Journal of Wildlife Diseases, 48(2), 2012, pp. 382–393

DETECTION OF AVIAN INFLUENZA VIRUSES FROM SHOREBIRDS: EVALUATION OF SURVEILLANCE AND TESTING APPROACHES

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ABSTRACT: Although influenza A viruses have been isolated from numerous shorebird species (Family: Scolopacidae) worldwide, our understanding of natural history of these viruses in this diverse group is incomplete. Gaining this information can be complicated by sampling difficulties related to live capture, the need for large sample sizes related to a potentially low prevalence of infection, and the need to maintain flexibility in diagnostic approaches related to varied capabilities and resources. To provide information relevant to improving sampling and testing of shorebirds for influenza A viruses, we retrospectively evaluated a combined data set from Delaware Bay, USA, collected from 2000 to 2009. Our results indicate that prevalence trends and subtype diversity can be effectively determined by either direct sampling of birds or indirect sampling of feces; however, the extent of detected subtype diversity is a function of the number of viruses recovered during that year. Even in cases where a large number of viruses are identified, an underestimate of true subtype diversity is likely. Influenza A virus isolation from Ruddy Turnstones can be enhanced by testing both cloacal and tracheal samples, and matrix real-time PCR can be used as an effective screening tool. Serologic testing to target species of interest also has application to shorebird surveillance. Overall, all of the sampling and diagnostic approaches have utility as applied to shorebird surveillance, but all are associated with inherent biases that need to be considered when comparing results from independent studies.

Key words: Delaware Bay, influenza A virus, PCR, Ruddy Turnstone, serology, shore birds, subtypes, virus isolation.

INTRODUCTION

Shorebirds (Family: Scolopacidae) were implicated in the natural history of influenza A viruses when a high prevalence of infection was detected in migratory populations at Delaware Bay, USA (Kawaoka et al., 1988). Infections in shorebirds have been consistently detected at this site since this initial detection in 1987 (Krauss et al., 2010) but prevalence is variable across species, time of sampling, and possibly location (Hanson et al., 2008; Krauss et al., 2010). Influenza A viruses have been reported from numerous shorebird species worldwide, but outside of Delaware Bay, prevalence estimates from these birds often have been very low (Olsen et al., 2006; Munster et al., 2007).

Because many shorebirds are long-distance migrants and could therefore provide a vehicle for intercontinental movement of influenza A viruses, understanding the epidemiology of these viruses in these diverse species is potentially important. However, there is limited information related to optimizing or improving sampling and testing methods needed to efficiently advance this knowledge. In ducks, the ability to detect influenza A viruses is dependent upon the diagnostic method (virus isolation versus molecular methods [Munster et al., 2009]) and sample selection (cloacal or oropharyngeal [OP] swabs; Hoye et al., 2010). Resulting inconsistencies in field sensitivity, related to either diagnostic performance or sampling approach, may bias results or create difficulties when comparing results from independent studies. In addition, it is often difficult to standardize virus detection and sampling

protocols because of logistic constraints or limited resources related to capture of birds and testing capabilities. In such cases, sampling and diagnostic methods need to remain flexible but their limitations related to viral detection and isolation need to be clearly understood to adequately interpret results.

An additional problem associated with shorebird surveillance relates to a low infection prevalence, which appears to be the norm outside of Delaware Bay (Munster et al., 2007; Hanson et al., 2008) and it is not uncommon for influenza surveillance efforts directed at shorebirds, even with large sample sizes, to yield negative results (Hlinak et al., 2006; Winker et al., 2008). Serologic testing for influenza A virus antibodies has traditionally played a minor supportive role in surveillance, especially with shorebirds. The potential application of serology to surveillance has recently been demonstrated (Brown et al., 2010), and with shorebirds, this potential utility not only relates to providing an additional perspective for understanding epidemiology but also to the detection of species that are likely to be infected with these viruses. Such information can be utilized as an inexpensive means to target species for subsequent and efficient virus detection efforts and to maximize surveillance data return.

Our objectives were to 1) retrospectively compare virus isolation results (prevalence and recovered subtype diversity) based on swab samples from live birds and fecal samples collected during the same weeks from 2000 to 2009 at Delaware Bay, 2) compare matrix real-time PCR (mRT-PCR) and virus isolation approaches for influenza A virus detection in shorebirds, 3) compare isolation success as applied to paired cloacal and OP swabs, 4) further demonstrate the utility of using serologic testing to identify species that may be infected with influenza A viruses, and 5) based on these results and the existing literature, identify strengths and weaknesses associated with these surveillance approaches as applied to diverse shorebird species and populations.

MATERIALS AND METHODS

Sampling strategies

Independent virus isolation results from shorebirds sampled at Delaware Bay from 2000 to 2009 by St. Jude Children's Research Hospital (SJCRH) and The University of Georgia (UGA) were compared. Comparisons were limited to the same week of sampling during each year and all samples were stored in the field in liquid nitrogen; all sampling and testing protocols have been described (Krauss et al., 2004; Hanson et al., 2008). Three sampling methods were evaluated in relation to influenza A virus prevalence and subtype diversity: 1) general shorebird sampling (all predominant species), 2) Ruddy Turnstone (*Arenaria interpres*) targeted sampling, and 3) fecal sampling directly from beach habitats used by these birds. Ruddy Turnstones were targeted because influenza A virus infections are routinely highest in this species at this location (Kawaoka et al; 1988; Hanson et al., 2008; Krauss et al., 2010).

Virus detection

To compare virus isolation results from cloacal and OP swabs of Ruddy Turnstones, 96 individual paired samples collected during 2009 were compared. These samples were tested by virus isolation only. During 2007, 350 cloacal swab samples were collected as previously described from Ruddy Turnstones (Hanson et al., 2008) and tested by virus isolation and mRT-PCR. Briefly, samples stored at −80 C following collection were thawed and inoculated into four 9-day-old, specific-pathogen-free, embryonating chicken eggs for virus isolation (Hanson et al, 2008). RNA was immediately extracted from swab samples using a modified commercial protocol (Ambion MagMAX AI/ND Viral RNA Isolation Kit, Applied Biosystems, Foster City, California, USA) as previously described (Das et al., 2009). Extracted RNA was maintained at 4 C and tested within 24 hr by mRT-RCR on a SmartCycler PCR machine (Cepheid, Sunnyvale, California, USA) for amplification of the avian influenza virus matrix gene as described (Spackman et al., 2002; Das et al., 2006).

Serologic testing

To further determine the utility of serologic testing to support surveillance efforts, serum samples were collected from Ruddy Turnstones

(n=160), Red Knots (*Calidris canutus*; n=56), and Sanderlings (*Calidris alba*; n=40) at Delaware Bay, and from 25 additional shorebirds from wintering areas in Georgia and Florida during 2010; these included Ruddy Turnstones (n=3), Sanderlings (n=4), Red Knots (n=9), and Short-billed Dowitchers (*Limnodromus griseus*; n=9). All serologic testing was done using a commercially available influenza A specific blocking enzyme-linked immunosorbent assay kit to detect antibodies against the nucleoprotein (FlockCheck AI MultiS-Screen Antibody Test Kit, IDEXX Laboratories, Westbrook, Maine, USA).

RESULTS

Sampling strategies

Prevalence data comparisons were limited to 2003–09; the 2000–02 samples from SJCRH were pooled (Table 1). As expected, prevalence estimates for the targeted Ruddy Turnstone sampling exceeded prevalence estimates based on sampling of multiple shorebird species during all years (Table 1). With the exception of 2009, prevalence estimates based on cloacal swabs from Ruddy Turnstones also were higher than those derived from fecal sampling. With two exceptions, prevalence trends (an increase or decrease from the preceding year) were consistent between all sampling strategies. Exceptions included a decreased prevalence in the all-bird sample during 15 May 2008 to 21 May 2008 and increasing prevalence in the fecal samples during 2009 (Table 1). Significant differences in prevalence estimates for the total sample were detected by chi square with Yates' correction between the total UGA all-birds and Ruddy Turnstone samples (χ^2=92.295, df=1, P<0.0001, Table 1). Within the overlapping sampling date, differences for prevalence estimates based on the totals were detected between the all-birds and Ruddy Turnstone samples (χ^2=37.71, df=1, P<0.0001), all-birds and fecal samples (χ^2=5.48, df=1, P<0.0192), and the Ruddy Turnstone and fecal samples (χ^2=19.65, df=1, P<0.0001; Table 1). Within-year comparisons were restricted to

TABLE 1. Influenza A virus prevalence estimates for total shorebirds, Ruddy Turnstones (RUTU), and fecal samples at Delaware Bay, USA, based on independent virus isolation results at St. Jude Children's Research Hospital (SJCRH, fecal samples) and The University of Georgia (UGA, all-bird, and RUTU samples). Arrows indicate an increase or decrease in antibody prevalence from the previous year.

Year:	2003 (%)	2004 (%)	2005 (%)	2006 (%)	2007 (%)	2008 (%)	2009 (%)	Total (%)
All birds[a,b]	48/669 (7.2)	20/534 (3.7) ↓	36/858 (4.2) ↑	63/768 (8.2) ↑	21/994 (2.1) ↓	103/1,645 (6.3) ↑	132/1,145 (11.5) ↑	423/6,613 (6.4)
RUTU[a]	46/441 (10.4)	19/256 (7.4) ↓	33/245 (13.5) ↑	53/293 (18.1) ↑	19/441 (4.3) ↓	95/584 (16.3) ↑	111/847 (13.1) ↓	376/3,107 (12.1)
Overlapping sampling date[c]	15–21 May	15–21 May	15–21 May	22–28 May	22–28 May	15–21 May	15–21 May	
All birds[c]	30/461 (6.5)	20/534 (3.7) ↓	13/337 (3.9) ↑	30/363 (8.3) ↑	10/147 (6.8) ↓	40/747 (5.4) ↓	12/264 (4.5) ↓	155/2,853 (5.5)
RUTU[c]	29/294 (9.9)	19/256 (7.4) ↓	13/116 (11.2) ↑	30/224 (13.4) ↑	10/96 (10.4) ↓	35/204 (17.2) ↑	9/158 (5.7) ↓	145/1,348 (10.8)
Feces[c]	19/300 (6.3)	14/500 (2.8) ↓	36/595 (6.1) ↑	43/575 (7.5) ↑	39/574 (6.8) ↓	47/610 (7.7) ↑	63/624 (10.1) ↑	261/3,778 (6.9)

[a] All birds sampled during May of each year by UGA.

[b] All birds included Ruddy Turnstone (*Arenaria interpres*), Red Knot (*Calidris canutus*), Semipalmated Sandpiper (*Calidris pusilla*), Sanderling (*Calidris alba*), Least Sandpiper (*Calidris minutilla*), Dunlin (*Calidris alpina*), and Short-billed Dowitcher (*Limnodromus griseus*).

[c] All collected during the same week where SJCRH and UGA sampling overlapped.

TABLE 2. Detection of influenza A virus subtypes by fecal and cloacal swab sampling of shorebirds during an overlapping 1-wk period at Delaware Bay, USA, 2000–09.

Parameter[a]	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Week of sampling	15–21 May	15–21 May	15–21 May	15–21 May	15–21 May	15–21 May	22–28 May	22–28 May	15–21 May	15–21 May
Total no. subtypes	11	9	11	12	5	4	12	15	15	13
Viruses (*n*)	60	77	88	47	34	49	73	48	84	68
UGA										
Total subtypes detected	7	3	7	9	3	3	5	2	8	3
Viruses (*n*)	23	8	17	28	20	13	30	9	40	11
Subtypes detected (%)	64	33	64	75	60	75	42	13	53	23
SJCRH										
Total subtypes detected	8	9	10	5	5	4	10	15	11	12
Viruses (*n*)	37	69	71	19	14	36	43	39	44	57
Subtypes detected (%)	73	100	91	42	100	100	83	100	73	92
No. subtypes/isolate UGA	0.30	0.38	0.41	0.32	0.15	0.23	0.17	0.22	0.20	0.27
No. subtypes/isolate SJCRH	0.21	0.13	0.14	0.26	0.36	0.11	0.23	0.38	0.25	0.21

[a] UGA = The University of Georgia; SJCRH = St. Jude Children's Research Hospital.

prevalence estimates for the Ruddy Turnstone and fecal samples taken during the overlapping sampling date; statistically significant differences ($P<0.05$) were only detected for the 2004 ($\chi^2=7.592$, df=1, $P=0.0059$), 2006 ($\chi^2=6.099$, df=1, $P=0.0135$), and 2008 ($\chi^2=14.051$, df=1, $P=0.0002$) prevalence estimates.

Overall, 628 influenza A viruses that were isolated during an overlapping 1-wk period were identified to subtype: 199 by UGA and 429 by SJCRH. With the exception of 2003, the SJCRC samples (fecal) yielded the highest diversity of identified subtypes during an individual year (Table 2). Subtype diversity, however, was a function of the number of viruses recovered during that year (Table 2, Fig. 1). The ability to capture subtype diversity did not vary by sampling method; based on the total results (2000–09); cloacal swabs (UGA) and fecal sampling (SJCRH) yielded an average of 0.265 and 0.225 subtypes per isolated virus per year, respectively. Based on the combined sample, the number of detected subtypes ranged from 4/yr to 15/yr (Table 3). In every year, the predominant hemagglutinin/neuraminidase (HA/NA) subtype combination (Table 3) and the predominant HA subtypes (Table 4) were detected by both UGA and SJCRH. In the case of HA diversity, viruses representing HA subtypes that were not detected by either UGA or SJCRH generally represented a very small proportion (≤8%) of the total isolates for that year.

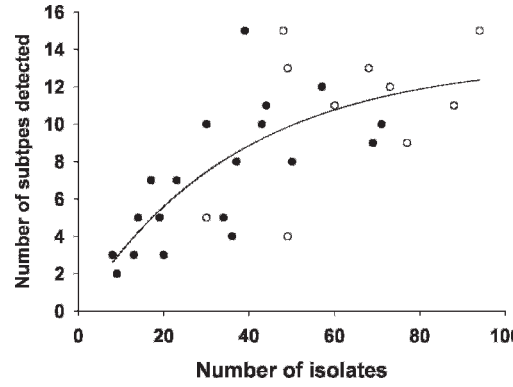

FIGURE 1. Diversity of influenza virus A subtypes collected from shorebirds at Delaware Bay, USA, 2000–2009 as a function of sampling effort. Solid circles are results from individual sampling efforts by the University of Georgia and St. Jude Children's Research Hospital. Open circles are the combined data for individual years. The nonlinear regression line ($y = 13.4412*(1-0.9734^x)$, $r^2 = 0.5460$) was calculated based on an exponential, two-parameter model using SigmaPlot 10 software (Systat Software, Inc., Richmond, California, USA).

Virus detection approaches

Virus isolation from 96 paired samples collected from Ruddy Turnstones resulted in 13 isolates from cloacal swabs and eight isolates from OP swabs. Only three birds tested positive on both cloacal and OP swabs. Prevalence estimates based on these results are 14% (95% confidence limits [CL]=6.8–20.38) for cloacal swabs only, 8% for OP swabs only (95% CL=2.8–13.9%), and 19% (95% CL= 10.9–26.6%) for the combined cloacal and OP results. However, differences between OP and cloacal swab results were not statistically significant (McNemar's test, $P=0.4533$). The subtypes for the three viruses recovered from both cloacal and OP swabs from individual birds were identical for each positive bird.

Overall, the utilization of mRT-PCR resulted in increased detection of influenza A viruses in these samples; however, this increased sensitivity was dependent on the cycle threshold (ct) value (Table 5). This variation affected prevalence estimates as follows: 73/350 (20.9% [95% CL, 16.6–25.1%]) positive based on mRT-PCR (all ct values ≤45), 49/350 (14.0%, [95% CL, 10.4–17.6%]) positive based on mRT-PCR (all ct values <40), 20/350 (5.7% [95% CL, 3.3–8.1%]) positive by virus isolation. There was only one virus isolation positive/mRT-PCR negative sample detected from these 350 samples.

Serologic testing

Antibody prevalence estimates for Ruddy Turnstones, Red Knots, and Sanderlings

TABLE 3. Diversity of subtypes of influenza A virus detected through fecal and cloacal swab sampling of shorebirds during an overlapping 1-wk period at Delaware Bay, USA, 2000–09.

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Week of sampling	15–21 May	15–21 May	15–21 May	15–21 May	15–21 May	15–21 May	22–28 May	22–28 May	15–21 May	15–21 May
Subtypes detected[a]	**H12N5 (20)**[b]	**H10N7 (45)**	**H1N9 (45)**	**H9N2 (12)**	**H10N7 (18)**	**H3N6 (24)**	**H7N3 (48)**	**H12N5 (26)**	**H4N6 (31)**	**H10N7 (32)**
	H12N4 (13)	**H12N7 (15)**	**H11N9 (12)**	**H9N5 (12)**	H6N8 (10)	**H3N8 (19)**	**H9N2 (5)**	H12N4 (4)	**H12N5 (25)**	H10N1 (10)
	H10N7 (8)	H9N2 (4)	**H1N4 (7)**	*H9N9* (6)	**H5N7 (2)**	H11N8 (4)	H1N4 (3)	H7N3 (4)	**H10N7 (13)**	H1N1 (6)
	H10N4 (5)	**H6N2 (3)**	**H9N9 (7)**	H9N1 (4)	**H5N8 (2)**	**H11N6 (2)**	**H7N4 (3)**	H5N4 (2)	H6N8 (3)	H6N1 (4)
	H5N4 (5)	H12N9 (3)	**H11N4 (7)**	*H9N8* (4)	H11N9 (2)		*H7N7* (3)	**H12N1 (2)**	**H3N2 (2)**	H11N1 (3)
	H6N4 (3)	H9N7 (2)	H7N3 (3)	H9N4 (3)			H16N3 (3)	H4N9 (1)	*H4N7* (1)	**H1N8 (3)**
	H7N9 (2)	H11N2 (2)	**H6N4 (2)**	*H5N9* (1)			H6N4 (2)	H5N1(1)	H4N8 (1)	H1N7 (2)
	H5N3 (1)	H12N2 (2)	*H11N2* (2)	*H6N8* (1)			H13N9 (2)	H5N9 (1)	H6N2 (1)	H11N4 (2)
	H9N7 (1)	H13N6 (1)	H9N4 (1)	*H9N7* (1)			H6N2 (1)	H6N2 (1)	*H10N6* (1)	*H11N9* (2)
	H11N6(1)		H1N5 (1)	H11N9 (1)			*H6N7* (1)	H6N4 (1)	H10N8 (1)	H1N9 (1)
	H13N6 (1)		H6N9 (1)	*H12N5* (1)			H6N8 (1)	H6N5 (1)	H10N9 (1)	H3N1 (1)
				H12N9 (1)			H7N5 (1)	H9N9 (1)	*H11N9* (1)	H8N4 (1)
								H11N9 (1)	H12N3 (1)	H11N8 (1)
								H13N9 (1)	*H13N9* (1)	
								H16N3 (1)	H16N3 (1)	

[a] Subtypes in bold were detected by The University of Georgia (UGA) and St. Jude Children's Research Hospital (SJCRH); normal font = isolated by SJCRH only; italics = isolated by UGA only.

[b] Subtype (number of isolates).

TABLE 4. Hemagglutinin (HA) subtype diversity for influenza A viruses detected in the combined sample during the overlapping 1-wk period at Delaware Bay, USA, 2000–09. Values represent the percentage of total subtyped isolates.

HA subtype	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
H1			***60***				4			*13**
H2										
H3						***88***			*2*	1
H4								2	***39***	
H5	*10*[a]			*2**	*12**			*8**		
H6	*5*	*4*	*3*	*2**	*29**		*7*	*6**	*5**	*6**
H7	3		*3**				***75***	*8**		
H8										1
H9	2	8	*9*	***89***			*7*	*1**		
H10	*22*	***58***			***53***				*19*	***62***
H11	*2**	*3**	*24*	*2*	6	*12**		2	*1**	*12*
H12	***55***[b]	*26*		*4**				***67***	*31*	
H13	2	1					3	2	*1**	
H16							4	2	1	

[a] Italicized values represent HA subtypes detected by St. Jude Children's Research Hospital and The University of Georgia; * indicate HA detected by The University of Georgia in May of that year but outside of the overlapping sampling period.

[b] Bold values represent the predominant HA subtype detected during that year.

at Delaware Bay were 55% (95% CL=47.3–62.7%), 86% (95% CL=76–95%), and 15% (95% CL=4–26%), respectively. Of the 25 birds sampled outside of Delaware Bay only Red Knots (five of nine, 56%, 95% CL=23–88%) had detectable antibodies to influenza A viruses.

DISCUSSION

The epidemiology of influenza A viruses in shorebirds at Delaware Bay is unique in that this is the only known "hotspot" where these viruses are reliably isolated from these species of birds (Krauss et al., 2010). Although data obtained from this system provided an opportunity to evaluate surveillance and diagnostic approaches as applied to shorebirds, the application of these results to global surveillance also needs to consider the possibility of a very low prevalence of infection in these populations.

TABLE 5. Virus isolation and matrix real-time PCR (mRT-PCR) results by cycle threshold (Ct) value for detection of influenza A virus in cloacal swabs collected from Ruddy Turnstones at Delaware Bay, USA, 2007.

Ct value range	No. positive by mRT-PCR	No. of mRT-PCR positive samples found positive by virus isolation (% positive)
25–29.9	11	9 (82)
30–34.9	18	4 (22)
35–39.9	20	6 (30)
40–44.9	24	0 (0)
All	73	19 (26)

Our results from shorebirds at Delaware Bay indicate that virus isolation from cloacal swabs and fecal samples both provide comparable prevalence estimates and trends over time. Overall, there were few instances where prevalence trends for the two sampling approaches were not in agreement, and these were likely related to small sample size during the overlap period where results were compared. Such differences also may have resulted from spatial/temporal variation as there was no attempt to standardize collection to specific beach locations or sampling times within the week of data overlap. Although differences in prevalence estimates derived from the three sampling approaches (all birds, Ruddy Turnstones, and fecal sampling) were relatively minor and statistically insignificant during most years,

differences as great as 12% could be attributed to sampling methodology (2008; Table 1). Such sampling-related variation needs to be considered when utilizing published data in meta-analyses or when comparing prevalence estimates from independent studies.

The three sampling approaches all were effective at capturing annual subtype diversity present in the population. At Delaware Bay, annual subtype diversity appears to be dominated by a single HA type each year that accounts for 35–90% of the total HA diversity (Table 4). The dominant HA subtype usually is represented by multiple HA/NA subtypes suggesting that these viruses undergo extensive genetic reassortment. Most of the missed HA/NA subtypes (either by UGA or SJCRH) were viruses representing low frequency HA subtypes or low frequency HA/NA subtypes that probably represented recent reassortment viruses. The extensive collection of viruses and data made available for this study gave a unique opportunity to clearly demonstrate that subtype diversity will almost always be underestimated in field studies, even those with a significant sample size and a high rate of virus recovery. As previously reported (Krauss et al., 2004), specific HA/NA combinations recovered from shorebirds and gulls often represent a very small proportion of the total viruses recovered. A similar result was recently demonstrated in duck populations in Minnesota, USA, where intensive surveillance over a 3-mo period that encompassed the entire staging and early migration periods during 2 yr resulted in isolation of most of the HA subtypes that are present in North America each year (Wilcox et al., 2011). In that study, which included results from more than 600 viruses, subtype-specific prevalence estimates ranged from 0.04% to 4.6%.

The predominant route of virus shedding of type A influenza viruses in birds is variable depending on host species (Hoye et al., 2010; Costa et al., 2011) and virus; there are consistent reports that highly pathogenic H5N1 influenza viruses are predominantly associated with OP rather than cloacal shedding (Sturm-Ramirez et al., 2004; Brown et al., 2006), whereas wild bird viruses in ducks are predominantly shed via the cloaca (Webster et al., 1978). Although recently evaluated in ducks (Parmley et al., 2011), there is little or no available information related to the detection of these viruses in cloacal and OP swabs of shorebirds. Although not statistically significant, our results with Ruddy Turnstones are similar to previous reports from Mallards (*Anas platyrhynchos*), in that more virus isolations were derived from cloacal swabs than from OP swabs (Munster et al., 2009). With ducks, detection success can be improved by combined OP/cloacal sampling (Parmley et al., 2011) and, based on the combined OP/cloacal results from our Ruddy Turnstone sample, prevalence estimates increased from 14% (cloacal swab results) to 19% (combined cloacal/OP results). Although this 5% increase is modest in relation to the prevalence estimates, it does represent a 36% error. This is another factor that needs to be considered when comparing results or utilizing data from independent studies. Due to potential species-related differences in shedding patterns (Hoye et al., 2010), this type of error may be species-dependent.

The virus isolation and mRT-PCR protocols that were compared in this study represent the predominant influenza virus detection approaches in use. It is well established that sensitivity can be enhanced with mRT-PCR, and this test is often used as a screening test for subsequent virus isolation attempts (Munster et al., 2009). The relationship between improved virus isolation success and lower ct values observed in our study is not surprising but underscores the need to clearly define positive threshold values and to use caution in the interpretation of positive results. For example, if we consider a ct value of <40 as the positive

threshold, prevalence estimates for our Ruddy Turnstone sample based on mRT-PCR (14%) would have been significantly higher than prevalence estimates based on virus isolation (5.7%; chi-square with Yates' correction, $\chi^2=12.61$, df=1, $P<0.0004$). Of the 24 samples testing positive by mRT-PCR at ct value of ≥40, no viruses were isolated. This failure probably relates to a very low viral titers (<10 infectious virions; Stallknecht et al., 2010) or possibly nonspecific results. The failure to isolate viruses from samples with ct values <40 is difficult to explain but may relate to the loss of infectivity related to the single freeze-thaw that is part of sample storage and processing, samples containing RNA and noninfective virus, or an insufficient quantity of virus to infect eggs. Regardless of cause, comparisons of prevalence estimates from independent studies based on mRT-PCR and virus isolation may prove difficult and this potential source of variation needs to be considered. That shortcoming aside, the utility of mRT-PCR as a screening tool is supported by our results, and if we had taken this approach, we would have missed only one of 20 (5%) of the viruses recovered in virus isolation attempts. Overall, we isolated virus from 19 of the 49 (39%) mRT-PCR-positive (<40 ct) samples, which is comparable to the 33.5% isolation rate reported for mRT-PCR-positive birds (primarily ducks and geese) sampled in Europe (Munster et al., 2007).

Serologic testing as currently applied to wild bird surveillance is underutilized, especially with shorebirds. Our results further demonstrate the utility of this approach as applied to shorebirds in two ways. First, results from individual populations can be replicated and reflect consistent trends between years. Reported antibody prevalence estimates (combined 2007–08 data) for Ruddy Turnstones, Red Knots, and Sanderlings sampled at Delaware Bay during May were 65%, 54%, and 3%, respectively (Brown et al., 2010). Although antibody prevalence was higher for all species tested during 2010 (Ruddy Turnstone, 55%; Red Knot, 86%; Sanderling, 15%), results are consistent with high prevalence in Ruddy Turnstones and Red Knots and lower prevalence in Sanderlings. Secondly, although our winter testing was minimal, serologic testing did identify Red Knots as antibody-positive prior to their arrival at Delaware Bay. This type of information has application to both identifying species that are commonly infected with influenza viruses (in the absence of direct virologic evidence) and in interpretation of virus detection results. Although antibody prevalence may underestimate true infection rates within the population (Hoye et al., 2011), with improved diagnostics this approach may lead to a much needed understanding of population immunity within wild bird populations.

As previously stated, sampling and testing protocols associated with field research or surveillance need to be selected in relation to surveillance objectives and expected prevalence in the target population, which may be very low in shorebird populations, especially outside of Delaware Bay. Based on our work at Delaware Bay, sampling of avian communities (direct and general sampling of all birds), targeted surveillance (direct sampling of Ruddy Turnstones), or indirect sampling (sampling of feces) for virus detection and serologic testing all can be effectively applied (individually or in combination) to meet specific objectives under the varied logistic constraints expected in the field and support laboratory. The primary disadvantage of sampling avian communities relates to capture efforts but, as with Delaware Bay, this may be offset if coordinated with existing biologic studies or banding efforts. A second disadvantage relates to scale. Avian assemblages often involve numerous species using unique habitat components, and this can make capture efforts costly and challenging. Advantages include the ability to collect both cloacal and OP swabs,

plasma or serum for supportive serologic testing, complete biologic data on sampled birds, and data and isolates from interacting species at the community level. This can be especially important for understanding results in situations where shorebirds share habitats and potentially influenza A viruses with waterfowl. Although challenging, this type of study is necessary to fully understand the natural history of these viruses.

Surveillance targeted to specific species requires a priori species-related information. This approach has the same advantages and limitations as community-based approaches, but as demonstrated in this study, can improve efficiency. It is ideally suited for long-term studies where precise prevalence estimates related to infection or immunity are needed or when efficient virus recovery is desired.

Although fecal sampling will provide less information related to the host population, it provides a very efficient approach for virus recovery and lower but reliable prevalence estimates, and does not require bird capture. This approach is ideally suited for testing species and populations of unknown status and the recent development of techniques to determine species from these fecal samples (Lee et al., 2010) has greatly increased its utility. With a priori information, as exists with Delaware Bay, this approach can be utilized at the community or individual species level. Although the most cost-efficient approach, the major disadvantage relates to the inability to collect relevant biologic data such as age, condition, or data retrieved from band recoveries.

As for testing, our results as applied to shorebirds support the use of mRT-PCR as an effective screening test; however, results should be confirmed by virus isolation when possible. This is important not only to provide subtype-specific data and field isolates for genetic or phenotypic characterization, but also in cases of low detected prevalence, to confirm positive results. When sampling birds, the collection of combined cloacal and OP swabs to enhance virus recovery and serum or plasma for type-specific antibody testing should also be considered.

All of these surveillance and testing approaches have the potential to yield useful data related to understanding the natural history of influenza A viruses, but as recently reported by Hoye et al. (2010), success is highly dependent on appropriate sample size, especially when prevalence is low. Our results from Delaware Bay add another sample size consideration related to subtype detection; the prevalence of specific subtypes can be extremely low, and because of this, subtypes can be easily missed. As occurs in ducks (Stallknecht and Brown, 2008), prevalence estimates for avian influenza in shorebirds are highly dependent on species and temporal/spatial variables (Hanson et al., 2008; Krauss et al, 2010). Our results demonstrate that sampling and testing techniques as applied to shorebirds also affect prevalence estimates. These inherent biases do not negate the value of these data and cannot always be corrected due to sampling and testing constraints, but need to be considered when evaluating data from comprehensive data bases or published independent studies.

ACKNOWLEDGMENTS

We gratefully acknowledge the field and lab efforts of K. Jones, B. Hanson, A. Maxted, J. Smith, and B. Wilcox. Funding for this work was provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contracts HHSN266200700005C, HHSN266200700007C, and N01-AI95357; Cancer Center Support grant CA-21765; the American Lebanese Syrian Associated Charities and Specific Cooperative Agreement 58-6612-2-0220 (Agricultural Research Service, US Department of Agriculture). The opinions expressed herein are those of the authors and do not necessarily reflect the views of any of the funding agencies.

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Submitted for publication 30 June 2011.
Accepted 2 November 2011.