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Absence of detection of highly pathogenic H5N1 in migratory waterfowl in southern France in 2005–2006

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Abstract

During fall 2005, the rapid and wide spread of highly pathogenic (HP) H5N1 avian influenza viruses (AIV) outside Asia alerted European health authorities. Because of abnormal and recurrent field mortality, wild migratory birds were considered to be the main dispersing agent of the virus at an intercontinental scale. European wintering wetlands, such as the Camargue (Rhône delta, France), are identified as potential hot spots for the risk of introduction and transmission of bird-borne diseases. In this study, we investigated the role of migratory waterbirds (mainly ducks) in the spread of HP H5N1 viruses. We combined molecular analysis of living and freshly killed birds with population surveillance (aerial censuses and death surveillance). We sampled 1345 birds belonging to 17 waterbird species (3 orders) in the Camargue between September 2005 and March 2006. The prevalence of AIV was 1.8%. We did not detect HP H5N1 virus. Population censuses did not reveal any population decreases nor abnormal mortalities. We discuss, in the light of these results, the implication of wild migratory ducks in the arrival of HP H5N1 AIV in Europe.

Keywords: Avian influenza; Anseriforms; Camargue

1. Introduction

The threat of an influenza pandemic following the recent spread of the H5N1 avian influenza viruses (AIV) is currently a matter of concern for health authorities. Wild birds, and especially waterbirds in the Anseriforms and Charadriiforms orders, are natural hosts for influenza A viruses (Webster et al., 1992; Olsen et al., 2006). Avian influenza viruses can be sorted on the basis of virulence (Garten and Klenk, 1999; Horimoto and Kawaoka, 2005). Low pathogenic avian influenza (LPAI) viruses lead to benign respiratory and/or intestinal tract infections. Highly pathogenic avian influenza (HPAI) viruses cause a multi-organ systemic infection responsible for high levels of mortality. These viruses are rarely isolated in wild birds (Swayne and Suarez, 2000). Conversely, domestic birds, especially poultry, are victims of recurrent outbreaks due to HPAI viruses of subtypes H5 and H7 (Alexander, 2000; Olsen et al., 2006). In poultry, AIV evolve independently of the wild bird reservoir (Suarez, 2000). Outbreaks caused by HPAI viruses are generally limited to small geographic areas, but cause the death of a considerable number of domestic birds and important economic losses.

Highly pathogenic (HP) H5N1 viruses have been detected in China since 1996 (Tang et al., 1998; Xu et al., 1999). Outbreaks were reported almost simultaneously in eight neighbouring Asian countries between December 2003 and January 2004, suggesting that the viruses had spread recently and rapidly (Sims et al., 2005). In July 2005, HP H5N1 viruses started their westward progression by crossing the Russian border (Kilpatrick et al., 2006; Gauthier-Clerc et al., 2007): outbreaks were first

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detected in Western Siberia, and almost simultaneously in Kazakhstan and Mongolia (O.I.E., 2006). In October, it was found in Romania, Croatia and the Western edge of Turkey. From February 2006 onwards a second phase started, characterized by numerous sporadic outbreaks affecting mainly wild birds in European countries, but also poultry in Africa (*e.g.* Nigeria) and the Middle East (*e.g.* Iraq).

Migratory waterbirds were at the top of the list of suspects for the spread of HP H5N1 viruses (Webster et al., 2006; Normile, 2005, 2006), especially after the discovery of thousands of bar-headed geese killed by HP H5N1 in Qinghai Lake (Western China, Chen et al., 2005; Liu et al., 2005). However, the relative contribution of migratory birds, and especially waterbirds, in the spread of HP AIV remains unclear (Gauthier-Clerc et al., 2007). For instance, the recent report by Chen et al. (2006) showed that only a small proportion of wild ducks and geese might be involved in the spread of the virus compared to domestic species. Nevertheless, the potential role of wild birds in the HP H5N1 crisis is still put forward (*e.g.* FAO, 2006; Gauthier-Clerc et al., 2007), calling for multi-disciplinary research towards a better understanding of the ecology of avian influenza viruses and to assess the risks linked to the circulation of HP viruses (Melville and Shortridge, 2006; Capua and Alexander, 2006; Munster et al., 2006).

The Camargue is an alluvial wetland covering some 140,000 ha in the Rhône delta (South of France). It is situated at the crossroads of numerous migratory routes of Palaearctic birds (Berthold, 2001; Blondel and Isenmann, 1981) and is recognized as one of their main Mediterranean wintering area. During fall and spring migrations, hundreds of thousands of wild waterbirds stop in the Camargue to forage, and during winter this area is considered to be a particularly important wintering site for species belonging to the Anseriform order (*e.g.* wild ducks, geese and swans; Tamisier and Dehorter, 1999). Anseriforms represent a central element of the ecology of avian influenza viruses because a particularly wide variety of subtypes have been isolated from these species (Deibel et al., 1985; Webster et al., 1992; Alexander, 2000), especially for the Mallard (*Anas platyrhynchos*, Munster et al., 2005).

The main objective of our study was to investigate the role of migratory waterbirds in the spread of HP H5N1 influenza viruses by combining different data sets (bird census, death surveillance and virus detection), closely taking into account the cascade of events characterising the first arrival of the HP H5N1 viruses in Western Europe.

2. Materials and methods

2.1. Bird sampling

Live birds were caught daily with two funnel live-traps placed at the periphery of a wintering marsh in the private natural reserve of "La Tour du Valat" (43°30′N, 4°40′E). Cloacal swabs were performed to collect faecal samples. Birds were marked with a steel ring and identified by sex and to species before being released. Sixty three percent of the Camargue wetland area corresponds to private hunting marshes, from which we also sampled freshly killed birds in seven locations (Fig. 1).

2.2. Molecular analyses

Cloacal swabs were collected using the Viral Pack kit (Biomedics, S.L.). Automatic RNA extraction was performed according to the manufacturers instructions with the BIOROBOT MDX (QIAGEN) using the QIAamp Virus BioRobot MDX kit (QIAGEN). The presence of influenza viruses was

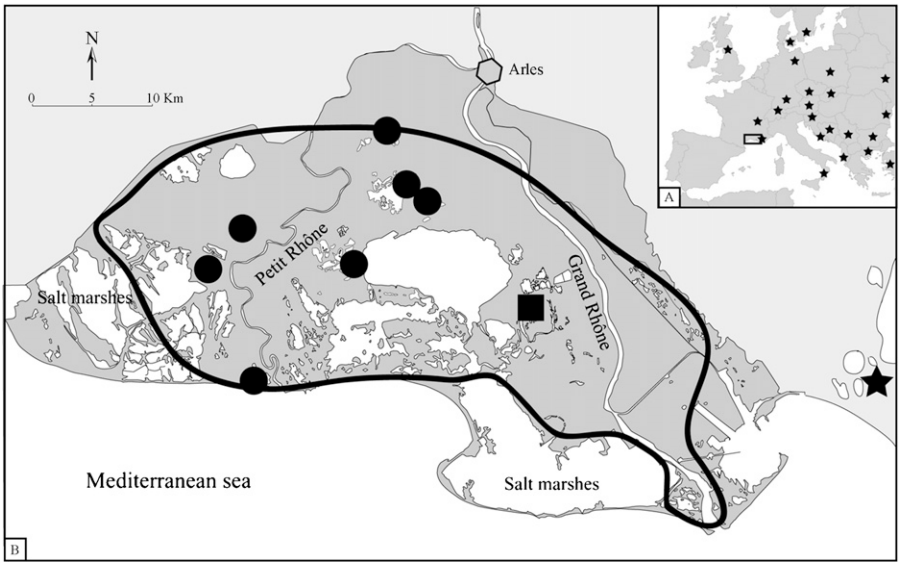

Fig. 1. (A) Schematic representation of locations where HP H5N1 viruses (represented by stars) have been detected in Europe (poultry or wild bird deaths) during the study period (September 2005–March 2006). The geographic situation of the Camargue in the Rhône delta, France, is shown by a rectangle. (B) Map of the Camargue. Waterfowl sampling sites are represented by circles (hunt marshes) and a square ("Tour du Valat" Natural Reserve). The heavy line marks outline of the geographical area covered by the waterbird population census. The black star represents the location where HP H5N1 virus was detected in a dead swan on February 28, 2006 (Saint Mitre les Ramparts, O.I.E., 2006).

detected by reverse transcription polymerase chain reaction (RT-PCR) targeting the Matrix gene segment. Amplification was performed on 5 μl RNA with the Superscript II kit (INVITROGEN) in the presence of oligonucleotides M52C: 5′-CTT CTA ACC GAG GTC GAA ACG-3′ and M253R: 5′-AGG GCA TTT TGG ACA AAK CGT CTA-3′ (Fouchier et al., 2000) using the following cycling conditions: 30 min at 45 °C, 15 min at 55 °C, 2 min at 94 °C, then 15 s at 94 °C, 30 s at 45 °C and 30 s at 72 °C repeated five times, 15 s at 94 °C, 30 s at 55 °C and 30 s (plus 2 s per cycle) at 72 °C repeated 35 times, and finally 5 min at 72 °C. The amplification products were analysed by 1% agarose gel electrophoresis and ethidium-bromide staining.

For the positive samples, detection by RT-PCR of the H5 gene was performed using oligonucleotide primers H5/+/1544-1563: 5′-CCG CAG TAT TCA GAA GAA GC-3′and H5/+/1664-1683: 5′-AGA CCA GCT ACC ATG ATT GC-3′ and the following cycling conditions: 30 min at 45 °C, 15 min at 55 °C, 2 min at 94 °C, then 15 s at 94 °C, 30 s at 58 °C and 30 s (plus 2 s per cycle) at 72 °C repeated 40 times, and finally 10 min at 72 °C. The N1 gene was detected by the procedure recommended by the World Health Organisation (WHO, 2005) using the N1-1: 5′-TTG CTT GGT CGG CAA GTG C-3′ and N1-2: 5′-CCA GTC CAC CCA TTT GGA TCC-3′ primers. The amplification products were analysed by 1% agarose gel electrophoresis and ethidium-bromide staining.

2.3. Long-term populations studies

Aerial counting techniques have been used in the Camargue to estimate the total population of the most abundant wintering waterfowl species since 1964 (Tamisier and Dehorter, 1999). Species censused are: common teal (*Anas crecca*), mallard (*Anas plathyrhynchos*), common pochard (*Aythya ferina*), northern shoveler (*Anas clypeata*), gadwall (*Anas strepera*), Eurasian wigeon (*Anas penelope*), northern pintail (*Anas acuta*), red-crested pochard (*Netta rufina*), tufted duck (*Aythya fuliga*), and common coot (*Fulica atra*). Censuses were conducted over 100 marshes used by ducks and coots as resting places (Fig. 1) on Tuesday after the 15th of each month, between September and March, from a single-engine monoplane flying at an altitude of 200 ft. If weather conditions precluded the plane from flying, the census was postponed to the next date with good weather conditions.

2.4. Statistical analysis

To test for differences of prevalence between species and orders, we used a Fisher exact test on an r x k table (Npstat software). The confidence interval of prevalence was computed by calculating the standard deviation of binomial law ($\sqrt{(p(1-p)n)}$ where p is the observed prevalence and n is the sample size).

Aerial censuses allowed us to assess the proportion of birds sampled for each species according to the mean of the total individuals counted in the Camargue (sampling effort estimated, SEE in Table 1). The mean abundance of each species was computed monthly between 1964 and 2004. The values recorded during winter 2005–2006 were compared with minimum and maximum abundance values recorded during the 1964–2004 period.

3. Results

3.1. Bird sampling and virological tests

We sampled 1345 wild waterbirds of 17 common species of the Camargue, belonging to 3 distinct orders (Table 1): 11 species of Anseriforms: common teal, mallard, common pochard, northern shoveler, gadwall, Eurasian wigeon, northern pintail, red-crested pochard, common shelduck (*Tadorna tadorna*), tufted duck and garganey (*Anas querquedula*); three species of Gruiforms: common coot, common moorhen (*Gallinula chloropus*) and water rail (*Rallus aquaticus*); and three species of Charadriiforms: common snipe (*Gallinago gallinago*), jack snipe (*Lymnocryptes minimus*) and northern lapwing (*Vannellus vanellus*).

Tests revealed that 1.8% (24/1345) of these birds were positive for avian influenza virus (*i.e.* excreting virus in faeces), but none were infected by an H5N1 virus (Table 1). Confidence intervals of prevalence in all individuals was 1.42–2.15%. Frequencies of infection were not significantly different between species ($P = 0.080$; when only Anseriforms were considered: $P = 0.067$), orders ($P = 0.91$), and between hunted versus living birds ($P = 0.41$).

Table 1
Detection of AIV in waterbirds during winter 2005–2006

Species		*N*	SEE (%)	NIB	%
Anseriforms					
Common teal	*Anas crecca*	303	1.1	11	3.6
Mallard	*Anas platyrhynchos*	275	1.0	8	2.9
Common pochard	*Aythya ferina*	138	1.6	0	–
Northern shoveler	*Anas clypeata*	149	1.4	4	2.7
Gadwall	*Anas strepera*	117	0.8	0	–
Eurasian wigeon	*Anas penelope*	62	0.7	0	–
Northern pintail	*Anas acuta*	50	5.1	0	–
Common shelduck	*Tadorna tadorna*	8	–	0	–
Red-crested pochard	*Netta rufina*	6	0.2	0	–
Tufted duck	*Aythya fuligula*	6	1.1	0	–
Garganey	*Anas querquedula*	5	–	1	20.0
Total		1119		24	2.1
Gruiforms					
Common snipe	*Gallinago gallinago*	94	–	0	–
Common coot	*Fulica atra*	83	0.2	0	–
Water rail	*Rallus aquaticus*	6	–	0	–
Total		183		0	–
Charadriiforms					
Common moorhen	*Gallinula chloropus*	17	–	0	–
Northern lapwing	*Vannellus vannellus*	16	–	0	–
Jack snipe	*Lymnocryptes minimus*	10	–	0	–
Total		43		0	–
Total		1345		24	1.8

Bird species, number of birds analysed (*N*), sampling effort estimate (SEE), number of infected birds (NIB) and percentage of infection (%).

3.2. Population census

We did not notice abnormal population size reductions during winter 2005–2006 (results of the aerial census during the study period are summarized in Supplementary Table). Dead swans (*Cygnus sp.*) were systematically noted during the aerial census in winter 2005–2006, however only one dead individual (*Cygnus sp.*) was recorded (on February 2006) from the eight aerial censuses performed during this period. Greylag goose (*Anser anser*) and swans (*Cygnus olor* and *C. columbianus*) population censuses from winter 2005–2006 were not compared with long-term monitoring surveillance data because such data were not available for these species.

4. Discussion

The aim of this study was to monitor the potential arrival and circulation of HP H5N1 viruses in the Camargue during the winter 2005–2006 by combined different data sets (bird census, death surveillance and virus detection). The Camargue area represents a stop-over site for a wide variety of bird species from all origins (Central Asia, Siberia, Northern and Eastern Europe, Western Africa and the Mediterranean basin). The Camargue is considered as a hot spot for the risks of introduction and transmission of bird-borne pathogens (Jourdain et al., 2007). There was a strong potential for waterbirds breeding in Northern and Eastern Europe and Siberia (Tamisier and Dehorter, 1999; Guillemain et al., 2005) to introduce HP H5N1 AIV to Europe in the winter of 2005–2006 during their annual migration and wintering in the Camargue.

The results of this study revealed that the proportion of waterbirds excreting avian influenza viruses in their faeces was low (1.8%) compared to previous reports from Europe (Fouchier et al., 2003; De Marco et al., 2004; Wallensten et al., 2006, 2007). Our peak of prevalence was found in September (Fig. 2), which is in accordance with seasonal AIV prevalences observed in European waterbirds (Wallensten et al., 2007).

No HP H5N1 virus was found among 24 samples positive for AIV. Although this result is in agreement with the idea that HP H5N1 viruses did not reach the Camargue, it could also be due to other reasons. Despite our effort to sample a large number of birds, the sample size ($N = 1345$) is small compared to that of other studies (*e.g.* Chen et al., 2006). Given the low prevalence of AIV infection in the wild bird population, we cannot exclude the hypothesis that we missed birds carrying HP H5N1 virus. A second potential problem is linked to the efficiency of virus detection given the way we collected samples, *i.e.* cloacal swabs. Indeed, experimental inoculation of domestic mallards with HP H5N1 strains revealed that the digestive tract is not the main site of replication for these strains and that viruses replicate rapidly in the trachea, suggesting that the respiratory tract might be the preferred sampling site for these viruses (Sturm-Ramirez et al., 2005).

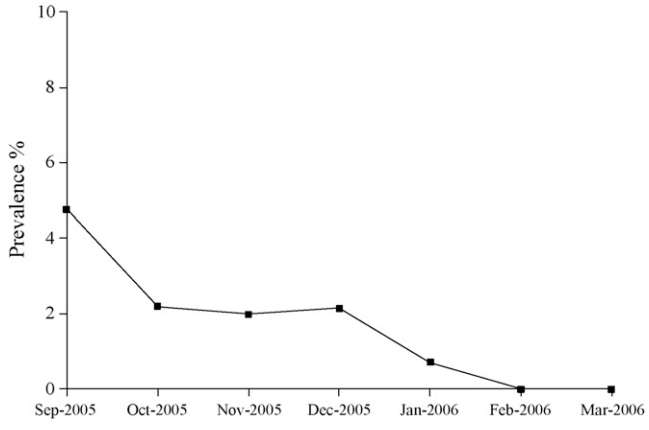

Fig. 2. Influenza A virus prevalence in Camargue waterbirds ($N = 1345$) between September 2005 and March 2006.

Further important information provided by the analysis of the long-term monitoring data of wintering ducks was that there was no abnormal population reduction. In other places, abnormal demographic variations have been shown to occur during HP H5N1 virus outbreaks. For instance, the epizootic recorded in Qinghai Lake (Chen et al., 2005; Liu et al., 2005) caused an estimated 10% decrease of the global population of bar-headed Geese (*Anser indicus*), highlighting the potentially devastating effect of HP H5N1 outbreaks on vulnerable wildlife (Olsen et al., 2006). Here, by combined data sets, we can conclude that despite the detection of the HP H5N1 virus near the Camargue (*i.e.* one case recorded from 30 km away; Fig. 1; O.I.E., 2006), circulation of HP H5N1 viruses during the winter 2005–2006 was either non-existent or negligible, with in any case no significant impact on local waterbird populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2007.05.009.

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