Short communication

Evaluation and optimization of a commercial blocking ELISA for detecting antibodies to influenza A virus for research and surveillance of mallards

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A B S T R A C T

The availability of a validated commercial assay is an asset for any wildlife investigation. However, commercial products are often developed for use in livestock and are not optimized for wildlife. Consequently, it is incumbent upon researchers and managers to apply commercial products appropriately to optimize program outcomes. We tested more than 800 serum samples from mallards for antibodies to influenza A virus with the IDEXX AI MultiS-Screen Ab test to evaluate assay performance. Applying the test per manufacturer's recommendations resulted in good performance with 84% sensitivity and 100% specificity. However, performance was improved to 98% sensitivity and 98% specificity by increasing the recommended cut-off. Using this alternative threshold for identifying positive and negative samples would greatly improve sample classification, especially for field samples collected months after infection when antibody titers have waned from the initial primary immune response. Furthermore, a threshold that balances sensitivity and specificity reduces estimation bias in seroprevalence estimates.

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Influenza A viruses (IAVs) are emerging and re-emerging pathogens of global significance due to their potential to impact wildlife, livestock, and human populations. The emergence of highly pathogenic Asian strain H5N1 in live bird markets in Southeast Asia in 1997 and its subsequent spread throughout much of Eurasia led to a proliferation of wild bird surveillance activities and research on avian IAVs (Spackman, 2009; Hoye et al., 2010). Since then, other detrimental strains have emerged and spread (e.g., H7N9, H5N8) and wild birds have been a common focus of research and epidemiologic investigation (Jeong et al., 2014; Jones et al., 2014; Miller et al., 2014). In particular, mallards (*Anas platyrhynchos*) have been identified globally as an important reservoir host and have been a common target of research and surveillance efforts aimed at understanding the natural ecology of IAVs (Jourdain et al., 2010; VanDalen et al., 2010). Several studies have shown that the IDEXX AI MultiS-Screen Ab test (Multi-S test) is a valid test for screening serum samples from wild bird species for antibodies to avian influenza A viruses (Brown et al., 2009, 2010; Claes et al., 2012). The Multi-S test has been found to outperform AGID tests (Brown et al., 2009) and to have comparable performance

compared with hemagglutination inhibition (HI) tests (Arsnoe et al., 2011). Subsequently, the MultiS test has been a test of choice to screen wild birds for IAV reactive antibodies in large-scale surveillance efforts, field studies, and experimental infection studies (e.g., Arsnoe et al., 2011; Hoye et al., 2011; Tolf et al., 2013; van Dijk et al., 2014; Samuel et al., 2015) where subtype specific information is not required.

The Multi-S test is an epitope blocking enzyme-linked immunosorbent assay (ELISA) that targets the IAV nucleoprotein, the antigenicity of which is highly conserved across viral strains; therefore the test detects antibodies to all avian IAV subtypes (Brown et al., 2010; Ciacci-Zanella et al., 2010). The test has been validated for a number of avian species, including ducks. While the availability of a reliable and validated commercial assay is an asset for wildlife investigations, these products are generally developed for use in livestock and are not necessarily optimized for wildlife research or surveillance applications (Claes et al., 2012). Furthermore, cutoff thresholds are generally optimized based on the relatively high antibody titers associated with a primary immune response from a recent infection or immunization. In contrast, surveillance and field research samples may be collected months post exposure when antibody titers are expected to be much lower. As titers wane over time since exposure, a clear demarcation between positive and negative samples may be harder to identify

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and an alternate threshold may be necessary to improve correct classification of samples. Consequently, a thorough investigation of the performance and characteristics of commercial assays for particular wildlife applications is important for optimizing research and surveillance outcomes. Brown et al. (2009) found that a cutoff threshold of 0.7 for the MultiS test optimized the number of samples correctly classified over a wide range of wildlife species. However, this threshold has not been widely adopted and deserves further investigation.

The agar gel immunodiffusion (AGID) assay is another general test commonly used to detect antibodies to IAV in birds, but evaluations of AGIDs have shown that they do not necessarily work well in mallards and have relatively low sensitivity compared to the Multi-S test (Cattoli and Capua, 2007; Brown et al., 2009, 2010). Hemagglutination inhibition (HI) tests are commonly used when subtype information is required, but the performance of HI tests can vary depending on whether the viral strain used in the test is a good match for the samples screened (Tse et al., 2012). Therefore, a general test like the Multi-S ELISA may be preferred for screening large numbers of samples or for testing where unknown subtypes or strains may be present. In this study, we evaluated the Multi-S test for application to mallard serum samples using a large dataset from naïve and experimentally infected mallards.

We collected serum samples from naïve, uninfected mallards ranging in age from 4 weeks to 14 months and from mallards experimentally infected with IAV and ranging in age from 6 weeks to 8 months (infections were initiated at 6 weeks or 6 months). Serum from infected mallards was collected on 0, 10 or 14, 21, 28, and 56 days post infection (DPI). All samples were collected and tested as part of several ongoing IAV studies (S. Shriner, unpublished data). Approximately equal numbers of male and female mallards were purchased as day-old ducklings from Murray McMurray Hatchery (Webster City, IA, USA). Upon arrival, all birds were held indoors in a BSL-2 room for at least 4 weeks and were then screened for antibodies to IAV to verify their negative status. Birds and samples were maintained and tested at the animal research facilities at the National Wildlife Research Center, United States Department of Agriculture (USDA), Fort Collins, CO. In total, we tested 824 serum samples from captive mallards. The dataset was comprised of 370 samples from naïve mallards (one sample per individual) and 454 samples from infected mallards (3–4 samples per individual across 126 individuals). In addition, we also screened serum, collected as part of USDA Wildlife Services surveillance activities, from 77 wild mallards.

All infected birds were inoculated with low pathogenic A/Wild Bird/IL/183983-24/2006 (H6N2) influenza virus via oralpharyngeal inoculation. The H6N2 virus was passaged in allantoic cavities of 9–11 day-old specific pathogen free embryonated chicken eggs at 37 °C. Allantoic fluid was harvested, pooled, and stored at −80 °C. Viral titers were determined as EID_{50} (Szretter et al., 2006) by the USDA National Veterinary Services Laboratory (Ames, Iowa, USA) and diluted to inoculation doses ranging from 10^3 EID_{50}/mL to 10^5 EID_{50}/mL. A bird was considered infected if viral RNA loads exceeded 10^2 EID_{50}/mL equivalents for two consecutive days for oral, cloacal, or fecal swabs on 2–10 DPI. All inoculated birds were classified as infected by these criteria. Viral RNA shedding was quantified via real-time RT-PCR on ABI 7900 Real-time PCR systems (Life Technologies) or CFX96 Touch instruments (Bio-Rad) with primers and probes developed by Spackman et al. (2003) and thermocycler conditions as previously described in Pepin et al. (2012).

All serum samples were tested for antibodies to IAV using the Multi-S test per manufacturer's recommendations. The sensitivity and specificity of the test were evaluated over a range of sample-to-negative (S/N) threshold cut-offs for determining the positive or negative status of a sample. S/N ratios were characterized using histograms, box plots, and linear regression as a function of DPI. All analyses were conducted in R 3.0.2 (R Development Core Team, 2010).

The mean S/N ratio for samples from naïve mallards (including 0 DPI samples from infected birds) was 0.91 (SE 0.005) and the mean for samples from infected mallards was 0.34 (SE 0.008). The distribution of S/N values for the 0 DPI samples did not statistically differ from the distribution of samples from naïve ducks so the samples were combined for analysis. The distribution of samples was strongly bimodal with limited overlap between positive and negative samples (Fig. 1). Using the manufacturer's recommended threshold of S/N ratio <0.5 for positive samples, the test correctly classified 91.26% of samples and exhibited 84.14% sensitivity (382/455 positive samples correctly classified) and 100% specificity (370/370 negative samples correctly classified, Table 1). Assay performance for mallard serum for alternative thresholds peaked at a threshold of S/N ratio <0.7 for positives, with 98.06% of samples correctly classified, 97.80% sensitivity (444/455 positive samples correctly classified), and 98.38% specificity (364/370 negative samples correctly classified).

Sample-to-negative ratios for samples from infected ducks varied by DPI (Fig. 2). Predicted values from a linear regression model of S/N ratio as a function of DPI showed samples collected within the first four weeks post infection had similar values, but values for samples collected at eight weeks (56 DPI) were significantly lower with nearly 40% of birds returning a negative status per the manufacturer's recommended threshold. Samples collected on days 10 or 14 DPI had the lowest S/N predicted estimate of 0.27, followed by 0.32 for samples collected on 21 and 28 DPI, and 0.50 for samples collected on 56 DPI. Applying the manufacturer's recommended threshold of S/N ratio <0.5 (optimized threshold of S/N ratio <0.7 in parentheses) resulted in the positive identification of 95.97% (99.19%) of 10 or 14 DPI samples, 88.89% (98.77%) of 21 DPI samples, 93.60% (99.20%) of 28 DPI samples and 59.68% (94.35%) of 56 DPI samples. Similar to our experimental samples, samples collected from wild birds had S/N values ranging from 0.15 to 1.01. Thirty-four (44%) of the samples had S/N ratios <0.5, 16 (21%) of the samples had S/N ratios between 0.5 and 0.7, and 27 (35%) samples had S/N ratios >0.7.

Consistent with previously published evaluations of the Multi-S test (Brown et al., 2009, 2010; Claes et al., 2012), our work demonstrates the test is appropriate for wild bird species and can be expected to provide excellent performance for mallards. An optimized threshold cut-off of S/N ratio <0.7 correctly classified more than 98% of samples and provided 98% sensitivity and specificity. This cut-off provides a better balance of sensitivity and specificity than the manufacturer's recommended threshold which resulted in 84% sensitivity and 100% specificity for the mallards we tested and correctly classified 91% of serum samples.

Balancing sensitivity and specificity is often appropriate for wildlife species research and surveillance activities where sample results are considered individually rather than grouped, as with flock testing. For flocks, weighting a threshold toward specificity may be warranted because many animals are tested at the same time to evaluate flock status such that reduced sensitivity may be an appropriate trade-off to avoid expensive confirmatory testing associated with false positive results. On the other hand, for research, a highly sensitive test is critical for screening animals prior to experimental infection studies to ensure that individuals have not been previously exposed. Detecting previous IAV infections is especially critical due to heterosubtypic cross immunity because prior infections with a different subtype are likely to influence experimental results (Jourdain et al., 2010; Pepin et al., 2012; Latorre-Margalef et al., 2013). For serosurveillance, two primary goals for wild birds are to detect prior IAV exposures and to estimate seroprevalence. Both of these objectives benefit from relatively high sensitivity and
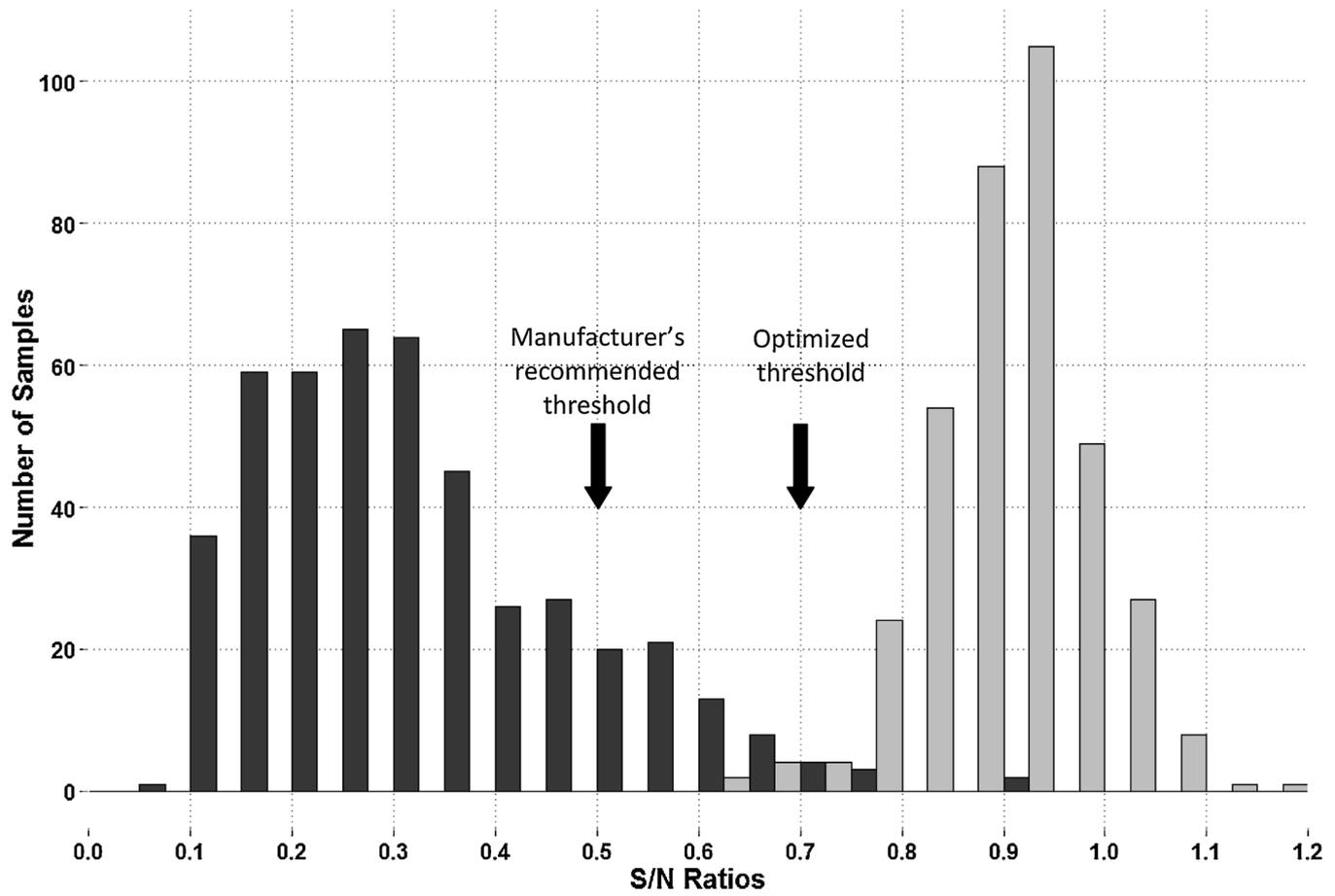
Fig. 1. Histogram of S/N ratios from tested serum samples from uninfected mallards (gray) and infected mallards (black). The manufacturer suggested threshold for a positive sample is S/N ratio <0.5 and the optimized threshold is S/N ratio <0.7 as shown by the respective arrows.

Table 1
Assay performance for different S/N thresholds based on test results for 370 serum samples from uninfected mallards and 454 serum samples from infected mallards. The manufacturer recommended threshold (S/N ratio <0.5) and the optimized threshold (S/N ratio <0.7) are displayed with shading.

Threshold Value	True Positives	False Positives	True Negatives	False Negatives	% Sensitivity	% Specificity	% Correctly Classified
<0.1	1	0	370	453	0.22	100.00	45.02
<0.2	96	0	370	358	21.15	100.00	56.55
<0.3	220	0	370	234	48.46	100.00	71.60
<0.4	329	0	370	125	72.47	100.00	84.83
<0.5	382	0	370	72	84.14	100.00	91.26
<0.6	423	0	370	31	93.17	100.00	96.24
<0.7	444	6	364	10	97.80	98.38	98.06
<0.8	451	36	334	3	99.34	90.27	95.27
<0.9	451	176	194	3	99.34	52.43	78.28
<1.0	453	330	40	1	98.69	10.81	59.83

using a threshold that balances sensitivity and specificity leads to unbiased estimates of seroprevalence. Consequently, using an optimized threshold would improve the accuracy of research and surveillance results.

In an investigation of the Multi-S test applied to 27 wild bird species, Brown et al. (2009) estimated the Multi-S to have only a 75% sensitivity for low pathogenic IAVs across species when using the manufacturer's recommended threshold. However, sensitivity was 92% for mallards at that threshold which is similar to our estimate of 91%. Brown et al. (2009) also found that a threshold cut-off of S/N ratio <0.7 optimized assay performance with a peak in samples correctly classified and a balance between sensitivity and specificity. While a thorough investigation of assay performance for each

species of wild bird studied with the Multi-S test would be ideal, the consistent finding of 0.7 as an optimized cut-off in both the current study and that of Brown et al. (2009) indicates that this threshold may be generally appropriate for a variety of species. An alternative to strictly applying the 0.7 threshold would be to use the manufacturer's suggested threshold of 0.5, but then designate individual tests that fall between 0.5 and 0.7 as suspect positive results.

While our study was limited to a single IAV subtype, Lebarbenchon et al. (2012) found the Multi-S test worked well across eight different IAV subtypes in experimentally infected mallards; accordingly, our results are likely generalizable across a wide variety of IAV strains. However, we did detect a significant drop in detectable antibodies at 56 DPI, with only 60% of samples
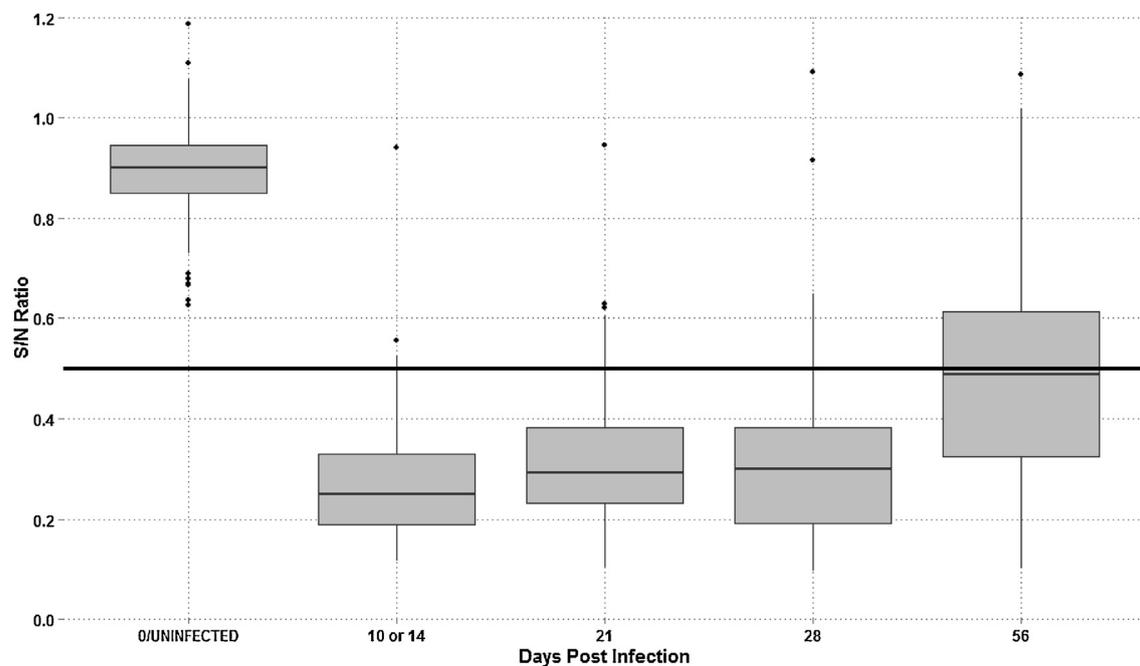
Fig. 2. Box plots for S/N ratios for samples collected on various days post infection and from 0 DPI/uninfected animals. The gray boxes show the interquartile range (IQR, middle 50% of values) for each group, horizontal lines represent medians, and vertical lines are values within 1.5*IQR. Outliers are plotted as individual points. The manufacturer suggested threshold for a positive sample is S/N ratio <0.5, as shown by the black horizontal line.

from infected birds eliciting a positive result per the manufacturer's recommended threshold. At the optimized threshold the drop was much less, to 94%, but since circulating antibodies are likely to fall even more for longer time periods post exposure, use of an optimized threshold is critical for field studies where samples may be collected for extended periods post infection. For example, in a study of pink-footed geese (*Anser brachyrhynchus*) tested with the Multi-S test, Hoye et al. (2011) found seroprevalence dropped by approximately 30% from spring to late summer. Thus, while the Multi-S test is expected to detect nearly all relatively recent influenza infections in mallards, samples collected from birds with longer times since infection are likely to exhibit lower sensitivity (Brown et al., 2009) as antibody titers decay. In our screen of wild birds using the Multi-S test, we did not have information on true exposure status or time since infection. However, 21% of the tested birds had S/N values between 0.5 and 0.7, indicating that these birds were likely exposed to influenza A viruses since these values are so much lower than the mean S/N value we found for uninfected experimental birds.

In conclusion, we recommend the use of an optimized threshold of S/N ratio <0.7 for the Multi-S test when it is applied to mallard serum samples. Moreover, this threshold should be evaluated across a broad spectrum of wild bird species.

Acknowledgements

References

Arsnoe, D.M., Ip, H.S., Owen, J.C., 2011. Influence of body condition on influenza A virus infection in mallard ducks: experimental infection data. PLoS ONE 6, e22633.

Brown, J.D., Luttrell, M.P., Berghaus, R.D., Kistler, W., Keeler, S.P., Howey, A., Wilcox, B., Hall, J., Niles, L., Dey, A., Knutsen, G., Fritz, K., Stallknecht, D.E., 2010. Prevalence of antibodies to type a influenza virus in wild avian species using two serologic assays. J. Wildl. Dis. 46, 896–911.

Brown, J.D., Stallknecht, D.E., Berghaus, R.D., Luttrell, M.P., Velek, K., Kistler, W., Costa, T., Yabsley, M.J., Swayne, D., 2009. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clin. Vaccine Immunol. 16, 824–829.

Cattoli, G., Capua, I., 2007. Diagnosing avian influenza in the framework of wildlife surveillance efforts and environmental samples. J. Wildl. Dis. 43, S35–S39.

Ciacci-Zanella, J.R., Vincent, A.L., Prickett, J.R., Zimmerman, S.M., Zimmerman, J.J., 2010. Detection of anti-influenza a nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. J. Vet. Diagn. Investig. 22, 3–9.

Claes, G., Vangeluwe, D., Van Der Stede, Y., Van Den Berg, T., Lambrecht, B., Marché, S., 2012. Evaluation of four enzyme-linked immunosorbent assays for the serologic survey of avian influenza in wild bird species. Avian Dis. 56, 949–954.

Hoye, B.J., Munster, V.J., Nishiura, H., Fouchier, R.A.M., Madsen, J., Klaassen, M., 2011. Reconstructing an annual cycle of interaction: natural infection and antibody dynamics to avian influenza along a migratory flyway. Oikos 120, 748–755.

Hoye, B.J., Munster, V.J., Nishiura, H., Klaassen, M., Fouchier, R.A.M., 2010. Surveillance of wild birds for avian influenza virus. Emerg. Infect. Dis. 16, 1827–1834.

Jeong, J., Kang, H.M., Lee, E.K., Song, B.M., Kwon, Y.K., Kim, H.R., Choi, K.S., Kim, J.Y., Lee, H.J., Moon, O.K., Jeong, W., Choi, J., Baek, J.H., Joo, Y.S., Park, Y.H., Lee, H.S., Lee, Y.J., 2014. Highly pathogenic avian influenza virus (H5N8) in domestic poultry and its relationship with migratory birds in South Korea during 2014. Vet. Microbiol. 173, 249–257.

Jones, J.C., Sonnberg, S., Koçer, Z.A., Shanmuganatham, K., Seiler, P., Shu, Y., Zhu, H., Guan, Y., Peiris, M., Webby, R.J., Webster, R.G., 2014. Possible role of songbirds and parakeets in transmission of influenza A(H7N9) virus to humans. Emerg. Infect. Dis. 20, 380–385.

Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Bröjer, C., Sahlin, S., Svensson, L., Waldenström, J., Lundkvist, Å., Olsen, B., 2010. Influenza virus in a natural host, the mallard: experimental infection data. PLoS ONE 5, e8935.

Latorre-Margalef, N., Grosbois, V., Wahlgren, J., Munster, V.J., Tolf, C., Fouchier, R.A.M., Osterhaus, A.D.M.E., Olsen, B., Waldenström, J., 2013. Heterosubtypic immunity to influenza A virus infections in mallards may explain existence of multiple virus subtypes. PLoS Pathog. 9, e1003443.

Lebarbenchon, C., Brown, J.D., Luttrell, M.P., Stallknecht, D.E., 2012. Comparison of two commercial enzyme-linked immunosorbent assays for detection of Influenza A virus antibodies. J. Vet. Diagn. Investig. 24, 161–165.

Miller, R.S., Sweeney, S.J., Akkina, J.E., Saito, E.K., 2014. Potential intercontinental movement of influenza A(H7N9) virus into North America by wild birds: application of a rapid assessment framework. Transbound. Emerg. Dis. 60, 650–668.

Pepin, K.M., VanDalen, K.K., Mooers, N.L., Ellis, J.W., Sullivan, H.J., Root, J.J., Webb, C.T., Franklin, A.B., Shriner, S.A., 2012. Quantification of heterosubtypic immunity between avian influenza subtypes H3N8 and H4N6 in multiple avian host species. J. Gen. Virol. 93, 2575–2583.

R Development Core Team, 2010. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Samuel, M.D., Hall, J.S., Brown, J.D., Goldberg, D.R., Ip, H., Baranyuki, V.V., 2015. The dynamics of avian influenza in western Arctic snow geese: implications for annual and migratory infection patterns. Ecol. Appl. 25, 1851–1859.

Spackman, E., 2009. The ecology of avian influenza virus in wild birds: what does this mean for poultry? Poult. Sci. 88, 847–850.

Spackman, E., Senne, D.A., Bulaga, L.L., Myers, T.J., Perdue, M.L., Garber, L.P., Lohman, K., Daum, L.T., Suarez, D.L., 2003. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis. 47, 1079–1082.

Szretter, K.J., Balish, A.L., Katz, J.M., 2006. Influenza: propagation, quantification, and storage. Curr. Protoc. Microbiol. Chapter 15, Unit 15:G.1.

Tolf, C., Latorre-Margalef, N., Wille, M., Bengtsson, D., Gunnarsson, G., Grosbois, V., Hasselquist, D., Olsen, B., Elmberg, J., Waldenström, J., 2013. Individual variation in influenza A virus infection histories and long-term immune responses in mallards. PLOS ONE 8, e61201.

Tse, M., Kim, M., Chan, C.H., Ho, P.L., Ma, S.K., Guan, Y., Peiris, J.S.M., 2012. Evaluation of three commercially available influenza A type-specific blocking enzyme-linked immunosorbent assays for seroepidemiological studies of influenza A virus infection in pigs. Clin. Vaccine Immunol. 19, 334–337.

van Dijk, J.G.B., Mateman, A.C., Klaassen, M., 2014. Transfer of maternal antibodies against avian influenza virus in mallards (*Anas platyrhynchos*). PLOS ONE 9, e112595.

VanDalen, K.K., Franklin, A.B., Mooers, N.L., Sullivan, H.J., Shriner, S.A., 2010. Shedding light on avian influenza H4N6 infection in mallards: modes of transmission and implications for surveillance. PLoS ONE 5, e12851.