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Human and animal integrated influenza surveillance: a novel sampling approach for an additional transmission way in the aquatic bird reservoir

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ABSTRACT

BACKGROUND: infectious low pathogenic avian influenza viruses (LPAIVs) have been recently detected on feathers of wild ducks. Laboratory trial results suggested that the preen oil gland secretion, covering waterbirds' feathers, may attract and concentrate virus particles from AIV-contaminated waters to birds' bodies. We evaluated whether ducks can become infected by the ingestion of preen oil-associated viral particles, experimentally smeared on their plumage. In addition, we compared virologic and serologic results obtained from mallards whose feathers were experimentally infected, with those from wild mallards naturally carrying AIVs on feathers.

METHODS: we experimentally coated 7 mallards (Anas plathyrynchos) using preen oil mixed with a LPAIV (H10N7 subtype), and housed them for 45 days with a control, uncoated duck. Cloacal, oropharyngeal and feather swabs were collected from all birds and examined for AIV molecular detection and isolation. Blood samples were also taken to detect influenza specific antibodies. In addition, sera from 10 wild mallards, carrying on feathers infectious LPAIV H10N7, were examined.

RESULTS: virologic and serologic results indicated that through self- and allopreening all the birds experimentally coated with the preen oil/AIV mix and the control duck ingested viruses covering feathers and became infected. Virus isolation from feathers was up to 32 days post-coating treatment. One out of 8 wild mallards showing antibodies against type A influenza virus was seropositive for H10 subtype too.

CONCLUSIONS: our experimental and field results show evidences suggesting that uninfected birds carrying viruses on their feathers, including immune ones, might play an active role in spreading AIV infection in nature. For this reason, routine AIV surveillance programs, aimed at detecting intestinal and/or respiratory viruses, should include the collection of samples, such as feather swabs, enabling the detection of viruses sticky to preened birds' bodies.

Key words: Avian influenza, Experimental infection, Infection route, Preening, Aquatic birds

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INTRODUCTION

Humans influence global ecology with new trends in animal production practices, changes in patterns of wildlife populations, demographic fluctuations, such as population growth, mobility and urbanization, and globalization of the food industry. Each of these factors has implications for the emergence of novel disease agents or re-emergence of pathogens which change their population dynamics (1).

This is particularly the case for influenza A viruses, since animal and human species have a key role in viral ecology and evolution. The cyclic spill over of influenza A viruses from the natural reservoir, represented by wild aquatic birds, to non-adapted hosts usually brings transitory self-limiting infections, but less frequently it represents the first step towards viral adaptations that can allow the emergence of a number of stable host-specific influenza lineages in non-reservoir species of mammals and birds, such as poultry, horses and humans (2). For instance, only three haemagglutinin subtypes (H1, H2, H3) and two neuraminidase subtypes (N1 and N2) originated stable lineages in the human population since 1918 (3).

To date, influenza A virus strains from 16 haemagglutinin subtypes (H1-H16) and 9 neuraminidase subtypes (N1-N9) have been detected in aquatic bird populations, where the co-evolution of the host/pathogen system has favoured, by natural selection, a well adapted bird/virus relationship in which low pathogenic avian influenza viruses (LPAIVs) cause asymptomatic infections. Sporadic transmissions of H5 and H7 subtypes of LPAIVs to poultry species can generate highly pathogenic avian influenza viruses (HPAIVs) such as the Eurasian lineage H5N1 HPAIV, which has important implications for both public and veterinary health (4).

Influenza A virus gene pool constantly circulates in animals and humans; suitable ecological interfaces allow interspecies transmission events. Among the wide range of susceptible animal species, intensively reared poultry and pigs represent primary hosts which enable the virus to cross the species barriers, that is the crucial step leading to the emergence of new viral strains in the human population. In such a context, the HPAI H5N1 virus and the 2009 pandemic swine origin H1N1 influenza virus are paradigmatic examples of pathogens arising from animal hosts, and showing a different ability to spread in the human population: in absence of an efficient inter-human transmission, the H5N1 virus has caused in about 14 years less than 600 cases whereas the 2009 H1N1 pandemic virus spread, within 1 year, to 214 countries causing >18 000 deaths worldwide (3).

To date, human cases of H5N1 HPAIV infection are rare and sporadic events. However the aggressive clinical course and high fatality rate (from late 2003 to December 15, 2011, 336 fatal cases out of 573 human infections) associated with widespread H5N1 HPAIV outbreaks in domestic and wild birds, highlight the importance of the large-scale surveillance efforts, carried out in avian species in several regions around the world (3, 5).

The H5N1 HPAI virus emerged in human population in 1997, when an H5N1 HPAI virus infected 18 persons in Hong Kong, killing 6 of them. Human infections, occurring during a serious poultry epidemic, were caused by the direct transmission of avian viruses to humans, opening at once new potential pandemic implications. After initial control measures, related to the effective mass slaughter of all poultry across the Hong Kong SAR (1.5 million birds), the HPAI H5N1 virus re-emerged in bird flocks in 2001, 2002, and in 2003 when further poultry outbreaks were associated with severe human cases. Between late 2003 and early 2004, the HPAI H5N1 virus started its inexorable spread among domestic birds throughout areas of East and Southeast Asia, later involving Europe, the Middle East and Africa (3).

At the present time, the H5N1 poultry outbreaks represent an unprecedented epidemiological situation, as characterized by both wide geographical extent and zoonotic potential, and the HPAI H5N1 virus is now endemic in areas of Asia and Africa where domestic ducks represent the main epidemiological reservoir (6, 7). In such a context, what is the involvement of wild birds in the spread of the H5N1 HPAI virus? From late 2003, H5N1 HPAIVs have been periodically detected in Asia in wild avian species, waterfowl included (8). Mortality of birds suggested a self-limiting dynamic of these infections, due to a viral spill over from infected reared poultry. However, in April 2005, thousands of wild waterfowl died at Qinghai Lake, in western China, from an H5N1 HPAIV outbreak (9). Subsequently, the H5N1 virus infected wild birds in Russia, Kazakhstan and Mongolia (mid 2005), in Romania, Turkey, and Croatia (October 2005), in Ukraine (December 2005). The proximity of concurrent epidemics in poultry and wild birds and the characterization of viral isolates, suggested that migrating waterbirds

play a role in the virus dissemination (10). In 2006, numerous lethal outbreaks occurred in wild birds, especially in European countries (11). In 2007 and 2008 European and Asian countries reported fewer deaths of wild avian species. However, the number of H5N1 HPAIV outbreaks in wild birds increased in 2009 and 2010 in Asia and Europe, and similarly, in 2011 a significant number of cases were reported in Asia and the Middle East areas (12).

The temporal and spatial spread of H5N1 HPAI virus shows an anomalous epidemiological situation in which, for the first time, wild aquatic birds are involved in the circulation of an HPAIV. Unlike what normally happens in this natural reservoir, in which LPAIVs are perpetuated by a well-adapted host/parasite relationship, wild ducks can become infected and die with H5N1 HPAI virus. However, how can a virus able to kill its reservoir host spread within and between countries via bird migration? To address this question we looked for an AIV circulation mechanism which does not affect the fitness of aquatic birds, enabling long distance movements of HPAIVs in nature (13). It is well known that replication of AIVs in ducks occurs primarily in the intestinal tract, with high concentrations of infectious virus shed, by infected faeces, in water. Hence, the faecal-oral route is believed to be an efficient, water mediated, transmission mechanism (14). Our field and laboratory studies demonstrated the existence of a natural concentration mechanism of AIVs from water onto birds' bodies, by which virus particles are captured by preened feathers and concentrated from the aquatic environment to bird bodies. AIV attraction and concentration on body surface has been associated with the preen oil gland secretion that aquatic birds spread on their feathers by the natural preening behaviour (13). In natural conditions, mallards spend 10.9% of their daily time in grooming behaviour (15, 16) including preening activities which are necessary for waterproofing, heat regulation, and also for provitamin D supply by preen oil ingestion (17). While preening themselves (selfpreening) or other cospecific birds (allopreening) waterbirds commonly ingest preen oil (15). Hence, this natural behaviour could facilitate a protracted ingestion of AIV particles stuck on birds' feathers, thus improving the efficiency of the indirect water-borne transmission route.

To determine whether ducks can become infected by ingestion of viral particles covering their feathers, we used the Mallard (*Anas plathyrynchos*) as experimental animal model because of its important role as AIV reservoir (18). Moreover, to verify whether infectious AIVs covering feathers may evade the duck's immune system, we compared virologic and serologic results obtained from mallards whose feathers were experimentally infected, with those from wild mallards naturally carrying AIVs on feathers.

METHODS

Animal model and experimental design

Eight mallards (6 months old) bred in captivity and purchased from commercial breeders were housed in a BioSafety Level 2 containment room (Department of Veterinary Medical Sciences, University of Bologna, Italy). The trial was approved by the Ethical Committee of Alma Mater Studiorum at Bologna University. Facilities were equipped such that duck bodies or faeces could not contaminate the drinking water, which was changed twice a day. Room temperature was maintained at 4 °C to 11 °C, thus reproducing Mediterranean winter temperature conditions. Each duck was individually identified by a leg ring.

To reproduce the hypothesised preening-mediated infection mechanism, 7 out 8 mallards were coated with a preen oil-AIV mix as follows. Uropygial secretions, tested AIV negative by reverse transcription (RT)-PCR (see below), were individually collected from 7 slaughtered mallards (commercial distribution chain) and dispensed in 7 tubes, weighed (range 0.6-1.15 g) and stored at –20 °C until use. A LPAI A/mallard/Italy/Unibo-403F/2006 (H10N7) virus strain, previously isolated from feathers of wild mallards (13), was used. The method of Reed and Muench (19) was used to calculate the virus titre. To prepare the virus suspension, 200 µL of infected allantoic fluid ($10^{7.9}$ EID_{50}/mL) was added to each of the above tubes containing uropygial secretions, vortexed (3 min), incubated (overnight at 4 °C), and vortexed again (3 min). This preen oil–virus mix, having an approximate final EID_{50}/mL between $10^{7.1}$ and $10^{7.4}$, was smeared on feathers of 7 of the 8 housed mallards: the infected uropygial secretion, quickly absorbed on feathers (Figure1), covered around 100 cm^2 of body surface at the duck waterline level. The AIV-coated area was the same we previously examined by feather swabs collected from wild mallards (13). The eighth, uncoated duck, was placed in the flock as a negative control. A progressive and delayed ingestion of virus particles was expected to occur via the preening activity. Thus, to coat ducks' bodies we used viral loads consistent with high EID_{50} titres, already used in experimental inoculation of mallards via the conventional oral route (20, 21).

FIG. 1

EXPERIMENTAL COATING OF MALLARD FEATHERS WITH PREEN OIL MIXED WITH INFLUENZA VIRUS

Cloacal and oropharyngeal swabs were taken daily until 8 days post-coating treatment (dpct), then on 10 dpct. Cloacal swabs were also taken on 18, 26, and 32 dpct. Feather swabs were sampled on 10, 18, 26, 32, and 45 dpct, and blood samples were collected on 7, 18, 26, 32, and 45 dpct; baseline measurements were taken on day 0 for comparison (Table 1). Collected swabs were stored at -80 °C in 1 mL PBS/glycerol transport media with antibiotics (13) until laboratory testing. During the experiment, mallards were visually checked for preening activities.

Virus detection and serologic assays

One-step RT-PCR specific for influenza A virus detection (matrix gene amplification) was used to initially screen collected samples; separate pools of cloacal, oropharingeal or feathers origin were prepared, treated and examined as described (13). When pooled samples were verified to be RT-PCR positive, each individual sample in that pool was retested by RT-PCR to identify the AIV positive duck.

To confirm virus infectivity, RT-PCR positive samples were inoculated into specific pathogen–free embryonated chicken eggs; then harvested allantoic fluids were tested by the hemagglutination (HA) assay (22) and an ELISA specific for influenza A nucleoprotein (23). HA- and ELISA-positive samples were further characterised with the hemagglutination inhibition serologic assay (HI) (22). The initially collected samples of RT-PCR positive cloacal, oropharingeal, feather swabs from which virus could not be isolated, were used to inoculate embryonated eggs again, as described (13).

Serum samples were tested for the presence of anti-nucleoprotein antibodies by a standard ELISA technique (NP-ELISA) performed with some modifications (24). To detect specific anti-H10 antibodies, the HI assay (22) was performed using as antigen the LPAIV H10N7 strain experimentally coated on mallards. In both serological tests, antibody titres of 8 of more were considered positive.

Free-living mallards

Sera from 10 out 345 wild mallards, trapped in wetland of the Orbetello Lagoon, World Wildlife Fund (WWF) oasis, Tuscany, Italy in 2006, and previously tested by virologic methods for AIV detection in cloacal and feather swabs (13) were examined by the above NP-ELISA and HI assays. To compare experimental and field data, we selected wild birds carrying on feathers infectious AIVs belonging to the LPAIV H10N7 subtype.

RESULTS

RT-PCR analysis of oropharyngeal and cloacal swabs showed that virus ingestion started at 1 dpct (Table 1). One duck became positive to oropharyngeal virus isolation (VI) 2 dpct, whereas five birds, including the negative control, became altogether positive to cloaca VI 6 dpct. All 8 ducks shed virus via oropharynx and cloaca 7-8 dpct and 8 dpct, respectively. One out of eight bird showed anti-nucleoprotein antibodies 7 dpct. All birds were seropositive with detectable hemagglutination-inhibiting (HI) and anti-nucleoprotein antibodies from 18 to 45 dpct. All feather swabs collected between 10 and 32 dpct were RT-PCR positive whereas the virus was isolated from 5 feather swabs between 18 and 32 dpct (Table 1). During the experiment, both self- and allopreening activities were observed in the bird group.

Table 2 summarizes virologic and serologic data from wild mallards. In particular, serologic results obtained from wild ducks showed that one out 8 mallards having antibodies directed against type A influenza virus, was seropositive for H10 subtype too.

DISCUSSION

The findings reported here document that ducks may become infected by AIV particles experimentally smeared on their feathers.

TABLE 1

DETAILS OF INFECTION DYNAMICS IN MALLARDS EXPERIMENTALLY COATED WITH PREEN OIL MIXED WITH AN LPAIV

DPCT	SAMPLE COLLECTED							
	OROPHARYNGEAL SWABS*		CLOACAL SWABS*		FEATHER SWABS*		SERUM SAMPLES*	
	RT-PCR	VI	RT-PCR	VI	RT-PCR	VI	NP-ELISA	HI
0	0/8	—	0/8	—	0/8	—	0/8	0/8
1	**7/8†**	0/8	**8/8†**	0/8	—	—	—	—
2	**6/8†**	**1/8**	**8/8†**	0/8	—	—	—	—
3	**8/8†**	**1/8**	**8/8†**	0/8	—	—	—	—
4	**8/8†**	**1/8**	**2/8**	0/2	—	—	—	—
5	**6/8†**	**1/8**	0/8	0/8	—	—	—	—
6	**8/8†**	**7/8†**	**8/8†**	**5/8†**	—	—	—	—
7	**8/8†**	**8/8†**	**8/8†**	**7/8†**	—	—	**1/8**	0/8
8	**8/8†**	**8/8†**	**8/8†**	**8/8†**	—	—	—	—
10	**8/8†**	**8/8†**	**8/8†**	**5/8**	**8/8†**	—	—	—
18	—	—	**7/8†**	0/8	**8/8†**	**1/8**	**8/8†**	**8/8†**
26	—	—	**4/8**	0/8	**8/8†**	**3/8†**	**8/8†**	**6/8**
32	—	—	0/8	0/8	**8/8†**	**1/8**	**8/8†**	**6/8**
45	—	—	—	—	0/8	—	**8/8†**	**6/8**

*Results are shown as the number of positive birds to the number of examined birds, and bold type indicates positive results; LPAIV, low pathogenic avian influenza virus (A/mallard/Italy/Unibo-403F/2006, H10N7); Dpct, day post-coating treatment on the body surface; —, no data; RT-PCR, reverse transcription PCR to amplify M gene of influenza A virus; VI, virus isolation in embryonated chicken eggs; NP-ELISA, nucleoprotein ELISA specific for antibodies against influenza A virus; HI, hemagglutination inhibition assay to test antibodies against the H10N7 LPAIV strain, A/mallard/Italy/Unibo-403F/2006.
†Positive results comprise the untreated control duck.

TABLE 2

VIROLOGIC AND SEROLOGIC RESULTS OBTAINED FROM WILD MALLARDS CARRYING ON FEATHERS INFECTIOUS LPAIV H10 STRAINS, ORBETELLO LAGOON, TUSCANY, ITALY, 2006

DUCK NO.	CLOACAL SWABS*		FEATHER SWABS*		SERUM SAMPLES†	
	RT-PCR	VI	RT-PCR	VI	NP-ELISA	HI
1	**+**	-	**+**	**+**	**> 64**	< 8
2	**-**	-	**+**	**+**	**16**	< 8
3	**+**	**-**	**+**	**+**	**> 64**	< 8
4	**+**	**-**	**+**	**+**	**16**	< 8
5	**-**	**-**	**+**	**+**	**> 64**	**64**
6	**-**	**-**	**+**	**+**	**64**	< 8
7	**-**	**-**	**+**	**+**	**> 64**	< 8
8	**-**	**-**	**+**	**+**	**> 64**	< 8
9	**-**	**-**	**+**	**+**	< 8	< 8
10	**-**	**-**	**+**	**+**	< 8	< 8

*Virologic results from both cloacal and feather swabs were obtained in previous field studies as described (13) and bold type indicates positive results; LPAIV, low pathogenic avian influenza virus; RT-PCR, reverse transcription PCR to amplify M gene of influenza A virus; VI, virus isolation in embryonated chicken eggs; NP-ELISA, nucleoprotein ELISA specific for antibodies against influenza A virus; HI, hemagglutination inhibition assay to test antibodies against the H10N7 LPAIV strain, A/mallard/Italy/Unibo-403F/2006; +, positive; -, negative.
† Antibody titres are expressed as reciprocal of serum dilution.

They also suggest that infection follows virus ingestion, which is in turn mediated by self- and allopreening. This is mostly supported by the early molecular detection of the virus in oropharyngeal and cloacal swabs of all the ducks, at 1 dpct. Cloacal samples collected from 1 to 4 dpct, showed very weak RT-PCR bands whose specificity was determined by sequencing the amplified product (not shown), whereas strong RT-PCR bands characterized virus isolation-positive cloacal samples from 6 to 10 dpct. The initial shedding of vRNA does not result in a concomitant virus isolation from cloacal swabs, obtained 6 dpct only. The faecal-oral transmission route plays a crucial role in the AIV perpetuation mechanism in nature (14). The timing of virus isolation from cloaca observed in the present study, is atypical if compared to other LPAIV experimental trials carried out in mallards, in which infectious AIVs have been detected in cloaca since day 1 post inoculation (25, 26).

Differences are obviously expected when comparing results of experimental trials performed by classic infection routes (20, 21) to results obtained by the preening-mediated virus ingestion. In particular, in traditional studies a single high virus dose was given to animals through oral route, whereas in our experiment a continual and progressive ingestion of lower doses of viral particles may have occurred.

Our virologic and serologic results, let us suppose the occurrence of a late efficient virus replication in ducks' intestinal tracts, resulting in cloacal virus isolation in five of eight ducks tested 6 dpct and seroconversion of all mallards examined 18 dpct. The 32 dpct virus isolation from feather swabs suggests a potentially longer host infectiousness via the feather route compared with the classical faecal-oral one (27, 28).

There have been previous reports indicating that feathers are potential fomites and source of AIV infection in gallinaceus birds and waterfowl (29-31). However, these studies show that HPAIVs can be isolated, after viremia, from follicles and calami of growing feathers (a suitable cell substrate for viral replication), whereas we substantiated the external origin and concentration of AIVs detected on mature feathers of ducks (13). Interestingly, both the viremia-mediated and preen oil-mediated mechanisms, allowing AIV concentration inside vascularised feathers and on feather surfaces respectively, seem to favour AIV persistence as recent reports (32, 33) and the present study demonstrated.

The experimental infection of mallards by a preening-mediated AIV ingestion adds new perspectives to AIV circulation mechanisms in waterbirds and may help explain long-distance movements and long-term infectivity of AIVs in wild migratory birds. Uninfected birds carrying viruses on their feathers, including immune ones, were observed in both experimental and field studies. These results might explain some unrecognized mechanisms of transmission of the H5N1 HPAI virus in Asia and Europe (34) providing insights on how the H5N1 HPAIV, that in 2002 began killing the wild waterfowl reservoir (35) may have circulated in wild bird populations of Asia and Europe. Moreover, the presence of Eurasian H5N1 HPAI virus on swan feathers, possibly due to the preen oil–virus interaction or faecal contamination, may also explain the only recorded human case of fatal infection passed from wild birds in February 2006 (36). All infected humans were involved in defeathering of dead wild swans after a massive die-off of these aquatic birds occurred in Azerbaijan.

The proposed preening-mediated mechanism would potentially provide a connection between large number of aquatic birds species which are spatially disconnected by ethological and ecological limits but which utilise a specific area contaminated with AIV. In this context, the virus can freely circulate in both reservoirs and epiphenomena and the water may contribute to virus tenacity as well as virus transmission, enabling the spread of the infection without direct contact between birds. However, our experimental conditions lack of environmental factors such as UV irradiation, desiccation, unfavourable temperatures, and the pH and salinity of water (37) that could, under natural conditions, counteract AIV persistence on feathers. Thus, additional work is required to determine how the previously undescribed preening-mediated mode of infection fits into the accepted faecal-water-oral as well as respiratory AIV transmission route in aquatic bird species (28).

Because of the existence of aquatic bird reservoir, influenza is not an eradicable disease and prevention and control are the only realistic goals. When tested by current AIV surveillance programs, based on collection of cloacal and tracheal swabs, birds carrying infectious virus on body surface could result negative. For this

reason, routine surveillance programs should include the collection of samples (e.g. feather swabs) enabling the detection of viruses stuck to preened birds' bodies.

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