AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

How Does Sampling Methodology Influence Molecular Detection and Isolation Success in Influenza A Virus Field Studies?

Neus Latorre-Margalef,[a]* Alexis Avril,[a] Conny Tolf,[a] Björn Olsen,[b,c] Jonas Waldenström[a]

Centre for Ecology and Evolution in Microbial Model Systems (EEMiS), Faculty of Health and Life Sciences, Linnaeus University, Kalmar, Sweden[a]; Section of Infectious Diseases, Department of Medical Sciences, Uppsala University, Uppsala, Sweden[b]; Zoonosis Science Center, IMBIM, Uppsala University, Uppsala, Sweden[c]

Wild waterfowl are important reservoir hosts for influenza A virus (IAV) and a potential source of spillover infections in other hosts, including poultry and swine. The emergence of highly pathogenic avian influenza (HPAI) viruses, such as H5N1 and H5N8, and subsequent spread along migratory flyways prompted the initiation of several programs in Europe, North America, and Africa to monitor circulation of HPAI and low-pathogenicity precursor viruses (low-pathogenicity avian influenza [LPAI] viruses). Given the costs of maintaining such programs, it is essential to establish best practice for field methodologies to provide robust data for epidemiological interpretation. Here, we use long-term surveillance data from a single site to evaluate the influence of a number of parameters on virus detection and isolation of LPAI viruses. A total of 26,586 samples (oropharyngeal, fecal, and cloacal) collected from wild mallards were screened by real-time PCR, and positive samples were subjected to isolation in embryonated chicken eggs. The LPAI virus detection rate was influenced by the sample type: cloacal/fecal samples showed a consistently higher detection rate and lower cycle threshold (C_t) value than oropharyngeal samples. Molecular detection was more sensitive than isolation, and virus isolation success was proportional to the number of RNA copies in the sample. Interestingly, for a given C_t value, the isolation success was lower in samples from adult birds than in those from juveniles. Comparing the results of specific real-time reverse transcriptase (RRT)-PCRs and of isolation, it was clear that coinfections were common in the investigated birds. The effects of sample type and detection methods warrant some caution in interpretation of the surveillance data.

The number of studies focusing on the role of wild birds as reservoir species for influenza A virus (IAV) has increased dramatically over the last 10 years (1). This increase was to a large extent caused by the emergence of a highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype in Southeast Asia in 1999 (2). This particular virus causes high mortality in domestic poultry and can be transmitted among wild birds, particularly waterfowl. It rapidly reached a large spatial distribution in Asia, Europe, and Africa in 2006 and has remained endemic in parts of this range. Several surveillance programs were initiated in response to the H5N1 spread (3), but standardized methods were not implemented everywhere. In addition, the emergence in Southeast Asia of other IAV subtypes in poultry and in humans, like H5N1 and H7N9, and the recent spread of H5N8 into Europe and North America (4, 5), point to the need for efficient and reliable screening methods (6). As IAV is an RNA virus and is sensitive to changes in the physical environment, such as temperature, pH, and salinity (7, 8), sampling and screening strategies need to be evaluated in order to provide best practice.

Traditionally, IAV surveillance has been based on cloacal swabs or fresh droppings (here called fecal samples) (9–11). The choice of cloacal and fecal samples as the basis for investigation was based on the infection patterns of low-pathogenicity avian influenza (LPAI) viruses, which typically infect and replicate in the lower gastrointestinal tract, including the colon, the cecum, and the bursa of Fabricius, and which are shed via feces (12). However, during the HPAI H5N1 epizootic, experimental studies showed that the virus had a higher detection rate in the respiratory tract of birds (3, 13); therefore, oropharyngeal or tracheal sampling was included in several screening programs (14–20). Most studies reported that LPAI detection was higher in cloacal than in oropharyngeal samples and that combined oropharyngeal and cloacal sampling increased overall detection rates.

Since 2002, we have run a long-term surveillance study on wild waterfowl in Northern Europe (21). Over time, the data collected have increased to encompass more than 26,000 samples, screened in the same laboratory by similar methods over the years. Here, we utilize a part of this data set (2002 to 2010) and analyze how biological and seasonal parameters shape the variation in virus detection, the infection intensity, and the likelihood of virus isolation. Moreover, we investigate the degree of coinfections detected by the combination of H5- and H7-specific molecular-screening results and the subtypes in retrieved isolates. We report these findings to improve the design of surveillance studies and the interpretation of data by different methods and to standardize procedures, from collection to analysis.

Received 7 October 2015 **Accepted** 20 November 2015

Accepted manuscript posted online 11 December 2015

Citation Latorre-Margalef N, Avril A, Tolf C, Olsen B, Waldenström J. 2016. How does sampling methodology influence molecular detection and isolation success in influenza A virus field studies? Appl Environ Microbiol 82:1147–1153. doi:10.1128/AEM.03283-15.

Editor: C. A. Elkins , FDA Center for Food Safety and Applied Nutrition

Address correspondence to Neus Latorre-Margalef, nlatorre@uga.edu.

* Present address: Neus Latorre-Margalef, Department of Population Health, College of Veterinary Medicine, Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, Georgia, USA.

This is contribution no. 288 from Ottenby Bird Observatory.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03283-15.

MATERIALS AND METHODS

Sampling methodology. Wild mallard ducks (*Anas platyrhynchos*) were trapped in a live-duck trap at the Ottenby Bird Observatory, located at the southern tip of the island of Öland in the Baltic Sea (56°12′N, 16°24′E). All handling of birds, including trapping, banding, and sampling, was approved by the Swedish authorities in accordance with national legislation (Linköping Animal Research Ethics Board, permit numbers 8-06, 34-06, 80-07, 111-11, and 112-11). The birds were aged and sexed based on plumage and grouped into three categories: juveniles (hatch year birds during fall), adults (post-hatch year birds), and unaged (birds that it was not possible to age with certainty).

The sampling season started in March or April after the ice melted and lasted until November or December, depending on the climatic conditions in different years. Three different sampling methodologies were used: (i) fresh droppings or fecal samples, (ii) cloacal swabs, and (iii) oropharyngeal swabs. For collection of fecal samples, the ducks were placed in single-use cardboard boxes, and the fecal material produced by the duck after a period spent in the box was collected with a sterile swab (22, 23). Individuals that did not defecate in the boxes were gently swabbed in the cloaca (14). Oropharyngeal swabs were collected together with fecal/cloacal samples from a proportion of the birds in 2006, 2008, 2009, and 2010. Individually packed rayon-tipped swabs were used (CP167KS01; Copan, Italy). The swabs were preserved in transport medium (Hanks' balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 μg/ml gentamicin, and 50 U/ml nystatin; Sigma) and stored at −70°C 1 to 4 h after sampling.

Virus detection, isolation, and typing. For molecular detection of IAV RNA, samples were thawed once for RNA extraction and immediately frozen again at −70°C. Screening of the samples was done using RNA extracted on different automated systems and subsequent real-time reverse transcriptase (RRT)-PCR. The screening methods for samples collected from 2002 to 2005 have been previously described (23). RNA extractions were performed either in the M48 robot (Qiagen, Germantown, MD, USA), using the MagAttract Viral RNA M48 kit, for samples collected in the years 2006 to 2009 or in the MagNA Pure 96 instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), using the MagNA Pure Viral NA kit, for samples collected in 2010. Different RRT-PCR assays based on amplification of the IAV matrix gene (18, 23–25) were used. Samples were screened by RRT-PCR assays on either a LightCycler 1.5 (Roche) or a StepOnePlus (Applied BioSystems, New Jersey, USA) as described previously (24) (see Table S1 in the supplemental material for a summary). Subsequent screening of the matrix RRT-PCR-positive samples using H5- and H7-specific RRT-PCR assays (26) were performed from 2005 on. The cleavage site of the hemagglutinin (HA) was sequenced on H5- and H7-positive samples in order to identify markers of pathogenicity before isolation. Cloacal samples confirmed positive by RRT-PCR were thawed a second time and further examined by viral isolation in 11-day-old embryonated chicken eggs, following standard procedures (27). The allantoic fluid was harvested after 2 days and tested by hemagglutination assay to detect viral antigens. Subsequent hemagglutinin (HA) typing of the isolates was done by hemagglutination inhibition assay (HI) (21).

Statistical analysis and comparison between sample type and variation in shedding. The cycle threshold (C_t) values from the matrix RRT-PCR runs were used as an indirect measure of viral shedding. Several methods and instruments were used for the screening of samples during the study period, and the methods tended to show increasing test sensitivity with instrument and kit upgrades (data not shown). Thus, in order to explore variation in the C_t values as a measure of relative virus shedding, we selected a period during which the same analysis method and laboratory equipment were used (the years 2006 to 2009).

We explored the influence of the sample type (oropharyngeal and cloacal swabs and fecal samples), the age of the birds (juveniles, adults, or unaged), and the year and date of sampling on the observed variation in C_t values using linear models. The C_t values were left-skewed distributed and right truncated, as high values above cycle 41 were considered RRT-PCR negative and were not reported in the database. Hence, the response variable "C_t value" was modified to fit normality assumptions following the transformation $\sqrt{-C_t \text{ value} + \max(C_t \text{ value}) + 1}$ (28). The seasonal variation in C_t values was modeled as a first- or second-order polynomial function of the number of days since 1 January each year (here referred to as the Julian day of the year), after having checked for the relevance of higher-power functions through diagnostic plots and residual variation. The durations of sampling periods were highly variable between years (due to variation in migration intensity), which precluded reliable modeling of a seasonal trend in some years. To avoid overfitting the data in those years, we examined first-order polynomial models of the whole data set, i.e., without including the year effect. We looked in detail at the seasonal variation of C_t values on a reduced data set based on the year 2009, as that year showed the longest time series of sampling, which in turn allowed reliable interpretation of seasonal trends. In both analyses, we accounted for repeated sampling of a given individual (some birds were released after sampling and retrapped later in the season) by adding a random intercept for each ring number (unique individual identifiers).

We also evaluated the relationship between C_t values and isolation success using generalized linear models (GLM). In these analyses, the response variable was binomial, describing virus isolation for each observation as either successful (1) or unsuccessful (0). We ran the model on year 2009 data only, because of the sparseness of the data for other years. The sample type and age of birds were also included in the model as explanatory variables. Due to convergence problems, we did not include interactions between variables or the influence of the date of sampling on isolation success.

All analyses were run with the lme4 package for R software (http://www.R-project.org/). Model selections were done using the Akaike information criterion corrected for small sample size (AICc) (29) with the package MuMln.

RESULTS

Screening results for oropharyngeal, fecal, or cloacal sampling. A total of 26,586 samples were tested for detection of viral RNA by RRT-PCR methods during the complete study period (2002 to 2010), of which 22,229 were fecal or cloacal samples and 4,357 were oropharyngeal samples (see Table S2 in the supplemental material). A total of 4,354 sample pairs with both oropharyngeal and fecal/cloacal samples were analyzed (including ~500 samples from a previous publication [14]) to compare detection depending on sample type. Detection was highest in fecal samples (12.4%), followed by oropharyngeal samples (6.4%), and was lowest in cloacal swabs (4.0%); however, only 25% of the samples were cloacal swabs. All positive detections were LPAI viruses, as among the H5- or H7-positive samples, no HPAI viruses were identified. The total prevalence for fecal/cloacal samples was 16.5%, while the corresponding value for oropharyngeal samples was 6.4%. Out of all the sample pairs, 3,507 (80.5%) were negative in both samples, and 153 (3.5%) were positive in both. Of the 691 sample pairs with mixed results, 565 (13.0%) were positive detections for the fecal/cloacal sample and negative for the oropharyngeal sample, while the opposite was true for 126 sample pairs. Consequently, 2.9% of the individuals would have been determined negative if the oropharyngeal swab had not been analyzed. For the 150 sample pairs that were positive for both sample types and where both C_t values were reported, the average C_t value for oropharyngeal samples was 35.65 ± 2.69 (mean ± standard deviation [SD]) cycles compared to 31.07 ± 4.74 cycles for cloacal samples (Wilcoxon's rank test for paired samples, $W = -9.61$; $P < 0.001$).

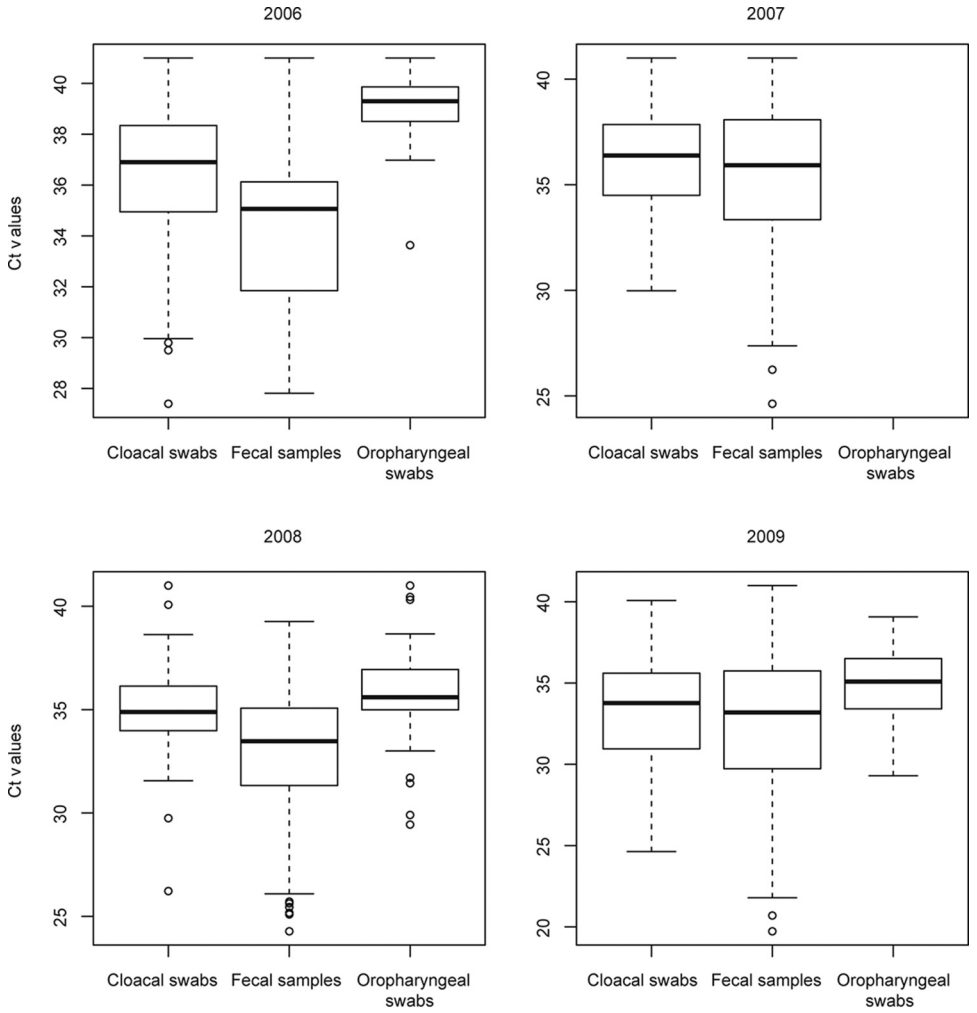

FIG 1 Variation in C_t values according to sample type and year. Box plots indicate the distribution of values, and medians are shown by the bold horizontal lines inside the boxes.

Variation in virus shedding. Overall, variation in C_t values was mainly explained by sample type and year (Fig. 1; see Table S3 in the supplemental material, model 1). On average, C_t values from oropharyngeal samples were higher than C_t values obtained by other sampling methods [mean predictions (95% confidence interval)]: oropharyngeal, 37.16 (36.70 to 37.59); fresh droppings, 34.71 (34.70 to 34.98); cloacal swabs, 35.34 (35.02 to 35.66) (predictions were derived from model 1 in Table S3 in the supplemental material). However, we could not find support for an age or a seasonal effect over all years (model 1 versus model 7, ΔAICc = 28 [see Table S3 in the supplemental material]). This was partly due to unbalanced sample sizes between years and a very short period when birds were captured and screened for AIV in 2006, 2007, and 2008. Therefore, we evaluated the data from year 2009 independently, as there were widespread sampling data to evaluate age and seasonal effects (see Table S4 in the supplemental material). For that year, seasonal variation in C_t values took the form of a quadratic function with season (day of sampling) (Fig. 2; see Table S4 in the supplemental material, model 1). However, the trend in C_t values did not depict clear increase or decrease of C_t values during the season. Average C_t values for juveniles and adults exhibited convex/constant and concave/decreasing trends, respectively, as the season progressed, whatever the sample type. In contrast, C_t values in unaged individuals tended to increase during the season, whatever the sample type. Note that these various trends between age categories were responsible for the age effect retained in model 1, whereas no significant difference in predictions of C_t value could be detected between the three age categories [mean predictions (95% confidence interval)]: juveniles, 33.59 (32.82 to 34.32); adults, 33.93 (33.02 to 34.80); unaged, 31.95 (31.57 to 32.32) (predictions were derived from model 1 in Table S4 in the supplemental material).

Regarding virus isolation, we found a significant ($P < 0.001$) negative relationship between isolation success and C_t values (slope = -0.167 ± 0.031; Wald score [z] = -5.36). Isolation success varied according to sample type, C_t value, and age in an additive way, as visualized in Fig. 3 (based on model 1 in Table S5 in the supplemental material). Low C_t values (a high number of initial RNA copies) had greater isolation success than higher C_t values. Additionally, the isolation success was greater in fecal samples than in cloacal swabs (intercept difference = $+0.70 \pm 0.031$; $z = 2.05$; $P = 0.04$) (Fig. 3) in such a way that samples from adults had less

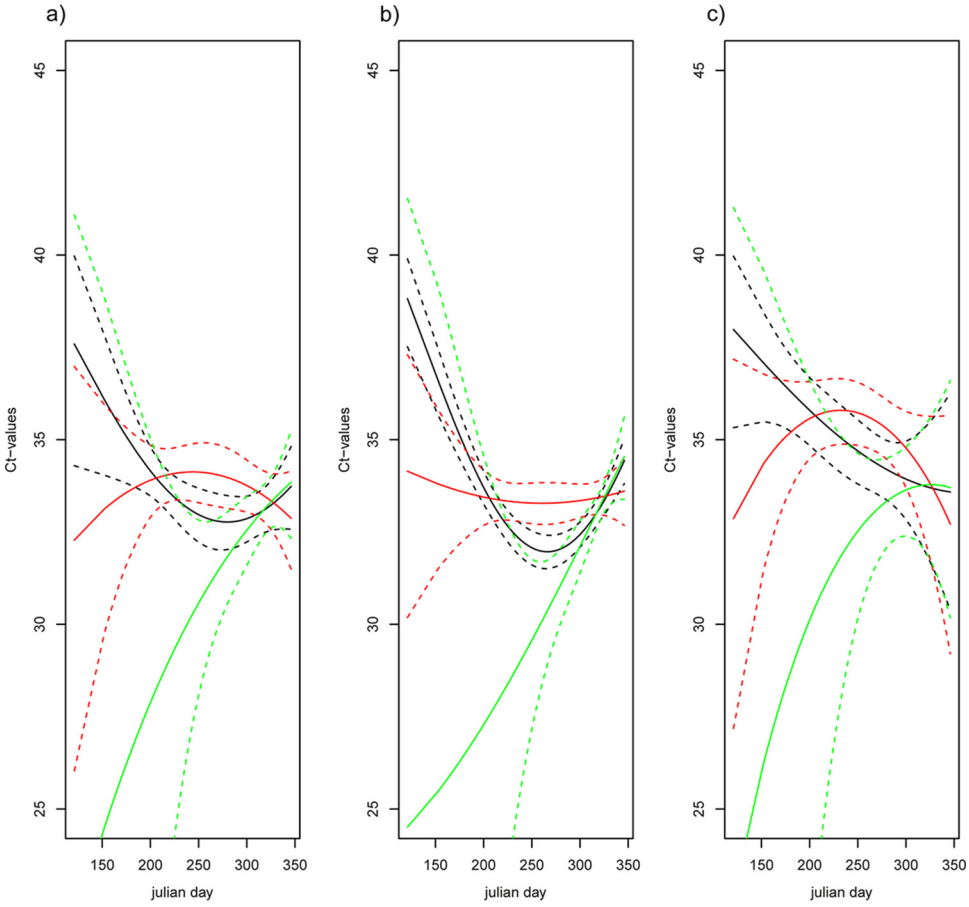

FIG 2 Seasonal variation in C_t values according to sample type and age of birds in 2009. Shown are mean predictions (solid lines) ± standard errors (SE) (dashed lines) derived from the best models presented in Table S4 in the supplemental material. Age classes are color coded black for juvenile birds, red for adults, and green for unaged birds. (a) Cloacal swabs. (b) Fecal samples. (c) Oropharyngeal swabs.

isolation success than those collected from juveniles or unaged individuals. For instance, the isolation success from RRT-PCR-positive cloacal swabs with a C_t value of 25 (mean predictions and 95% confidence interval) was 31% (12 to 51) for adults, 54% (32 to 76) for unaged, and 42% (23 to 62) for juveniles, while for fecal samples, the isolation success was 48% (32 to 65) for adults, 70% (56 to 84) for unaged, and 60% (46 to 73) for juveniles.

Detection of coinfections. By comparing the results of H5- and H7-specific RRT-PCRs (26) with the HA subtypes from isolated viruses, we could identify putative coinfections. For H7 RRT-PCR-positive samples, we detected 3 coinfections, with H1N6, H4N6, and H10N7. Moreover, an H7N2 virus was isolated from a sample that was negative in the H7 screening. Of 67 samples positive for H5 by RRT-PCR and where a virus was later successfully propagated, only 43% resulted in an H5 virus isolate. Of the remaining 38 samples, coinfections by H5 (as detected by RRT-PCR) with the H1, H2, H3, H4, H6, H10, or H11 subtype were found. This indicates that 57% of the H5-positive samples represented coinfections. We examined whether the frequency of HA variants in coinfections with H5 viruses followed their abundance in the viral population or if specific pairs of HA subtypes would be present in coinfections, as specific associations were overrepresented in an early study (30). To do this, we constructed a contingency table with two columns, one for the frequency of non-H5 subtypes isolated from H5 RRT-PCR-positive samples and one for the frequency of non-H5 subtypes isolated from H5 RRT-PCR-negative samples (Table 1). The null hypothesis that the two frequency distributions were similar could not be rejected ($\chi^2 = 7.089$; df = 6; $P = 0.312$), and the interpretation is that the frequency of coinfections followed the abundance of isolation of these subtypes in the population.

DISCUSSION

We used long-term IAV data from a well-studied population of mallards (21) to investigate how methodology and host factors affect the likelihood of retrieving IAV-positive samples by RRT-PCR and isolation. The sampling scheme at Ottenby Bird Observatory was initiated in 2002 and follows a standardized methodology, which allowed an evaluation of the long-term data. Trained ornithologists participated in the field sampling, and the workflow from sampling to laboratory analysis had been optimized to keep a cold chain (samples were stored and transported frozen until analysis).

Examination of molecular screening in this study showed that detection frequencies varied with the sampling methodology, where the number of RRT-PCR-positive detections in oropharyngeal swabs was lower and they contained a smaller amount of viral RNA than cloacal swabs or fecal samples taken from the same

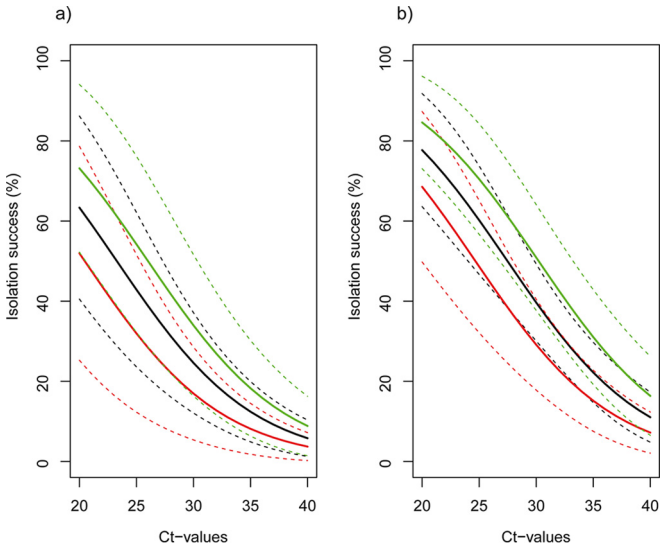

FIG 3 Isolation success according to C_t values, sample type, and age of birds in 2009. Shown are mean predictions (solid lines) ± SE (dashed lines) derived from the best models presented in Table S5 in the supplemental material. The black lines represent juvenile birds, the red lines represent adults, and the green lines represent unaged birds. Isolation was performed only for cloacal swabs and fecal samples (droppings). (a) Cloacal swabs. (b) Fecal samples.

individuals. These findings corroborate earlier studies (7, 14), where a combination of cloacal and oropharyngeal samples resulted in the highest total IAV detection (14–20). Fecal samples, on average, contained a larger amount of viral RNA than cloacal swabs, possibly as a consequence of the fact that fecal samples contained more material from the beginning. A question that still needs attention is whether positive detection in oropharyngeal samples proves actual replication, as a recent study in mallards found no evidence for replication of LPAI virus in the oropharyngeal tract even if there was a positive detection by molecular techniques in the birds (31). Nevertheless, different hosts (i.e., taxonomic groups) may vary in predominant replication sites based on differential expression of viral receptors, and at the same time, different virus strains can vary in tissue tropism, like the HPAI H5N1virus, which replicates primarily in the respiratory tract (12, 13).

The isolation success of IAV matrix RRT-PCR-positive samples varied with sample type and C_t value (i.e., the amount of viral RNA template in the sample or the viral load), where cloacal samples with low C_t values (indicative of larger amounts of viral RNA) had the highest likelihood of being isolated. A surprising result was the effect of bird age on isolation, where similar C_t values from the different age categories had differences in isolation success. Isolation success in samples from adults with comparable viral loads was approximately 10 to 20% lower than in juvenile or unaged individuals for all sample types. The viral load is usually correlated with transmission; however, if isolation success is used as a proxy for infectivity, it means that infected adults have a lower transmission potential than younger individuals in the population. These findings are important for our interpretation of positive results based on molecular screening and their significance in terms of infectivity (32), as they could be interpreted as adults being less infectious than juveniles. These results strengthen the view that juvenile birds are important drivers of IAV dynamics, as per capita transmission would be higher for infections in this age category, since the amount of active viruses is larger and infections last longer (33) than in adults. The observed differences in isolation and in shedding of infectious particles suggest that mechanisms like the development of mucosal immunity and production of cross-reactive neutralizing antibodies due to previous IAV exposure in adults influence infectivity. Thus, the clear reduction in shedding of infectious particles in adults likely impacts transmission parameters in a population.

During the fall migration, IAV prevalence and subtype diversity were high at the sampling site (21). Consequently, coinfections with two or more strains are expected to be common in naturally infected waterfowl (17, 30, 34–36). The detection of coinfections described above results from endpoint data for H5 and H7 RRT-PCR-positive samples compared to virus propagation results in eggs. Of the H5-positive samples, 57% were coinfections with other subtypes. It is likely that many virus coinfections have been missed, either by chance during isolation, through a higher likelihood of retrieving the most abundant virus in the sample, or due to propagation differences in standard isolation methods based on egg or cell cultures. While different approaches have been used to detect potential coinfections (by isolation [34, 35] or by PCR [35]), all are labor-intensive. The increasing use of sequencing-based techniques may help to overcome some of these earlier limitations (by amplifying different gene copies in a sample). However, the segmented genome of IAV makes the reconstruction of parental virus gene constellations a challenge in mixed samples.

H5 RRT-PCR-positive samples were quite frequent in our study, and the viruses isolated from those samples were related to the frequencies of the HA subtypes in the population. Sharp and colleagues observed patterns of coinfection indicating that the HA/neuraminidase (NA) subtypes involved in coinfections were not random (30), which in turn suggests reassortment may not be random, either. Other studies showed high frequency of reassortment but little segment association in avian isolates obtained from wild waterfowl (34) or from sentinel ducks (37). Further studies are needed to identify coinfections and to increase our understanding of reassortment processes and their crucial role in virus evolution.

How can IAV field sampling and data interpretation be improved? Combining the knowledge from earlier studies and the present study, we make the following recommendations. Storage conditions are decisive to successfully retrieve infectious virus particles, since IAV is sensitive to high temperatures (7), and any mistreatment of the samples, like freeze and thaw cycles, will com-

TABLE 1 Contingency table for H5 coinfections with other subtypes[a]

HA isolate	H5 PCR (no. of samples)	
	Positive	Negative
H1	1	44
H2	2	24
H3	2	28
H4	18	111
H6	5	26
H10	2	12
H11	8	44

[a] Based on results from specific H5 RRT-PCR and HI typing of the resulting virus isolates.

promise further analysis (6). Moreover, due to the problems in amplifying sequences from original samples and the segmented nature of the genome, obtaining virus isolates is essential for full characterization of viruses. Samples should ideally be frozen at the field site (using liquid nitrogen tanks or ultra-low-temperature freezers) or, if the necessary facilities are lacking, kept at +4°C for a limited time until they are processed in the laboratory. Virus transport media, such as Hanks' balanced salt solution supplemented with protein or brain heart infusion (BHI) broth, increase the stability of the viruses (7, 38) and are a preferred choice over phosphate-buffered saline (PBS) or dry swabs. The transport medium should be supplemented with antibiotics to avoid bacterial contamination during isolation. The swab material also influences the recovery of IAV, and flocked swabs are better than nonflocked swabs (38). Fixation of samples in ethanol (35) and sampling using FTA cards (22) are other preservation methods suitable for IAV surveillance and show advantages for biosafety and transportation, but analysis is limited to molecular screening and sensitivity between methods may vary.

The combination of cloacal and oropharyngeal samples, optimally in separate tubes, is recommended to maximize virus detection; however, this needs to be balanced with cost issues and animal health issues, and if only a single sample type can be taken, cloacal samples are preferred for detection of LPAI virus. However, if the screening is intended to maximize the chances of HPAI virus detection, both types of swabs should be collected, as these viruses show higher tropism to the respiratory tract.

When implementing surveillance studies, it is important that metadata (host species and age, sample type and age, location, date, etc.) be collected in parallel with the sampling. The clear reduction in shedding of infectious particles observed in adults, which likely impacts transmission parameters in a population, is a good example of the relevance of using metadata for epidemiological investigations. Therefore, age ought to be reported when possible and included in models of IAV transmission in wild hosts or in risk assessment for HPAI virus spread in the wild.

ACKNOWLEDGMENTS

We thank the staff at Ottenby Bird Observatory, who trapped and sampled all the mallards in this study, and also all laboratory staff for technical assistance over the years.

This study was supported by grants from The Swedish Environmental Protection Agency (V-124-01 and V-98-04), the Swedish Research Council (2008-58, 2010-3067, and 2011-48), the Swedish Research Council Formas (2007-297 and 2009-1220), and the Sparbanksstiftelsen Kronan. The surveillance at Ottenby was part of the European Union wild-bird surveillance and has received support from the Swedish Board of Agriculture and from the EU NP6-funded New Flubird project.

REFERENCES

1. **Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus ADME, Fouchier RAM.** 2006. Global patterns of influenza A virus in wild birds. Science **312:**384–388. http://dx.doi.org/10.1126/science.1122438.
2. **Webster RG, Peiris M, Chen H, Guan Y.** 2006. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis **12:**3–8. http://dx.doi.org/10.3201/eid1201.051024.
3. **Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TSP, Chen H, Ellis TM, Guan Y, Peiris JSM, Webster RG.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci U S A **102:**10682–10687. http://dx.doi.org/10.1073/pnas.0504662102.
4. **Lee DH, Torchetti MK, Winker K, Ip HS, Song CS, Swayne DE.** 2015. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J Virol **89:**6521–6524. http://dx.doi.org/10.1128/JVI.00728-15.
5. **Verhagen JH, van der Jeugd HP, Nolet BA, Slaterus R, Kharitonov SP, de Vries PP, Vuong O, Majoor F, Kuiken T, Fouchier RA.** 2015. Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways. Euro Surveill **20:**21069. http://dx.doi.org/10.2807/1560-7917.ES2015.20.12.21069.
6. **Eisfeld AJ, Neumann G, Kawaoka Y.** 2014. Influenza A virus isolation, culture and identification. Nat Protoc **9:**2663–2681. http://dx.doi.org/10.1038/nprot.2014.180.
7. **Munster VJ, Baas C, Lexmond P, Bestebroer TM, Guldemeester J, Beyer WEP, de Wit E, Schutten M, Rimmelzwaan GF, Osterhaus A, Fouchier RAM.** 2009. Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. J Clin Microbiol **47:**666–673. http://dx.doi.org/10.1128/JCM.01625-08.
8. **Stallknecht DE, Shane SM, Kearney MT, Zwank PJ.** 1990. Persistence of avian influenza-viruses in water. Avian Dis **34:**406–411. http://dx.doi.org/10.2307/1591428.
9. **Easterday BC, Trainer DO, Tumova B, Pereira HG.** 1968. Evidence of infection with influenza viruses in migratory waterfowl. Nature **219:**523. http://dx.doi.org/10.1038/219523a0.
10. **Slemons RD, Easterday BC.** 1978. Virus-replication in digestive-tract of ducks exposed by aerosol to type-A influenza. Avian Dis **22:**367–377. http://dx.doi.org/10.2307/1589291.
11. **Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG.** 1978. Intestinal influenza: replication and characterization of influenza-viruses in ducks. Virology **84:**268–278. http://dx.doi.org/10.1016/0042-6822(78)90247-7.
12. **Ito T, Suzuki Y, Suzuki T, Takada A, Horimoto T, Wells K, Kida H, Otsuki K, Kiso M, Ishida H, Kawaoka Y.** 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J Virol **74:**9300–9305. http://dx.doi.org/10.1128/JVI.74.19.9300-9305.2000.
13. **Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TSP, Chen H, Ellis TM, Guan Y, Peiris JSM, Webster RG.** 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol **79:**11269–11279. http://dx.doi.org/10.1128/JVI.79.17.11269-11279.2005.
14. **Ellstrom P, Latorre-Margalef N, Griekspoor P, Waldenstrom J, Olofsson J, Wahlgren J, Olsen B.** 2008. Sampling for low-pathogenic avian influenza A virus in wild mallard ducks: oropharyngeal versus cloacal swabbing. Vaccine **26:**4414–4416. http://dx.doi.org/10.1016/j.vaccine.2008.06.027.
15. **Gaidet N, Caron A, Cappelle J, Cumming GS, Balança G, Hammoumi S, Cattoli G, Abolnik C, Servan de Almeida R, Gil P, Fereidouni SR, Grosbois V, Tran A, Mundava J, Fofana B, Ould El Mamy AB, Ndlovu M, Mondain-Monval JY, Triplet P, Hagemeijer W, Karesh WB, Newman SH, Dodman T.** 2012. Understanding the ecological drivers of avian influenza virus infection in wildfowl: a continental-scale study across Africa. Proc Biol Sci **279:**1131–1141. http://dx.doi.org/10.1098/rspb.2011.1417.
16. **Ip HS, Dusek RJ, Heisey DM.** 2012. The effect of swab sample choice on the detection of avian influenza in apparently healthy wild ducks. Avian Dis **56:**114–119. http://dx.doi.org/10.1637/9832-061311-Reg.1.
17. **Krauss S, Pryor SP, Raven G, Danner A, Kayali G, Webby RJ, Webster RG.** 2013. Respiratory tract versus cloacal sampling of migratory ducks for influenza A viruses: are both ends relevant? Influenza Other Respir Viruses **7:**93–96. http://dx.doi.org/10.1111/j.1750-2659.2012.00359.x.
18. **Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WEP, Schutten M, Olsen B, Osterhaus ADME, Fouchier RAM.** 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog **3:**e61. http://dx.doi.org/10.1371/journal.ppat.0030061.
19. **Parmley EJ, Soos C, Breault A, Fortin M, Jenkins E, Kibenge F, King R, McAloney K, Pasick J, Pryor SP, Robinson J, Rodrigue J, Leighton FA.** 2011. Detection of low pathogenic avian influenza viruses in wild ducks from Canada: comparison of two sampling methods. J Wildl Dis **47:**466–470. http://dx.doi.org/10.7589/0090-3558-47.2.466.
20. **Jindal N, de Abin M, Primus AE, Raju S, Chander Y, Redig PT, Goyal**

SM. 2010. Comparison of cloacal and oropharyngeal samples for the detection of avian influenza virus in wild birds. Avian Dis **54:**115–119. http://dx.doi.org/10.1637/9032-082109-Reg.1.

21. **Latorre-Margalef N, Tolf C, Grosbois V, Avril A, Bengtsson D, Wille M, Osterhaus A, Fouchier RAM, Olsen B, Waldenstrom J.** 2014. Long-term variation in influenza A virus prevalence and subtype diversity in migratory mallards in northern Europe. Proc Biol Sci **281:**20140098. http://dx.doi.org/10.1098/rspb.2014.0098.
22. **Kraus RHS, van Hooft P, Waldenstrom J, Latorre-Margalef N, Ydenberg RC, Prins HHT.** 2011. Avian influenza surveillance with FTA cards: field methods, biosafety, and transportation issues solved. J Vis Exp **54:**2832. http://dx.doi.org/10.3791/2832.
23. **Wallensten A, Munster VJ, Latorre-Margalef N, Brytting M, Elmberg J, Fouchier RAM, Fransson T, Haemig PD, Karlsson M, Lundkvist A, Osterhaus ADME, Stervander M, Waldenstrom J, Olsen B.** 2007. Surveillance of influenza A virus in migratory waterfowl in northern Europe. Emerg Infect Dis **13:**404–411. http://dx.doi.org/10.3201/eid1303.061130.
24. **Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol **40:**3256–3260. http://dx.doi.org/10.1128/JCM.40.9.3256-3260.2002.
25. **Karlsson M, Wallensten A, Lundkvist A, Olsen B, Brytting M.** 2007. A real-time PCR assay for the monitoring of influenza a virus in wild birds. J Virol Methods **144:**27–31. http://dx.doi.org/10.1016/j.jviromet.2007.03.013.
26. **Veterinary Laboratories Agency.** 3 September 2007. One-step RT PCR for detection of H5 & H7 avian influenza & cleavage site sequencing. http://www.flu-lab-net.eu/docs/pub-protocol-ai-vi545.pdf. Accessed 28 September 2015.
27. **WHO.** 2005. Manual on animal influenza diagnosis and surveillance. WHO, Geneva, Switzerland. http://www.wpro.who.int/emerging_diseases/documents/docs/manualonanimalaidiagnosisandsurveillance.pdf. Accessed 28 September 2015.
28. **Osborne J.** 2002. Notes on the use of data transformations. Practical Assessment Res Eval **8**(6)**:**11.
29. **Kenneth P, Burnham DRA.** 2002. Model selection and multimodel inference: a practical information-theoretic approach, 2nd ed. Springer Science + Business Media. Inc., Berlin, Germany.
30. **Sharp GB, Kawaoka Y, Jones DJ, Bean WJ, Pryor SP, Hinshaw V, Webster RG.** 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. J Virol **71:**6128–6135.
31. **Wille M, van Run P, Waldenstrom J, Kuiken T.** 2014. Infected or not: are PCR-positive oropharyngeal swabs indicative of low pathogenic influenza A virus infection in the respiratory tract of mallard Anas platyrhynchos? Vet Res **45:**53. http://dx.doi.org/10.1186/1297-9716-45-53.
32. **Brown JD, Poulson R, Carter DL, Lebarbenchon C, Stallknecht DE.** 2013. Infectivity of avian influenza virus-positive field samples for mallards: what do our diagnostic results mean? J Wildl Dis **49:**180–185. http://dx.doi.org/10.7589/2011-11-322.
33. **Costa TP, Brown JD, Howerth EW, Stallknecht DE.** 2010. The effect of age on avian influenza viral shedding in mallards (Anas platyrhynchos). Avian Dis **54:**581–585. http://dx.doi.org/10.1637/8692-031309-ResNote.1.
34. **Dugan VG, Chen R, Spiro DJ, Sengamalay N, Zaborsky J, Ghedin E, Nolting J, Swayne DE, Runstadler JA, Happ GM, Senne DA, Wang RX, Slemons RD, Holmes EC, Taubenberger JK.** 2008. The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathog **4:**e1000076. http://dx.doi.org/10.1371/journal.ppat.1000076.
35. **Wang RX, Soll L, Dugan V, Runstadler J, Happ G, Slemons RD, Taubenberger JK.** 2008. Examining the hemagglutinin subtype diversity among wild duck-origin influenza A viruses using ethanol-fixed cloacal swabs and a novel RT-PCR method. Virology **375:**182–189. http://dx.doi.org/10.1016/j.virol.2008.01.041.
36. **Hatchette TF, Walker D, Johnson C, Baker A, Pryor SP, Webster RG.** 2004. Influenza A viruses in feral Canadian ducks: extensive reassortment in nature. J Gen Virol **85:**2327–2337. http://dx.doi.org/10.1099/vir.0.79878-0.
37. **Wille M, Tolf C, Avril A, Latorre-Margalef N, Wallerström S, Olsen B, Waldenström J.** 2013. Frequency and patterns of reassortment in natural influenza A virus infection in a reservoir host. Virology **443:**150–160. http://dx.doi.org/10.1016/j.virol.2013.05.004.
38. **Spackman E, Pedersen JC, McKinley ET, Gelb J.** 2013. Optimal specimen collection and transport methods for the detection of avian influenza virus and Newcastle disease virus. BMC Vet Res **9:**35. http://dx.doi.org/10.1186/1746-6148-9-35.