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Influenza A Virus H5–specific Antibodies in Mute Swans (*Cygnus olor*) in the USA

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ABSTRACT: The use of serologic assays for influenza A virus (IAV) surveillance in wild birds has increased because of the availability of commercial enzyme-linked immunosorbent assays (ELISAs). Recently, an H5-specific blocking ELISA (bELISA) was shown to reliably detect H5-specific antibodies to low- and high-pathogenic H5 viruses in experimentally infected waterfowl. Mute Swans (*Cygnus olor*) were frequently associated with highly pathogenic H5N1 outbreaks in Europe and may have a similar role if highly pathogenic H5N1 is introduced into North America. We measured the prevalence of antibodies to the nucleoprotein and H5 protein in Mute Swans using three serologic assays. We collected 340 serum samples from Mute Swans in Michigan, New Jersey, New York, and Rhode Island, US. We detected antibodies to the IAV nucleoprotein in 66.2% (225/340) of the samples. We detected H5-specific antibodies in 62.9% (214/340) and 18.8% (64/340) using a modified H5 bELISA protocol and hemagglutination inhibition (HI) assay, respectively. The modified H5 bELISA protocol detected significantly more positive samples than did the manufacturer's protocol. We also tested 46 samples using virus neutralization. Neutralization results had high agreement with the modified H5 bELISA protocol and detected a higher prevalence than did the HI assay. These results indicate that North American Mute Swans have high nucleoprotein and H5 antibody prevalences.

Key words: *Cygnus olor*, H5-specific ELISA, hemagglutination inhibition, Mute Swan, serology.

Surveillance for influenza A viruses (IAVs) in wild birds has traditionally been based on detection of viral shedding using virus isolation or PCR. However, recently, there has been an increase in the use of serologic assays for IAV surveillance because of the development and validation of several commercially available enzyme-linked immunosorbent assays (ELISAs) in several wild bird species (Brown et al. 2009). These commercial assays detect antibodies to the IAV nucleoprotein (NP) and have been effectively used for IAV surveillance in wild birds (Brown et al. 2010; Kistler et al. 2012). However, the commercial assays validated for use in wild birds detect antibodies to all IAVs and not subtype-specific antibodies.

Historically, hemagglutination inhibition (HI) has been the most frequently used assay to detect subtype-specific antibodies in wild birds (Winkler et al. 1972). The HI assay can detect IAV antibodies in multiple species and has been used in large-scale surveillance studies (Niqueux et al. 2010). However, there are disadvantages to the HI assay; the most important of which is a lack of standardization of antigen and antisera across laboratories, making it difficult to compare results among studies (Stephenson et al. 2007). The development of a blocking ELISA (bELISA) with high sensitivity and specificity for H5-specific antibodies in experimentally infected Mallards (*Anas platyrhynchos*; Lebarbenchon et al. 2013) offers the potential for standardization of results across laboratories.

The emergence of highly pathogenic (HP) H5N1 in poultry in 1997 resulted in an increase in the availability of H5-

specific antibody tests. During the past 15 yr, HP H5N1 viruses have been reported in wild birds across Asia and Europe. In particular, Mute Swans (*Cygnus olor*) were frequently associated with outbreaks in Europe (Feare 2010). Mute Swans may play a role if HP H5N1 is introduced to North America. We tested Mute Swans in the US for H5 IAV antibodies using three H5-specific serologic assays.

Blood samples were collected from Mute Swans (n=340) in collaboration with the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service, during wildlife damage management operations in Michigan, New Jersey, New York, and Rhode Island, US. Blood samples were collected postmortem from the jugular vein or by cardiocentesis within 2 h of euthanasia, were placed in Vacutainer® tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), and were kept cold in the field. Samples were centrifuged for 15 min at 1,500 × G, and sera were stored at −20 C until testing.

Antibodies to all IAV serotypes were detected using a commercial NP bELISA (IDEXX Laboratories, Westbrook, Maine, USA) per manufacturer's instructions (Brown et al. 2009). We detected H5-specific antibodies using three assays: 1) bELISA (ID VET, Montpelier, France); 2) H5 HI assay; and 3) an H5 virus microneutralization (MN) assay. We used two protocols with the H5-specific bELISA: 1) the manufacturer's protocol, and 2) a modified protocol using a 1:2 serum dilution with an 18-h incubation at 36 C (Lebarbenchon et al. 2013). We tested 182 serum samples with both H5 bELISA protocols, and the remaining 158 samples with only the modified H5 ELISA protocol. Samples with sample to negative ratios >0.35 were considered negative. We tested all 340 serum samples with the HI assay using A/mallard/AI08-3532/H5N2 as the antigen and antisera obtained from the USDA National Veterinary Service Laboratories (Ames, Iowa, USA). Serum samples were treated with receptor-destroying enzyme (RDE [II] Denka Seiken, Tokyo, Japan) at a 1:3 dilution and incubated for 18 h at 36 C and 56 C for 1 h. The HI assay was performed as described (Pedersen 2008) using four hemagglutination units per 25 μL of antigen, and a titer ≥ eight was considered positive (Curran et al. 2014). We tested 46 serum samples using the MN assay as described by Ramey et al. (2014) using low-pathogenic A/mallard/MN/AI11-3933/2011(H5N1) virus.

Statistical analyses were conducted with R version 3.0 (R Development Core Team 2014). Agreement between the two H5 bELISA protocols was evaluated using κ statistics and percentage of agreement. Interpretation of the κ value was based on the criteria of Landis and Koch (1977). We used McNemar's χ^2 to test for a significant difference in the number of positive samples detected among the assays.

We detected NP antibodies in 66.2% of serum samples using the NP bELISA (Table 1). In a comparison of the two H5 bELISA protocols, we detected antibodies in 33.0% (60/182) of the birds using the manufacturer's protocol and in 69.2% (126/182) using the modified protocol. There was 62% agreement between the two H5 bELISA protocols with moderate agreement (κ=0.3; 95% confidence interval=0.1–0.5); the modified protocol detected significantly more positive samples than did the manufacturer's protocol (McNemar's χ^2=63; P<0.001). Compared with the two H5 bELISA protocols, we detected a relatively low prevalence with the HI assay (Table 1). Therefore, we tested a subset (n=46) of samples with the MN assay. The 46 samples were selected based on HI results (23 negatives and 23 positives) to evaluate the efficacy of the tests and do not accurately represent the study population. The H5 antibody prevalence for this subset was 65% (30/46) using the MN assay and the modified H5 bELISA

Table 1. Prevalence of influenza A virus antibodies to the nucleoprotein (NP) and to the H5-subtype in Mute Swans (*Cygnus olor*) from four states using three serologic assays.[a]

State	NP bELISA, positive/*n* (%)	H5 bELISA modified protocol, positive/*n* (%)	H5 hemagglutination inhibition assay, positive/*n* (%)
Michigan	145/182 (79.7)	136/182 (74.7)	28/182 (15.4)
New Jersey	12/68 (18)	27/40 (67)	10/68 (15)
New York	11/12 (92)	9/12 (75)	3/12 (25)
Rhode Island	57/78 (73)	42/78 (54)	23/78 (29)
Total	225/340 (66.2)	214/340 (62.9)	64/340 (18.8)

[a] bELISA = blocking enzyme-linked immunosorbent assay.

protocol and 50% (23/46) using the HI assay.

The high IAV NP antibody prevalence we detected in Mute Swans was similar to the 45% prevalence reported by Pederson et al. (2014). This high antibody prevalence is likely related to the persistence of antibodies, which may be >1 yr in the absence of virus circulation (Fereidouni et al. 2010), and age of the sampled birds because Mute Swans are a long-lived waterfowl species (Reese 1980). The high prevalence of H5-specific antibodies was unexpected because H5 IAVs are not frequently reported from waterfowl in North America (Wilcox et al. 2011). A similarly high H5 antibody prevalence was reported in Mute Swans in France (Niqueux et al. 2010). The higher prevalence of H5-specific antibodies, compared with NP antibodies, in New Jersey may be related to increased persistence of subtype-specific antibodies (Burger et al. 2012).

Lebarbenchon et al. (2013) detected an increased sensitivity with the H5 bELISA using the modified H5 bELISA protocol in experimentally infected ducks. The data from our study also suggest that the modified protocol for the H5 bELISA has increased sensitivity for detection of H5-specific antibodies in Mute Swans and possibly other birds. The low prevalence detected with the HI assay was surprising because Niqueux et al. (2010), in France, detected H5-specific antibodies in >45% of Mute Swans using the HI assay. However, the HI assay has performed poorly in experimentally infected Mute Swans (Kalthoff et al. 2008) and did not detect antibodies in naturally exposed sentinel ducks (Globig et al. 2013). The data from the MN assay were similar to those from the modified H5 bELISA protocol; both detected a higher prevalence of antibodies than did the HI assay. Increased sensitivity of the MN assay compared with the HI assay has been reported in detection of H5 antibodies (Rowe et al. 1999). In addition, HI is likely not the most sensitive assay to detect subtype-specific antibodies in Mute Swans and, if used, confirmatory assays should be performed.

Our results suggest that Mute Swans in the US have high NP and H5 antibody prevalences. The significance of this high prevalence is unknown, but if this reflects high existing population immunity to H5 viruses, the probability of disease or successful introduction of highly pathogenic H5N1 viruses to the US may be low. Alternatively, these antibodies may protect Mute Swans from disease associated with highly pathogenic H5N1 viruses and allow these to circulate without noticeable mortality.

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