isolated during this period consistently matched the serological results except for the H1 isolate from coots as no
H1 antibodies were detected in this species (Table 5). The low isolation percentages compared to the frequency of influenza antibodies suggests that also in the Mediterranean area influenza infection occurs chiefly towards the end of the breeding season, during the seasonal peak in AIV activity (Stallknecht and Shane, 1988). However, in a previous study based on combined data from seroconversions and virus isolations in overwintering birds, we had already shown that the frequency of influenza infections in ducks in the winter seems to be higher than that estimated on the basis of virus isolation rates only (De Marco et al., 2003).

Finally, with regards to the role of wild ducks as a source of viruses potentially pathogenic for domestic poultry, virological and serological data highlighted the continuous circulation of H5 viruses in ducks during all the sampling seasons, as evidenced by the serological data and confirmed by the isolation of the strain. HA sequence of the A/Mallard/Italy/80/93 (HSN2) isolate showed a high degree of homology with the HPAI H5N2 viruses which caused outbreaks in backyard poultry flocks in Italy, during 1997–1998, but did not possess any additional basic residue at the cleavage site sequence, a feature typical of low pathogenicity viruses (L. Campitelli, personal communication; Capua et al., 1999; Donatelli et al., 2001). A more comprehensive comparison of the antigenic and genetic features of these viruses is in progress. In contrast with results previously obtained from sentinel ducks in North Europe (Süss et al., 1994) and from wild mallards in other sites of the Mediterranean Basin (Lipkind and Weisman, 1989), no evidence of H7 virus circulation was found either at the serological or virological level in the area under study. The results of the influenza circulation monitoring carried out after the period reported here, and still ongoing, will enable us to evaluate the occurrence of H7 infection in wild wader and shorebird populations in Italy, during the years of the catastrophic Italian poultry epidemic due to the serovar H7N1 of HPAI virus (Capua and Marangon, 2000).

Our data also indicate some differences in the influenza virus circulation between sympatric ducks and coots; further studies on the viruses isolated from these birds will permit us to verify whether distinct influenza gene pools are maintained in ducks and coots, as already observed by Kawakita et al. (1988) in gulls, shorebirds and ducks.

In conclusion, our results emphasise the continuous need to monitor wild avian populations to better understand the natural cycle of influenza A viruses, and underline the importance of surveillance activities aimed at studying the circulation of virus strains with epidemiological implication in domestic animal and/or human influenza.

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