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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 oral-pharyngeal 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