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Original Article

Respiratory tract versus cloacal sampling of migratory ducks for influenza A viruses: are both ends relevant?

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Background Early studies in dabbling ducks showed that cloacal swabs yielded a larger number of avian influenza virus (AIV) isolates than did respiratory tract swabs. Historically, AIV surveillance has been performed by collecting cloacal or environmental fecal samples only. Highly pathogenic avian influenza H5N1 virus emerged in 1996 and replicated to higher titers in the respiratory rather than the gastrointestinal tract of ducks, prompting the collection of respiratory samples in addition to cloacal swabs from wild birds. Studies confirmed that some virus subtypes, especially H9 and highly pathogenic H5, are shed primarily through the respiratory tract and may not be detected in cloacal swabs.

Objectives To examine prevalence and subtype differences for AIV isolates from cloacal or respiratory swabs of wild ducks and to determine whether individual respiratory tract samples should be included in AIV surveillance studies in wild birds.

Methods Individual respiratory tract and cloacal swabs were collected from each of 1036 wild ducks in Alberta, Canada, during the month of August from 2007 to 2010 in an ongoing surveillance study. Virus isolation in eggs and subtype identification by antigenic and molecular methods were performed.

Results and conclusions Respiratory tract and cloacal swabs yielded ten influenza virus HA subtypes representing 28 HA–NA combinations. Three HA–NA subtype combinations were found exclusively in respiratory tract samples. Only four HA subtypes (H1, H3, H4, and H7) were recovered from respiratory samples, but respiratory shedding was associated with the dominance of 1 year's subtype. Might respiratory shedding provide a risk assessment indicator?

Keywords Avian influenza virus, cloaca, migratory ducks, oropharyngeal, respiratory tract, risk assessment.

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Introduction

Surveillance studies throughout the world have demonstrated that an influenza A virus reservoir exists in wild aquatic birds.[1–3] In the natural reservoir, avian influenza viruses (AIV) replicate primarily in the gastrointestinal tract, are excreted mainly in feces, and are transmitted via the fecal–oral route.[1] Early studies in dabbling ducks showed that cloacal swabs yielded a larger number of AIV isolates than did respiratory tract swabs.[4–6]

Highly pathogenic avian influenza (HPAI) H5N1 virus emerged in 1996 and replicated to higher titers in the respiratory rather than the gastrointestinal tract of ducks,[7,8] prompting the collection of respiratory samples in addition to cloacal swabs from wild birds. Several studies in recent years have incorporated tracheal or oropharyngeal sampling of poultry and wild birds.[9–11] These studies confirmed that low-pathogenic avian influenza (LPAI) viruses are shed predominantly via the gastrointestinal route, but they found that some virus subtypes, especially H9, are shed primarily through the respiratory tract and may not be detected in cloacal swabs.[12,13] Here, we provide evidence suggesting that a restricted number of LPAI virus subtypes are shed from the respiratory tract of wild ducks and that a high level of oral shedding may contribute to seasonal prevalence.

Methods

During the month of August from 2007 to 2010, we collected paired respiratory tract and cloacal swabs from wild ducks in Alberta, Canada, in an ongoing surveillance study that began in 1976.[14] We obtained 2277 samples from 1242 birds, including 1036 pairs of samples (Table 1).

Table 1. Comparison of influenza virus prevalence estimates from respiratory and cloacal swabs of migratory ducks

	2007		2008		2009		2010	
	No.	Positive (%)	No.	Positive (%)	No.	Positive (%)	No.	Positive (%)
Total samples*	499	92 (18·4)	578	15 (3·0)	600	15 (2·5)	600	173 (28·8)
Total birds*	353	82 (23·2)	289	14 (4·8)	300	14 (4·7)	300	113 (37·7)
Total cloacal samples*	351	53 (15·1)	289	9 (3·1)	300	13 (4·3)	300	78 (26·0)
Total respiratory samples*	149	38 (25·5)	289	5 (1·7)	300	2 (0·67)	300	96 (32·0)
Birds swabbed respiratory and cloaca*	147	53 (36·1)	289	14 (4·8)	300	14 (4·7)	300	113 (37·7)
Birds positive, cloacal only**	–	15 (10·2)	–	9 (3·1)	–	12 (4·0)	–	19 (6·3)
Birds positive, respiratory only**	–	28 (19·0)	–	3 (1·0)	–	1 (0·33)	–	31 (10·3)
Birds positive, respiratory and cloacal**	–	10 (6·8)	–	1 (0·35)	–	1 (0·33)	–	59 (19·7)

*Pearson's chi-square test, $P < 0{\cdot}001$.
**Fisher's exact test, $P < 0{\cdot}001$.

Swabs were obtained from hatch-year and after-hatch-year ducks from the following species: Mallard (*Anas platyrhynchos*), Northern Pintail (*Anas acuta*), Blue-winged Teal (*Anas discors*), Green-winged Teal (*Anas crecca*), Cinnamon Teal (*Anas cyanoptera*), American Wigeon (*Anas americana*), Canvasback (*Aythya valisineria*), Redhead (*Aythya americana*), Lesser Scaup (*Aythya affinis*), Common Goldeneye (*Bucephala clangula*), Bufflehead (*Bucephala albeola*), and Gadwall (*Anas strepera*). AIV was isolated predominately from mallard, but was also recovered from Northern Pintail, Blue-winged Teal, Green-winged Teal, Cinnamon Teal, American Wigeon, Redhead, and Bufflehead. The following eight lakes served as sample sites (although not all during a single season): Frank Lake (2007), Hay Lake (2007–2010), Buffalo Lake (2007–2009), Cardinal Lake (2008–2010), George Lake (2008, 2010), Little Burnt Lake (2008), Sitting Stone Lake (2009), and 'Ducks Unlimited' Lake (2008). Sample collection and transport and virus isolation and subtyping were described previously.[15] In summary, swabs were collected and put into vials containing 1·0 ml of transport medium containing 50% glycerol in phosphate-buffered saline pH 7·2 with antibiotics. The sample vials were placed immediately into a dewar containing liquid nitrogen and transported by air to St. Jude Children's Research Hospital where they are stored at −80°C upon arrival. Viruses were isolated in the allantoic cavity of 11-day-old embryonated chicken eggs after incubation at 35°C for 72 hours. Virus subtypes were determined by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays using monospecific antibodies and/or by RT-PCR and subsequent sequence analysis of the hemagglutinin (HA), neuraminidase (NA), and matrix (M) genes.

Pearson's chi-square test and Fisher's exact test were used to compare the positivity rate (i.e., prevalence) for AIV over time. Three analyses were performed: (i) comparison of the positive rate over time by total samples, total birds, total cloacal swabs, and total respiratory swabs; (ii) comparison of the positive rate over time by birds with both respiratory and cloacal swabs (birds with paired samples); and (iii) comparison of the positive rate over time between birds with paired samples from which virus was detected in cloacal only, respiratory only, or both cloacal and respiratory. Analysis was performed using the pasw Statistics (spss) 18 software (IBM, Armonk, NY, USA). *P*-values <0·001 were considered significant.

Results

Avian influenza virus prevalence estimates are shown in Table 1, as is the cloacal, respiratory, or cloacal and respiratory source of isolates. When based on total birds sampled, prevalence was highest during the years 2007 (23·2%; 36·1% if only paired samples are considered) and 2010 (37·7%) and lowest during 2008 (4·8%) and 2009 (4·7%; $P < 0{\cdot}001$). Virus was isolated from total respiratory samples most frequently in 2007 (25·5%) and 2010 (32·0%) and least frequently in 2008 (1·7%) and 2009 (0·67%; $P < 0{\cdot}001$). Virus isolation from total cloacal swabs showed a similar pattern (2007, 15·1%; 2008, 3·1%; 2009, 4·3%; 2010, 26·0%; $P < 0{\cdot}001$). In paired samples, AIV was isolated from both the cloaca and respiratory tract of a single bird most frequently in 2007 (6·8%) and 2010 (19·7%), when overall prevalence was high (Table 1). Furthermore, viruses were isolated from the respiratory tract only (as compared to the cloaca only) most frequently in 2007 (19·0% versus 10·2%) and 2010 (10·3% versus 6·3%), corresponding to the 2 years of high prevalence estimates. Conversely, during 2008 and 2009, the years of low prevalence, shedding from the cloaca only was more frequent (3·1% versus 1·0% and 4·0% versus 0·33%, respectively).

The difference in prevalence among respiratory only, cloacal only, or both cloaca and respiratory over time was statistically significant ($P < 0{\cdot}001$).

The HA subtypes of the isolates were H1–H7 and H9–H11, and the NA subtypes were N1 and N3 through N9; there were 28 HA–NA combinations (Figure 1). Twenty-five subtypes were isolated from cloacal swabs and 12 from respiratory swabs. Respiratory shedding was limited to viruses of the subtypes H1, H3, H4, and H7. Sixteen HA–NA combinations were unique to cloacal samples, and three combinations (H3N5, H3N6, and H4N5) were found only in respiratory samples.

Avian influenza virus was isolated from 18·7% (194) of the 1036 birds, from which paired respiratory and cloacal swabs were obtained. AIV shedding from both the cloaca and respiratory tract was observed in 71 ducks and co-infection (two subtypes) was observed in 15 (21·1% of ducks with positive paired samples) (Figure 2). The subtypes isolated in co-infections were H3N1 (1), H3N5 (1), H3N6 (1), H3N8 (10), H4N3 (1), H4N4 (1), H4N5 (3), H4N6 (10), and H4N8 (2).

	2007		2008		2009		2010	
Subtype	C[a]	R[b]	C	R	C	R	C	R
H1N4	4		3	1				
H1N5	1							
H1N6							1	
H1N9					1			
H2N3					5			
H2N9	1				1			
H3N1	1							
H3N5								1
H3N6		3						
H3N8	17	17	3		3	2	35	51
H3N9	3	1						
H4N3	1	2						
H4N4							2	3
H4N5								5
H4N6	15	11	2	4			30	22
H4N8	2	4					1	
H4N9	2						1	
H5N5					1			
H6N1					1			
H6N4					1			
H7N3							4	6
H7N8							2	2
H9N5	2							
H10N4	1						1	
H10N5	1							
H10N6	1							
H10N7	1		2					
H11N9							2	
Total	53	38	10	5	13	2	79	90

recovered only from cloacal swab
recovered only from respiratory swab
recovered from both cloacal and respiratory swabs
[a]C, cloacal swab
[b]R, respiratory swab

Figure 1. The number of influenza virus subtype isolates in ducks during each study year. Colors indicate the type of sample from which influenza virus was recovered: cloacal (gray), respiratory (pink), or both cloacal and respiratory (yellow) swabs. Note: Co-infections in individual ducks are included in the tabulation so that total numbers may differ from those found in Table 1.

		Respiratory								
		H3N5	H3N6	H3N8	H4N3	H4N4	H4N5	H4N6	H4N8	H7N3
Cloaca	H3N1			1						
	H3N8		1	32	1			3		
	H4N6	1		2		1	3	21		
	H4N8			2					1	
	H7N3									2

co-infection

Figure 2. The total number and combination of subtypes isolated from 71 ducks that shed influenza virus via both the respiratory and cloacal routes. Yellow boxes indicate the number of ducks that shed two different subtypes simultaneously, indicating co-infection.

Virus titers of six oral (five H3N8 and one H4N4) versus seven cloacal (four H3N8 and three H7N3) field samples were compared for 13 swabs from 2010. Only two oral swabs from which H3N8 influenza virus was isolated had detectable titers (2·0 and 2·5 log 10 EID50/ml). The remaining samples had titers below the detectable limit (0·5 log 10 EID50/ml).

Discussion

Here, we determined the frequency with which AIV was isolated from the respiratory tract and/or cloaca of migratory ducks in Canada during 2007–2010. Of the 28 HA–NA subtype combinations detected, three were found only in the respiratory tract (H3N5, H3N6, H4N5), nine in both respiratory tract and cloaca (H1N4, H3N8, H3N9, H4N3, H4N4, H4N6, H4N8, H7N3, H7N8), and the remaining 16 in cloacal samples only. Of the influenza viruses shed by both the respiratory and cloacal routes, it is noteworthy that two subtypes, H3N8 and H4N6, represented 72% of all isolates, and these subtypes were recovered from both cloacal and respiratory samples in 3 of 4 years. The H3N8 subtype was the most prevalent strain during 2010, comprising 86 of the 169 isolates and was recovered more frequently from respiratory samples than from cloacal samples (51 isolates versus 35 isolates, respectively). Each subtype that was shed exclusively by the respiratory tract occurred in only one sampling season – H3N6 in 2007; H3N5 and H4N5 in 2010.

The significance of respiratory shedding and the nature of any associated host or virus genetic factors remain to be determined. We speculate that respiratory shedding favors the spread of virus to mammals, as evidenced by the detection of H3N8 equine influenza virus and H4N6 AIV infection in swine.[16] It is noteworthy that the influenza viruses that transmit in domestic poultry (H9N2) and those that

have become pathogenic for domestic poultry (H5N1, H5N2, H7N7, H7N3) are preferentially shed in the respiratory tract of ducks and gallinaceous poultry.[7,12] Although no H5N1, H5N2, or H7N7 strains were detected in this study, 10 H7N3 viruses were recovered in 2010 – four isolates from respiratory swabs, and six from cloacal swabs.

Because field surveillance and virus isolation are relatively expensive, it is valid to question whether both respiratory and cloacal sampling of a bird is merited. Our analysis shows that only nine viruses from three low-prevalence subtypes (H3N5, H3N6, H4N5) would have been missed by cloacal sampling only. However, information potentially relevant to AIV risk factor assessment (e.g., potential for reassortment and transmission) may be missed if only cloacal sampling is performed. Similarly, information can be lost when respiratory and cloacal samples are combined to increase per-sample isolation rates.[11] Further studies are required to determine whether respiratory sampling provides any predictive advantage in determining potential risk of transmission to mammals.

Long-term surveillance of the influenza virus reservoir in ducks in Alberta, Canada, shows the dominant AIV subtypes 'wax and wane'.[15] The question is whether respiratory shedding is associated with dominance, as suggested by the dominance of H3N8 viruses in 2010, in our study. Additional studies are merited to determine whether the cyclic pattern of subtype dominance in nature is related to respiratory tract shedding.

Because of increasing evidence of genetic interplay between influenza viruses in wild and domestic duck species[10] and because AIV shed by the respiratory route may have spread back to wild birds from domestic birds, genomic analysis of these viruses is warranted and may provide insight into the role of respiratory shedding in the spread of AIV. We strongly recommend that both ends of the bird be swabbed in influenza surveillance studies.

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