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# Serological evidences showing the involvement of free-living pheasants in the influenza ecology

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## ABSTRACT

From 1995 to 2002, 219 sera were collected in Northern Italy from wild pheasants, in order to establish the possible involvement of these *Galliformes* birds in the influenza ecology. A serological survey for avian influenza viruses (AIVs) was carried out by ELISA test in order to detect type A influenza antibodies. The overall seroprevalence was 12.3%, with yearly values ranging from 0% to 42.5%. No antibodies against either H5 or H7 AIV subtypes were found by hemagglutination-inhibition test. Data from 16 recaptured birds, among 113 animals banded for individual identification, showed seroconversions in 2 pheasants. Our results indicate AIV circulation in free-living pheasants; the involvement of this land-based bird species in influenza ecology is discussed.

*Key Words:* Pheasant, Avian influenza, Influenza ecology, Serological survey.

## RIASSUNTO

### DIMOSTRAZIONE SU BASE SIEROLOGIA DEL COINVOLGIMENTO DI FAGIANI A VITA LIBERA NELL'ECOLOGIA DELL'INFLUENZA

*Nel periodo compreso tra il 1995 e il 2002, 196 fagiani a vita libera sono stati catturati in un'area protetta dell'Italia Settentrionale, situata in Emilia-Romagna. Le catture, durante le quali 113 fagiani sono stati inanellati per un'identificazione individuale e quindi rilasciati nell'area di studio, sono avvenute durante cinque periodi invernali di cui 4 consecutivi e hanno consentito di raccogliere 219 emosieri. I campioni ottenuti sono stati sottoposti ad esami sierologici per valutare: i) il possibile coinvolgimento di una popolazione di fagiani a vita libera caratterizzata da un'elevata densità di animali sul territorio, nell'ecologia dei virus influenzali di tipo A; ii) l'eventuale circolazione tra questi volatili di virus influenzali appartenenti ai sottotipi H5 o H7. La ricerca è stata eseguita esaminando i campioni di siero tramite una metodica ELISA in grado di evidenziare anticorpi nei confronti della nucleoproteina dei virus influenzali di tipo A (NP-ELISA): la sieroprevalenza totale era uguale al 12,3%, mentre i risultati osservati annualmente variavano da una percentuale pari allo 0% (durante il 1995) a un valore massimo del 42,5% (durante il 2001). I campioni risultati positivi alla metodica NP-ELISA sono stati testati mediante la prova di inibizione dell'emoagglutinazione (IEA) effettuata impiegando come antigeni 5 ceppi di virus influenzali a bassa patogenicità, isolati in Italia e appartenenti ai sottotipi H5N2, H5N3, H5N9, H7N1, H7N3. Tutti i campioni esaminati mediante IEA sono risultati sieronegativi per i sottotipi H5 e H7.*

*L'esame degli emosieri ottenuti da 16 fagiani inanellati e catturati più di una volta, hanno consentito di dimostrare in 2*

soggetti sier conversionsi verso i virus influenzali di tipo A, indice di una recente infezione legata alla contemporanea circolazione virale nell'area di studio.

Dai risultati della presente indagine si evince come nella popolazione naturale di fagiani esaminata siano circolati, per i quattro anni consecutivi del campionamento, virus influenzali di tipo A e come questi non appartenessero ai sottotipi H5 e H7, responsabili delle recenti epidemie italiane del pollame. L'esistenza di zone umide nell'area di studio costituisce una possibile interfaccia ecologica tra specie aviarie serbatoio naturale dei virus influenzali (uccelli acquatici) e fagiani che utilizzando l'acqua per l'abbeverata condividono questa probabile fonte di infezione. Ulteriori indagini consentiranno di approfondire il ruolo epidemiologico di questo galliforme selvatico nell'ecologia dei virus influenzali.

Parole chiave: Fagiano, Influenza aviare, Ecologia dell'influenza, Indagine sierologica

## Introduction

Land-based birds belonging to the *Galliformes* Order include species, such as turkey, chicken, and quail, that are highly susceptible to avian influenza viruses (AIVs) primarily harboured in wild aquatic birds (Webster *et al.*, 1992). In addition to heavy economic losses due to influenza epidemics in poultry, important public health implications could arise from AIV circulation in land-based birds, recently indicated as a potential source of pandemic strains (Perez *et al.*, 2003).

AIV infections have been described in Italy in reared pheasants (*Phasianus colchicus*) both as limited outbreaks (Rinaldi *et al.*, 1967) or associated to severe poultry epidemics (Capua *et al.*, 2003). Although sporadic isolations of AIVs have been reported in free-living pheasants (Romváry *et al.*, 1976), the epidemiological role played by wild populations of this *Galliformes* species is not well understood, to date.

Aims of this serological survey, carried out on wild pheasants trapped in northern Italy, were: i) to establish the occurrence of type A influenza infection; ii) possibly, to detect the circulation of H5 and H7 AIV subtypes.

## Material and methods

### Sampling

Free-living pheasants were monitored on an estate (about 35 hectares) located in a protected lowland area (Bologna province, Emilia Romagna region). The number of birds occupying the study area ranged from 150 to 40 in autumn and spring, respectively. From 1995 to 2002 a total of 196 pheasants were trapped; 113 of them were banded for individual identification then released into the wild. Overall, 219 sera were collected (Table 1)

including 23 recapture samples (Table 2). Serum samples were stored at  $-20^{\circ}\text{C}$  until tested. Bird sex and age were recorded whenever possible. Phenotypic characteristics allowed sex determination. The age of pheasants was determined as described (Cattadori *et al.*, 1997): juveniles were birds hatched during the last breeding season, adults were birds hatched any year before the last breeding season.

### Serological test

Sera were assayed for antibodies against influenza A virus nucleoprotein using an ELISA test (NP-ELISA) performed as described (De Marco *et al.*, 2003b).

Available NP-ELISA positive sera were assayed as described (De Marco *et al.*, 2004) by hemagglutination-inhibition (HI) test, in order to detect antibodies against 5 different Italian strains belonging to both H5 and H7 subtypes of AIVs (Table 1).

Recapture data (Table 2) were analysed in order to evidence a significant increase in antibody titres (De Marco *et al.*, 2003b).

### Statistical analysis

Chi-square test was performed in order to test non-random associations between the overall seroprevalences and: i) pheasant age; ii) pheasant sex. The significance level was set at a  $P < 0.05$ .

## Results and discussion

As shown in Table 1, the overall NP-ELISA antibody frequency to avian influenza viruses (AIVs), including data from 23 recaptures, was 12.3% (27/219). Pheasants seropositive for influenza A viruses were found in 4 of the 5 sampling periods, and the prevalence of sera found positive

Table 1. Serological results for antibodies against avian influenza viruses in 196 free-living pheasants trapped in a protected area of the Emilia Romagna region (Northern Italy). Seroprevalences were calculated on overall sera sampled, including 23 sera obtained from 16 recaptured bird.

Sampling period	NP-ELISA prevalence % (positive/tested sera)	HI antibody frequencies (positive/tested sera) calculated on NP-ELISA positive sera against the following AIV subtypes(*)				
		H5N2	H5N3	H5N9	H7N1	H7N3
Mar. 1995	0 (0/34)	nd	nd	nd	nd	nd
Jan. 1999/Feb. 1999	3.8 (2/53)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Feb. 2000/Mar. 2000	5.6 (3/54)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Jan. 2001/Mar. 2001	42.5 (17/40)	0 (0/15)	0 (0/16)	0 (0/16)	0 (0/15)	0 (0/16)
Jan. 2002	13.2 (5/38)	0 (0/4)	0 (0/5)	0 (0/5)	0 (0/4)	0 (0/5)
Total 1995/2002	12.3 (27/219)	0 (0/23)	0 (0/25)	0 (0/25)	0 (0/23)	0 (0/25)

(\*) Low-pathogenic AIV strains used: *A/mallard/Italy/80/93(H5N2)*; *A/mallard/Italy/208/00(H5N3)*; *A/chicken/Italy/9097/97(H5N9)*; *A/turkey/Italy/6423-1/99(H7N1)*; *A/mallard/Italy/33/01(H7N3)*; nd = not done.

Table 2. Serological data for antibodies against influenza A viruses obtained from 16 free-living pheasants trapped between 1995 and 2002 in the study area, and recaptured once or twice.

Bird n.	Bird age <sup>^</sup>	1 <sup>st</sup> capture time (NP-ELISA result)	1 <sup>st</sup> recapture time (NP-ELISA result)	2 <sup>nd</sup> recapture time (NP-ELISA result)
1	Ad	14/03/95 (-)	02/02/00 (-)	nd
2	Ad	03/02/99 (-)	22/02/00 (-)	02/03/00 (-)
3	Ad	04/02/99 (-)	22/02/00 (-)	29/02/00 (+) <sup>^^</sup>
4	Ad	09/02/00 (-)	18/02/00 (-)	24/02/00 (-)
5	Ad	09/02/00 (-)	14/02/00 (-)	29/02/00 (+) <sup>^^</sup>
6	Juv	11/02/00 (-)	15/02/00 (-)	nd
7	Un	11/02/00 (-)	22/02/00 (-)	01/03/00
8	Ad	11/02/00 (-)	28/01/02 (-)	nd
9	Ad	22/02/00 (-)	24/02/00 (-)	29/02/00 (-)
10	Ad	23/02/00 (-)	29/02/00 (-)	nd
11	Ad	23/02/00 (-)	08/02/01 (-)	nd
12	Juv	23/02/00 (-)	01/03/00 (-)	nd
13	Juv	24/02/00 (-)	29/01/02 (-)	nd
14	Ad	29/02/00 (-)	06/02/01 (-)	28/01/02 (-)
15	Ad	13/02/01 (-)	27/02/01 (-)	nd
16	Un	01/03/01 (+) <sup>^^</sup>	06/03/01 (+) <sup>^^</sup>	nd

Ad: adult; Juv: juvenile; Un: unknown; (+) positive; (-) negative; nd: not done;  
<sup>^</sup>: determined during the 1<sup>st</sup> capture; <sup>^^</sup>: positive antibody titre = 8.

ranged from 0% (in 1995) to 42.5% (in 2001). Samples were not collected during 1996, 1997 and 1998, thus it is not possible to establish precisely the first occurrence of AIV infection, however seropositive birds were constantly found between 1999 and 2002. No sex-related differences were found whereas the age-related NP-ELISA seroprevalences resulted significantly higher in the juvenile birds, compared to the adult ones, thus suggesting a higher susceptibility of juvenile pheasants that congregate in post-breeding periods to AIV transmission.

No H5 and H7 positive sera were found by HI assay, performed using 5 different low-pathogenic AIV strains recently isolated in Italy from both wild and domestic avian species (Table 1).

Among 16 birds captured more than once, seroconversion for type A influenza viruses (Table 2, data evidenced with ^^) was observed in pheasants n. 3 and n. 5, indicating that AIVs circulated in the study area during the winter 2000.

## Conclusions

Our findings indicate the occurrence of avian influenza virus (AIV) infection in free-living pheasants. In general wild birds are potentially exposed to AIVs perpetuated by natural reservoirs, in particular the pheasants examined in the present study drank from small ponds located in the study area and occasionally used by migrating ducks. Faecal contamination of waters could represent an ecological interface between primary hosts of AIVs and other susceptible bird species (Webster *et al.*, 1992).

The high population density characterising the study area could facilitate the virus circulation; indeed the data we observed are in contrast with seronegative results obtained from pheasants living in protected sites characterised by a lower population density (De Marco *et al.*, 2003a).

Fortunately, our results indicate that neither H5 nor H7 subtypes of AIV, recently involved in Italian poultry epidemics (Capua *et al.*, 2003; Campitelli *et al.*, 2004), circulated within the examined bird population. Further surveillance studies will enable us to acquire information to better understand the dynamics of influenza infection in pheasants, a land-based bird species poten-

tially implicated in the interspecies transmission of AIVs harboured in natural reservoirs (Wood *et al.*, 1985; Perez *et al.*, 2003).

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