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Subtype-specific influenza A virus antibodies in Canada geese (*Branta canadensis*)

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ABSTRACT

Historically, surveillance for influenza A viruses (IAVs) in wild birds has relied on viral detection assays. This was largely due to poor performance of serological assays in wild birds; however, recently developed commercial serological assays have improved the ability to detect IAV antibodies in wild birds. Serological surveillance for IAV antibodies in Canada geese (*Branta canadensis*) has shown that, despite a low prevalence of virus isolations, Canada geese are frequently exposed to IAVs and that exposure increases with latitude, which follows virus isolation prevalence patterns observed in dabbling ducks. The objectives of this study were to further evaluate IAV antibodies in Canada geese using a subtype-specific serological assay to determine if Canada geese are exposed to subtypes that commonly circulate in dabbling ducks. We collected serum samples from Canada geese in Minnesota, New Jersey, Pennsylvania, and Wisconsin and tested for antibodies to IAVs using a blocking ELISA. Positive samples were further tested by hemagglutination inhibition for 10 hemagglutinin IAV subtypes (H1–H10). Overall, we detected antibodies to NP in 24% (714/2919) of geese. Antibodies to H3, H4, H5, and H6 subtypes predominated, with H5 being detected most frequently. A decrease in H5 HI antibody prevalence and titers was observed from 2009 to 2012. We also detected similar exposure pattern in Canada geese from New Jersey, Minnesota, Washington and Wisconsin. Based on the published literature, H3, H4, and H6 viruses are the most commonly reported IAVs from dabbling ducks. These results indicate that Canada geese also are frequently exposed to viruses of the same HA subtypes; however, the high prevalence of antibodies to H5 viruses was not expected as H5 IAVs are generally not well represented in reported isolates from ducks.

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1. Introduction

Wild birds in the orders Anseriformes and Charadriiformes are considered the natural reservoirs for influenza A viruses (IAVs) (Olsen et al., 2006) and historical surveillance for these viruses in wild birds has relied on viral detection by either virus isolation or RT-PCR (Hinshaw et al., 1985; Wallensten et al., 2007). However,

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serological assays have been developed recently that have a high sensitivity at detecting antibodies to IAVs, thus these assays can be used to improve surveillance approaches (Brown et al., 2009; Lebarbenchon et al., 2012). The duration of detectable antibodies can be >1 year in naturally infected ducks (Tolf et al., 2013), and with repeated infections, they may persist for the life of the bird. In contrast, viral shedding is of short duration, often <10 days (Costa et al., 2011). The long duration of antibodies allows for sampling during times when birds are more easily captured (e.g. summer molting) or in species where information about their role in the maintenance of IAVs is limited. Serology has been recently used to supplement virus isolation data and advance our current understanding of IAVs in Canada geese (*Branta canadensis*) (Kistler et al., 2012).

Traditionally, Canada geese have not been implicated in an important role in the epidemiology of IAVs. Although Canada geese have a near ubiquitous distribution in the United States (US) and share aquatic habitats with known IAVs reservoir species (Hestbeck, 1995), IAV isolations from Canada geese are rare (Harris et al., 2010). This perceived low prevalence of viral isolation is likely due to brief and infrequent viral shedding patterns reported in experimentally infected Canada geese (Berhane et al., 2014; Pasick et al., 2007) and sample timing which often occurred during a 3–4-week flight-less molting period during June and early July (Harris et al., 2010). Using serologic testing, Canada geese were found to be frequently exposed to IAVs and the prevalence of antibodies increased with latitude (Kistler et al., 2012). This increase in antibody prevalence in geese followed a similar trend of virus shedding data in dabbling ducks (Hinshaw et al., 1985; Stallknecht et al., 1990).

Results from these previous studies suggests that serological surveillance of IAVs in Canada geese may provide an inexpensive sentinel system to monitor or supplement surveillance efforts to understand spatial and annual trends in IAV transmission in waterfowl populations. However, subtype-specific serological data are needed to understand if antibodies detected in Canada geese are representative of the predominant subtypes detected in waterfowl, especially dabbling ducks. Based on virus isolation results from dabbling ducks, hemagglutinin subtypes H3, H4, and H6 are most commonly reported during peak IAV transmission in late summer and early fall (Wilcox et al., 2011). The objectives of this study were to determine long term trends in IAVs antibodies to the nucleoprotein (NP) and to detect subtype-specific antibodies in Canada geese.

2. Materials and methods

In June and July 2010–2012, we collected blood samples ($n = 2225$) from Canada geese from 116 locations (Fig. 1) in Pennsylvania during banding and nuisance removal programs. Blood samples were collected from the medial metatarsal vein from geese being released and by cardiocentesis from birds that were euthanized. Blood samples were placed in Vacutainer® serum separator tubes (BD, Franklin Lakes, NJ, USA) and placed on wet ice in the field. After transport to a laboratory (<1 day) blood samples were centrifuged (15 min at $1200 \times g$) and serum was removed and stored at −20 C until testing.

We first screened serum samples for presence of antibodies to the IAV NP using a commercial blocking enzyme-linked immunosorbent assay (bELISA; IDEXX Laboratories, Westbrook, ME, USA). Samples that had antibodies to the IAV NP were then screened by a hemagglutination inhibition (HI) assay using antigen from the Southeastern Cooperative Wildlife Disease Study (University of Georgia, Athens, GA, USA; Table 1) and positive control serum from specific pathogen-free chickens (National Veterinary Service Laboratories, United States Department of Agriculture, Ames, IA, USA). Canada goose serum was first treated with 10% chicken red blood cells (1:1 dilution), incubated at room temperature for 1 h, and then centrifuged for 10 min at $800 \times g$. The supernatant was then removed and used for the HI assays. The HI assays for all subtypes were conducted as previously described (Pedersen, 2008) using 4 HA/25 μl and a positive cut-off titer of ≥32.

We also included Canada goose samples collected in 2009 during a previous study (Kistler et al., 2012). These

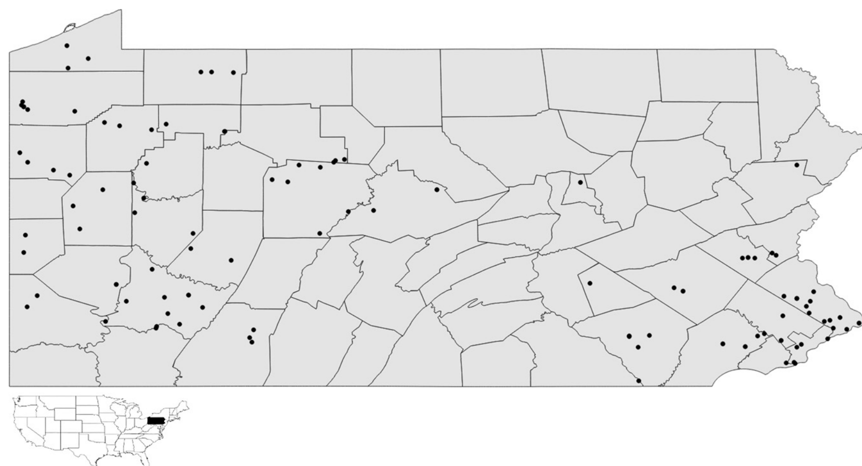

Fig. 1. Sample location distribution in Pennsylvania 2009–2012.

Table 1
Viruses used to make antigen for the hemagglutination inhibition assay.

Hemagglutinin subtype	Virus
H1	A/Mallard/Minnesota/sg-00627/2008(H1N1)
H2	A/Mallard/Minnesota/AI08-2755/2008(H2N3)
H3	A/Mallard/Minnesota/Sg-00627/2008(H3N8)
H4	A/Mallard/Minnesota/Sg-01049/2008(H4N6)
H5	A/Mallard/Minnesota/AI08-3532/2008(H5N2)
H6	A/Mallard/Minnesota/Sg-00796/2008(H6N1)
H7	A/Mallard/Minnesota/AI09-3770/2009(H7N9)
H8	A/Mallard/Minnesota/Sg-00689/2008(H8N4)
H9	A/Mallard/Arkansas/AI09-5649/2009(H9N2)
H10	A/Mallard/Minnesota/Sg-00689/2008(H10N7)

2009 samples had antibodies to IAV NP as determined by the bELISA and were from New Jersey (n = 63), Minnesota (n = 14), Pennsylvania (n = 134), and Washington (n = 26). In addition, samples (n = 80) with IAVs NP antibodies collected from 11 locations in Wisconsin during 2010 and 2011 were tested. All techniques were reviewed and approved by the IACUC committee at the University of Georgia.

3. Results

Overall, we detected antibodies to the IAV nucleoprotein in 24% (714/2919) of Canada geese in Pennsylvania. Of the 714 samples with NP antibodies, we used 653 samples for subtype-specific antibody testing. We detected the highest antibody prevalence to the H5 subtype with 60% testing positive (Table 2 and Fig. 2). There was a decrease in both H5-specific antibody prevalence and geometric mean titer from 2009 through 2012 (Table 2). Antibodies to the H3, H4, and H6 subtypes were also detected in >20% of geese, but there was little fluctuation in antibody prevalence across years (Fig. 2 and Table 3). There was also little fluctuation in antibody prevalence to H7 and H9 subtypes (Table 3). Antibody prevalence to the remaining four subtypes (H1, H2, H8 and H10) were estimated at <1%.

Table 2
H5-specific antibody titers and prevalence across years in Canada geese from Pennsylvania as determined by the hemagglutination inhibition assay.

Year	Positive/sampled (%; 95% CI)	Geometric mean titer
2009	127/134 (95; 91–99)	163
2010	101/113 (89; 84–95)	130
2011	125/222 (56; 48–65)	37
2012	42/184 (23; 16–30)	33
Total	395/653 (60; 52–69)	

We further evaluated if subtype-specific antibodies were consistent on a larger scale. We tested an additional 183 IAV antibody positive samples from four states (NJ, MN, WA, and WI) for antibodies to H3, H4, H5, and H6 subtypes. Antibodies to the H5 subtype were the most frequently detected in these states (Fig. 3). While antibodies to the H3 and H6 subtypes were >20% and H4-specific antibodies were <10%. We did not detect any H4-specific antibodies from Wisconsin in 2011.

4. Discussion

This study was a continuation of previous serological survey conducted in Canada geese (Kistler et al., 2012). We detected slight variations of IAVs nucleoprotein antibody prevalence among years; however, these changes were not significant. In a previous study, antibody prevalence

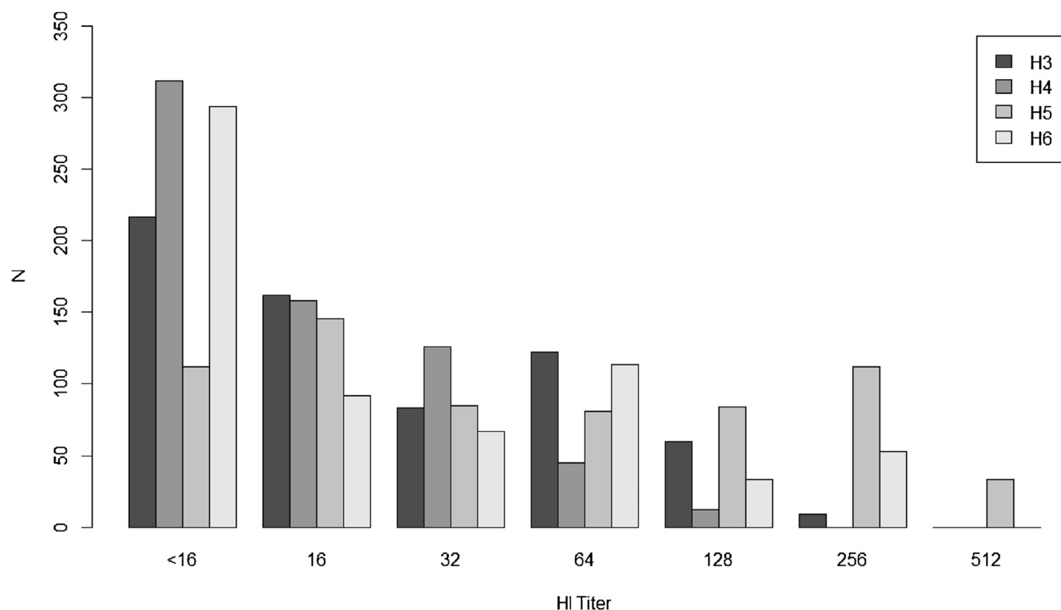

Fig. 2. Influenza A virus subtype-specific antibodies from (n = 653) Canada geese in Pennsylvania as determined by the hemagglutination inhibition assay. A titer ≥32 was considered positive.

Table 3
Antibody prevalence to H3, H4, H7, and H9 influenza A virus subtypes in Canada geese from Pennsylvania as determined by hemagglutination inhibition assay.

Year	H3 positive/sampled (%; 95% CI)	H4 positive/sampled (%; 95% CI)	H6 positive/sampled (%; 95% CI)	H7 positive/sampled (%; 95% CI)	H9 positive/sampled (%; 95% CI)
2009	41/134 (31; 23–38)	44/134 (33; 25–41)	50/134 (37; 29–46)	15/134 (11; 6–17)	4/134 (3; 0–6)
2010	54/113 (48; 39–57)	28/113 (25; 17–33)	35/113 (31; 22–29)	7/113 (6; 2–11)	6/113 (5; 1–9)
2011	98/222 (44; 37–51)	51/222 (23; 17–29)	107/222 (48; 42–55)	11/222 (5; 2–8)	27/222 (12; 8–16)
2012	81/184 (44; 37–51)	60/184 (33; 26–39)	75/184 (41; 34–48)	19/184 (10; 6–15)	8/184 (4; 1–7)
Total	274/653 (42; 38–46)	183/653 (28; 25–31)	267/653 (41; 37–45)	52/653 (8; 6–10)	45/653 (7; 5–9)

estimates from Canada geese were shown to increase with latitude (Kistler et al., 2012), which corresponds with IAV isolation data from dabbling ducks (Stallknecht et al., 1990; Wilcox et al., 2011). These data suggested that Canada geese share a common exposure to IAVs with dabbling ducks. Although IAVs from all avian HA subtypes, except for the H16 subtype, have been detected from dabbling ducks, the H3, H4, and H6 subtypes are the most frequently isolated (Olsen et al., 2006). In our study, antibodies to H3, H4, and H6 were frequently detected which supports a common source of exposure in Canada geese. This source is likely through direct contact of dabbling ducks or through the environment contaminated with IAVs. The low prevalence of H1, H2, H8, and H10 was not surprising as these subtypes are often under-represented in IAVs detected in dabbling ducks (Sharp et al., 1993).

Influenza A viruses of the H5 subtype are usually detected in low prevalence from dabbling ducks and often account for <1% of detected IAVs (Wilcox et al., 2011). However, local variation in the prevalence of H5 IAVs in ducks has been reported (Lindsay et al., 2013). The high H5 antibody prevalence we detected in Canada geese is not understood, but could be related to this subtype circulating outside the northern pre-migration staging areas when most sampling occurs. A higher prevalence of H5 IAVs has been detected in resident ducks in California (Hill et al., 2012). Alternatively, the high H5-specific antibody prevalence we detected could be due to a more robust immune response associated with the H5 subtype as Canada geese experimentally infected with H5 viruses develop higher subtype specific antibody titers than when infected with other subtypes (Berhane et al., 2014). These higher titers are likely due to H5 viruses replicating more efficiently in geese than other subtypes. In experimental infections, geese shed virus and develop antibodies after a single inoculation with H5 viruses, but often need to be inoculated more than once with other subtypes for viral shedding and a detectable antibody response (Berhane et al., 2010). The high H5 antibody prevalence we detected across multiple states and locations indicates this subtype circulates across the country.

We detected a decrease in H5-specific antibody titer and prevalence every year in Pennsylvania from a peak in

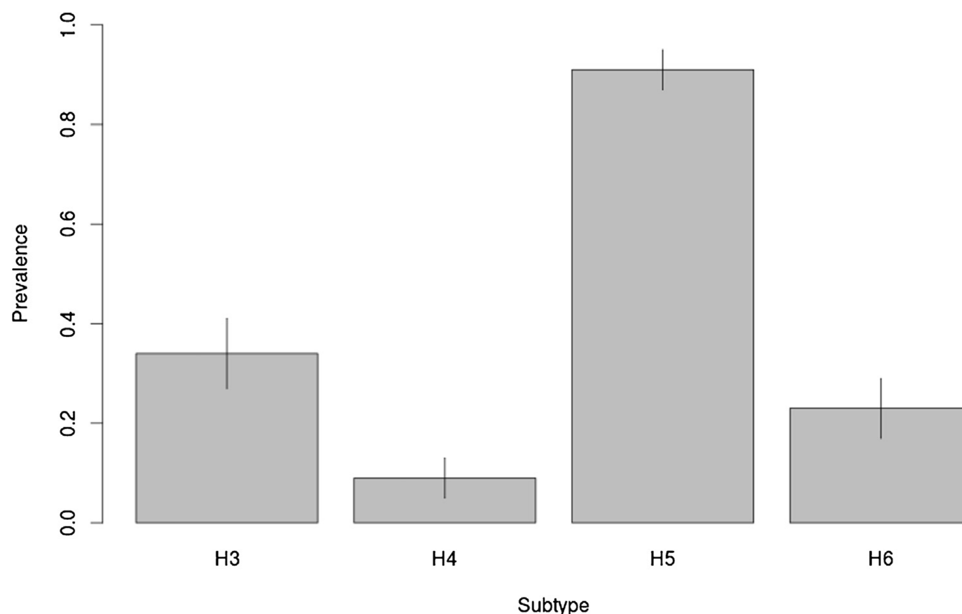

Fig. 3. Prevalence of influenza A subtype-specific antibodies from ($n = 184$) Canada geese in New Jersey, Minnesota, Washington, and Wisconsin as determined by the hemagglutination inhibition assay.

2009. This decrease in antibody titer and prevalence is likely due to a decrease in exposure to this subtype, population recruitment, and a waning subtype-specific antibody response. Annual variation in HA subtype prevalence has been reported in dabbling ducks (Krauss et al., 2004). Although there is very little information on the persistence of subtype-specific antibodies in wild birds, antibodies to the IAV nucleoprotein have been shown to persist for 1-year in the absence of detected virus circulation (Tolf et al., 2013). The observed variation could also have resulted from our use of one H5 antigen, regardless of year. Antigenic drift of the circulating H5 viruses may have resulted in poor antibody match to our antigen. The extent of H5 antigenic diversity in North American IAVs is not known but any antigenic changes could have altered our prevalence and titers detected. Although antigenic drift has been associated with highly pathogenic H5N1 viruses and H5N2 viruses (Lee et al., 2004; Zhong et al., 2014) it is poorly documented with low pathogenic H5 viruses from wild birds.

We detected similar subtype-specific antibody prevalence estimates in Canada geese sampled in several states. Although, we only tested for antibodies to four HA subtypes, geese from these states had similar proportion of antibodies to these subtypes with antibodies to the H5 subtype predominating; however, we did not detect any antibodies to the H4 subtype in Wisconsin in 2011. This may be likely to low circulation of H4 IAVs during that year. Yearly variation in circulation of viral subtypes has been shown in wild ducks (Wilcox et al., 2011).

Overall, we detected little variation in IAV antibody prevalence in Canada geese from Pennsylvania. In addition, geese were frequently exposed to subtypes that commonly circulate in dabbling ducks. We detected a high prevalence of H5-specific antibodies and these viruses are not well represented among viruses isolated from dabbling ducks. The H5-specific antibody prevalence could be due to increased antibody response to this subtype in Canada geese or circulation of this subtype after peak viral shedding is seen in dabbling ducks. Alternatively, the H5 results may mean that the subtype is better adaptive to circulate in geese as H13 and H16 subtypes are more commonly associated with Charadriiformes than Anseriformes (Krauss et al., 2004; Munster et al., 2007). These results support that Canada geese can be used as a serologic sentinel for IAV distribution on a regional scale; however, additional information related to antibody response in this species and seasonal variation in subtype prevalence are needed to fully interpret serologic data.

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