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Letter to the Editor

Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: Oropharyngeal versus cloacal swabbing

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To the Editor,

Since the emergence in SE Asia in 1997 of the H5N1 highly pathogenic avian influenza (HPAI) virus many countries have started virus surveillance schemes. Although targeted primarily at detecting HPAI H5N1, these schemes have also been issued to achieve a greater understanding on the general circulation of low-pathogenic avian influenza (LPAI); in particular viruses of hemagglutinin (HA) subtypes H5 and H7, for their potential to mutate into HPAI viruses in poultry.

The traditional way of sampling wild birds have been through cloacal swabbing. The rational for this method stems from the 1970s when it was noted that cloacal samples gave higher isolation rates compared to tracheal samples, in dabbling ducks (*Anas* spp.) [1,2]. However, the methodology for influenza virus detection changed dramatically with the appearance of PCR-based assays. The current standard for detection in bird samples is RNA extraction and real time RT-PCR (RRT-PCR) targeted at the virus' matrix gene [3,4]. Compared to direct egg culture, this method has increased sensitivity, is less time-consuming and allows the screening of greater sample sizes in surveillance studies.

Wild waterfowl (Anseriformes) constitute the main reservoir for LPAI viruses [5]. HPAI viruses are normally found only in poultry, but during the expansion of HPAI H5N1 several reports of dead wild birds came from affected countries. Experimental infections with HPAI H5N1 showed that dabbling ducks (*Anas*), particularly Mallards, had little or no symptoms of disease and yet could shed large number of viruses [6,7], factors that make them potential vectors for the spread of H5N1 HPAI. Generally noted in such studies is the fact that virus recovery and titers are much higher from the upper respiratory tract compared to the cloaca [6–8]. As a consequence, it has been suggested that oropharyngeal sampling should be used routinely in the surveillance of HPAI H5N1 in Mallards [7].

Avian influenza surveillance of wild birds is a costly enterprise and requires a full logistic chain from ornithologists in the field to virologists in the laboratory. Therefore it is essential that the sampling methodology is chosen to fit its purpose, regardless if this is screening for highly or low-pathogenic strains.

In this study, we compared the efficiency of LPAI virus detection between oropharyngeal and cloacal swabs from 534 wild Mallards captured and sampled at Ottenby Bird Observatory, Sweden (56°12′N 16°24′E), between 15 August and 1 December 2006 [9,10]. The oropharyngeal sample was taken by gently swabbing the epithelial surfaces of the bird's oropharynx. Virus detection was performed by RRT-PCR, detecting the matrix gene [3,4]. RNA was extracted from 100 μl of the original sample using the MagAttract virus mini kit (Qiagen) on an M48 extraction robot (Qiagen).

We found that the detection of LPAI in wild Mallards was much higher in cloacal ($n = 84$, 15.7%) than in oropharyngeal ($n = 27$, 5.1%) samples. The cloacal samples had significantly lower C_t-values than the oropharyngeal samples (cloacal: mean 36.1, S.D. 3.0 cycles; oropharyngeal: mean 39.9, S.D. 1.3; Mann–Whitney $U = 244.0$, $n = 111$, $p < 0.001$). This is similar to studies using egg inoculation instead of RRT-PCR as screening method, and conforms to the dogma of the field [1,2]. However, 3% ($n = 16$) of the sampled birds were only positive in the oropharynx and would have been missed with only cloacal sampling.

Matrix positive samples, 26 from oropharynx and 8 correspondent cloacal samples were inoculated (200 μl) into the allantoic cavity of two 11-day-old embryonated chicken eggs. The samples were first tested for H5, H7 and pathogenicity according to the protocols from the EU Community Reference Laboratory [4]. The eggs were harvested after 2 days and hemagglutination titers were determined with hen erythrocytes using standard procedures. Negatives were inoculated for a second passage. From 34 samples, 20 virus isolates were obtained, giving an isolation rate of 59%. This is remarkably high compared to other studies [11,12], and can most probably be attributed to the transport medium and the unbroken freeze chain from the sampling site to the lab. Interestingly, the isolation rate of oropharyngeal samples was nearly as high as the cloacal samples, despite their higher C_t values.

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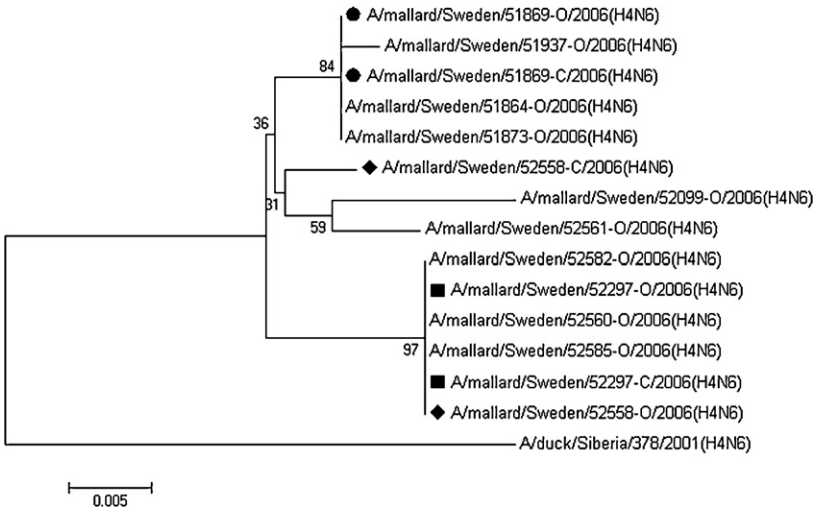

Fig. 1. A Kimura 2-parameter Neighbor-Joining tree illustrating the genetic relatedness of the H4N6 isolates of this study. Samples from the same birds are marked with symbols. C denotes a cloacal origin, whereas O denotes oropharyngeal. A/duck/Siberia/378/2001(H4N6) was used as a outgroup (accession number in GenBank: AB295610).

The HA subtype of the virus isolates were characterized using a hemagglutination inhibition (HI) assay with hen erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes [13]. Neuraminidase (NA) subtypes were determined by sequencing [14] and BLAST analysis to those deposited in GenBank [15].

Table 1 lists the subtypes found in the Mallards that were PCR positive in both the cloaca and oropharynx. In total, six different subtypes were found, of which the H4N6 was the most prevalent (14 isolates, all from the same week in late October). Four samples were H5 positive by RRT-PCR. In seven birds, we were able to characterize the virus in both the oropharynx and the cloaca (Table 1). Five of the ducks were infected with the same virus at both sites: three individuals carried H4N6 and two carried H5, suggesting that it was one and the same virus infecting each bird. However, two exceptions to this were observed, with one Mallard carrying a H4N6 in the oropharynx and a H1N9 virus in the cloacae, and another carrying a H3N8 in the oropharynx and a H2 virus in the cloacae. In these cases, we do not know whether both viruses were present at both sites simultaneously, *i.e.* an ongoing double infection, or if the infection in the oropharynx had not yet reached the intestines. Co-infections by different viruses are reported to be common in ducks [16,17]. These are an important source of variability within the genetic pool of influenza viruses, facilitating reassortment when different viruses infect the same cells. Sampling only one end of the bird would have missed the above cases.

Table 1
Isolation results and subtype information for Mallard individuals, PCR positive for influenza A in both oropharyngeal and cloacal samples

Sampling date	Mallard individual	Oropharyngeal	Cloacal
21 August 2006	50138	H3N8	H2
22 August 2006	50155	Neg	ND
27 August 2006	50267	Neg	H2N3
22 October 2006	51869	H4N6	H4N6
23 October 2006	51932	Neg	H1N1
27 October 2006	52297	H4N6	H4N6
28 October 2006	52447	H5[a]	H5[a]
29 October 2006	52558	H4N6	H4N6
29 October 2006	52582	H4N6	H1N9
1 October 2006	52981	H5N3	H5[a]
8 November 2006	58789	Neg	Neg

ND, not determined.
[a] Subtype only determined by RRT-PCR.

The NA sequences of all N6 subtypes were aligned and exported to MEGA [18], where a Neighbor-Joining tree with 1000 bootstrap was produced using the Kimura 2-parameter model to illustrate similarities and differences between strains. The 14 N6 virus sequences were similar to each other and many were identical over the 489 bp sequenced fragment and all clustered within the Eurasian N6 NA clade (Fig. 1). In total there were 20 variable sites (4%), 7 of which were non-synonymous. Two of the birds had identical viral sequences in the oropharynx and the cloaca, while one (52558) differed by 6 synonymous substitutions between the two isolates, further supporting that the same strain was found both in the oropharynx and the cloaca.

In this study, using modern methods for RNA extraction and RRT PCR analysis with optimally treated samples, we confirmed a higher prevalence of LPAI virus in cloacal than in oropharyngeal samples from wild Mallards. Even though oropharyngeal sampling has been recommended for the surveillance of the H5N1 HPAI, we propose to combine cloacal with oropharyngeal sampling in surveillance studies, for optimal detection of both LPAI and HPAI viruses.

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