



HHS Public Access

Author manuscript

Avian Dis. Author manuscript; available in PMC 2022 July 06.

Published in final edited form as:

Avian Dis. 2016 May ; 60(1 Suppl): 341–345. doi:10.1637/11103-042115-Reg.

Antibodies to Influenza A Viruses in Gulls at Delaware Bay, USA

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SUMMARY.

Gulls are the known reservoir for H13 and H16 influenza A viruses (IAV) but also host a diversity of other IAV subtypes. Gulls also share habitats with both ducks and shorebirds, increasing the potential for cross-species IAV transmission. We serologically tested laughing gulls (*Leucophaeus atricilla*) collected at Delaware Bay during May when they were in direct contact with IAV-infected shorebirds; both species feed on horseshoe crab (*Limulus polyphemus*) eggs on beaches during this month. From 2010 to 2014, antibody prevalence as determined by competitive blocking enzyme-linked immunosorbent assay ranged from 25%–72%. Antibodies to H13 and H16 were detected by hemagglutination inhibition (HI) tests in 12% and 24% of tested gulls, respectively. Results from virus microneutralization (MN) tests for antibodies to H1–H12, H14, and H15 varied among years but the highest prevalence of neutralizing antibodies was detected against H1 (24%), H5 (25%), H6 (35%), H9 (33%), and H11 (42%) IAV. The subtype diversity identified by serology in gulls was dominated by Group 1 HA subtypes and only partially reflected the diversity of IAV subtypes isolated from shorebirds.

RESUMEN.

Anticuerpos contra el virus de la influenza A en gaviotas en la Bahía de Delaware, en los Estados Unidos. Las gaviotas son el reservorio conocido de los virus de la influenza H13 y H16 (IAV), pero también son los hospederos de una diversidad de otros subtipos del virus de la influenza. Las gaviotas también comparten hábitat con patos y aves costeras, lo que aumenta la posibilidad de que transmisión cruzada de virus de la influenza entre las especies. Se analizaron de manera serológica las gaviotas reidoras (*Leucophaeus atricilla*) recolectadas en la bahía de Delaware durante el mes de Mayo cuando estaban en contacto directo con aves playeras infectadas por el IAV; ambas especies se alimentan de los huevos de cangrejos de herradura (*Limulus polyphemus*) en las playas durante este mes. De 2010 a 2014, la prevalencia de anticuerpos determinada por un ensayo de inmunoabsorción con enzimas ligadas de tipo competitivo, osciló entre de 25% a 72%. Los anticuerpos para H13 y H16 fueron detectados por pruebas de inhibición de la hemaglutinación (HI) en el 12% y 24% de las gaviotas ensayadas, respectivamente. Los resultados de pruebas de microneutralización viral (MN) para anticuerpos contra H1-H12, H14 y H15 varió entre años, pero la mayor prevalencia de anticuerpos neutralizantes se detectó en contra virus de

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influenza H1 (24%), H5 (25%), H6 (35%), H9 (33%) y H11 (42%). La diversidad de subtipo identificada por serología en gaviotas estaba dominado por subtipos HA grupo 1 y refleja sólo parcialmente la diversidad de subtipos de virus de influenza aislados de aves playeras.

Keywords

antibodies; influenza A virus; laughing gull; *Leucophaeus atricilla* microneutralization; serology

Gulls are a recognized reservoir for influenza A viruses (IAV) and, worldwide, most avian HA subtypes of IAV (HA1-12, H13, H16) have been detected from more than 20 gull species (1). Some HA subtypes, such as H13 and H16, are commonly isolated from gulls and are maintained exclusively within gull populations (5); other subtypes are shared with shorebird and waterfowl species and are reported from gulls less frequently (19). With the exception of the H13 and H16 viruses in fledgling gulls (5,20,21), the reported prevalence of IAV based on virus isolation is generally low (<5%) but can vary with season (1,11,13). In contrast, IAV antibody prevalence estimates in numerous gull species have consistently been high, generally exceeding 50% (2,18).

Like many gull species, laughing gulls (*Leucophaeus atricilla*) can be infected with numerous IAV HA subtypes and are long-distance intercontinental migrants that can range from the northeastern United States to northern South America. Laughing gulls also are an extremely common species at Delaware Bay where a high prevalence of IAV is annually observed in shorebirds that share feeding habitats with this species (9). The source(s) of the viruses that annually infect shorebirds at this site, and the extent of IAV sharing between laughing gulls and shorebirds during this period, are not well described.

Although reports of high IAV antibody prevalence in gulls suggest that serologic data could be utilized to better understand the natural history of IAV in gull populations and provide insight into possible cross-species interactions, recent serologic testing has centered on detecting antibodies to IAV-reactive antigens such as the nucleoprotein (NP). The objectives of this study were to document the presence of IAV neutralizing antibodies in laughing gull populations at Delaware Bay and to determine if subtype diversity, as determined by serology, reflects the IAV subtype diversity present in laughing gulls and cohabiting shorebirds, primarily ruddy turnstones (*Arenaria interpres*), as determined by virus isolation.

MATERIALS AND METHODS

During May 2010–2014, 199 adult laughing gulls (*Larus argentatus*) and six herring gulls (*Scientific name*) were captured with cannon nets on the beaches of Delaware Bay in New Jersey. Combined cloacal and oropharyngeal swabs were collected and tested by virus isolation in chicken eggs (7). Additional virus isolation results from laughing gulls from 1986–2009 were obtained through the Influenza Research Database (IRD) (16). Comparison virus isolation data from cohabiting shorebirds during 2010–2014 were compiled from data provided by St. Jude Children’s Research Hospital and the Southeastern Cooperative Wildlife Disease Study; these birds were sampled and tested as previously described (17).

Serum samples were collected via jugular venipuncture. Serum samples were tested by commercial blocking enzyme-linked immunosorbent assay (bELISA; IDEXX Laboratories, Westbrook, ME) for IAV antibodies (2). Samples testing positive by bELISA were also tested by virus microneutralization (MN). Antigens for MN were prepared in Madin Darby canine kidney cells (MDCK; American Type Culture Collection, Manassas, VA). During virus propagation, and in all MN test procedures, cells were maintained in minimal essential media (MEM; Sigma-Aldrich, St. Louis, MO) containing L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (final concentration of 1 µg/ml; Worthington Biochemical Corporation, Lakewood, NJ) and antibiotics (final concentration of 100 units penicillin, 0.1 mg streptomycin, and 0.25 mg amphotericin B/ml; Sigma-Aldrich). Antigens were stored at -80 C until used. For antibody testing, sera were diluted 1:10 in MEM and heat inactivated at 57 C for 30 min. Serum samples were screened at a 1:20 dilution against all antigens. For the screen, 25 µl of the diluted serum (1:10) were placed into a single well of a 96-well, v-bottom plate corresponding to each antigen. An additional well for each serum sample served as a serum control to determine potential toxicity. A positive control well using chicken antisera to each antigen (provided by the National Veterinary Services Laboratory [APHIS, USDA]) and a negative control well using MEM were also included. Each antigen (25 µl containing 100 median tissue culture infective doses [$10^{2.0}$ TCID₅₀]) was added to each well, not including the serum control wells which received 25 µl of MEM. Plates were incubated for 2 hr at room temperature after which 25 µl from each well was transferred to a second 96-well tissue culture plate with a confluent monolayer of MDCK cells. Prior to transfer, the tissue culture plate containing the MDCK cells was washed two times with Dulbecco's phosphate-buffered saline (Sigma-Aldrich), and 150 µl of trypsin-supplemented MEM was added to each well. The inoculated tissue culture plate was incubated at 5% CO₂ at 37 C and was visually read at 48–72 hr. For the test result to be considered valid, all controls (serum, positive, and negative) had to meet their expected negative or positive status. In addition, based on back titration in MDCK cells (four replicates per dilution), the viral titer of the antigen had to fall within $10^{1.5}$ and $10^{2.5}$ TCID₅₀/25 µl. Sera were considered positive on the screen if complete neutralization (no cytopathic effect [CPE]) was observed. All positive serum samples were titrated. Each positive serum sample was diluted 2-fold in MEM on a 96-well, v-bottom plate (final volume of 25 µl/well at dilutions 1:20 to 1:640) and tested as described above. If CPE was observed at the minimum 1:20 dilution, the sample was classified as negative; if not, the positive titer was recorded as the highest dilution at which no CPE was observed. Viruses used as antigens in the MN assays included A/mallard/NJ/AI10-4263/2010 (H1N1), A/mallard/MN/AI08-2755/2008 (H2N3), A/mallard/MN/AI10-2593/2010 (H3N8), A/mallard/MN/AI10-3208/2010 (H4N6), A/mallard/MN/AI11-3933/2011 (H5N1), A/mallard/MN/AI08-2721/2008 (H6N1), A/mallard/MN/AI08-3770/2009 (H7N9), A/mallard/MN/SG-01048/2008 (H8N4), A/RUTU/DE/AI11-809/2011 (H9N2), A/mallard/MN/SG-00999/2008 (H10N7), A/mallard/MN/SG-00930/2008 (H11N9), A/mallard/MN/SG-3285/2007 (H12N5), A/blue-winged teal/TX/AI13-1028/2013 (H14N5), and A/wedge-tailed shearwater/Western Australia/2327/1983 (H15N6).

Because representative H13 and H16 IAVs could not be propagated in MDCK cells supplemented with trypsin, hemagglutination inhibition (HI) tests were used to test for antibodies against these viruses, as described (14). Viruses used for HI tests included A/AWPE/MN/AI07-1819/2007 (H13N9) and A/ring-billed gull/DE/AI10-1144/2010 (H16N3).

RESULTS

From 2009–2014, 13 IAV were isolated from laughing gulls including H5N3 ($n = 1$), H10N2 ($n = 2$), H10N7 ($n = 2$), H10N8 ($n = 2$), H11N2 ($n = 1$), H11N4 ($n = 1$), H11N9 ($n = 2$), H12N2 ($n = 1$), and H16N3 ($n = 1$). Additionally, 24 IAV isolates reported from laughing gulls from 1986–2008 included H1N9 ($n = 2$), H2N7 ($n = 1$), H3N6 ($n = 1$), H4N6 ($n = 1$), H6N3 ($n = 1$), H6N8 ($n = 4$), H7N3 ($n = 1$), H9N1 ($n = 1$), H9N5 ($n = 1$), H9N9 ($n = 1$), H10N2 ($n = 1$), H11N1 ($n = 2$), H11N2 ($n = 1$), H11N9 ($n = 1$), H13N2 ($n = 1$), H13N9 ($n = 1$), and H16N3 ($n = 4$) (16). Overall, of the 1296 virus isolation attempts from laughing gulls reported in the IRD that were sampled in Delaware, New Jersey, and New York from 1986 to 2014, 40 IAV were isolated representing a prevalence of 3.1%.

Antibodies to IAV were detected in 61% of 199 laughing gulls sampled from 2010–2014 (Table 1). Antibody prevalence estimates varied by year ranging from 25%–72%. Of the six herring gulls sampled during 2012 and 2014, three (50%) tested positive for IAV antibodies. Results from MN ($n = 104$) and HI ($n = 82$) testing of bELISA-positive serum samples from laughing gulls are shown in Table 2. Positive MN results were detected in 82 of the 104 bELISA-positive samples. Of these 25, 18, 22, 5, 7, 3, and 2 samples tested positive to 1, 2, 3, 4, 5, 6, and 7 HA subtypes by MN, respectively. Antibody prevalence varied by subtype and was highest for H1, H5, H6, H9, H11, H13, and H16 (Table 2; Fig. 1). As shown in Figure 1 where HA subtypes are arranged based on their phylogenetic relatedness (6), all of these HA subtypes are included in Group 1.

Serologic results from gulls only partially reflect the subtype diversity present in shorebirds. As shown on Table 2, H5, H6, and H11 IAV were isolated from the shorebird population during most years, and in 2010 and 2011, H6 and H9 represented the predominant subtypes isolated from shorebirds, respectively. In both of those years, neutralizing antibodies to these HA subtypes were detected in gulls. In contrast, although a high prevalence of H1 antibodies was detected in gulls, this subtype was poorly represented in shorebird isolates during this same period. With the H10 and H12 viruses which together represent predominant shorebird isolates in three of the five years (Table 2), antibody prevalence in gulls was consistently low, with neutralizing antibodies detected in less than 6% of the 104 bELISA-positive laughing gulls. During years when H10 IAV (2013) and H12 IAV (2012 and 2014) predominated in shorebirds, antibodies to these HA subtypes were not detected in gulls (Table 2).

DISCUSSION

The low prevalence of reported virus isolation-positive laughing gulls and the high prevalence of antibodies to NP were consistent with previous reports from gull studies. The observed annual variation in prevalence (25%–72%) cannot be explained but may have

resulted from variations in the prevalence and subtype diversity present in the shorebird populations, variation in adult gull age structure during an individual year, or the timing of gull sampling. At Delaware Bay, subtype diversity varies by year, and peak prevalence associated with these annual outbreaks can be short-lived and limited to a single week (12).

Subtype diversity present in the reported virus isolates from laughing gulls from 1986–2014 included representatives of all HA subtypes except H8, H14, and H15. Antibodies to all HA subtypes except H15 were also detected in laughing gulls sampled at Delaware Bay from 2010–2014. Predominant subtypes identified by serologic results, however, were skewed toward Group 1 HAs, specifically H1, H5, H6, H9, H11, H13, and H16 (Table 2; Fig. 1). All of these subtypes, except H1, H9, and H13, were represented in recent (2009–2014) virus isolation data from laughing gulls sampled at Delaware Bay. The predominance of these Group 1 subtypes in laughing gulls also is consistent with virus isolation results reported for ring-billed gulls (*Larus delawarensis*) sampled in Maryland during 1977–1979; the 70 virus isolations reported in that study included H2, H5, H6, H9, H11, and H13 IAV (6). A total of 234 IAV isolates from species in the family Laridae worldwide (including Canada, Republic of Georgia, Iceland, Netherlands, and the United States) are reported in the IRD (16). Of these, 145 are H13 and H16 IAV. The remaining 89 isolates include H1–H7 and H9–H12 (Fig. 2); however, H1, H2, H6, and H11 IAVs represented 68% of these 89 isolates. Although the H1, H5, H6, H9, H11, and other Group 1 HA subtypes appear to be prevalent in gulls, they are not restricted to gulls and occur frequently in both waterfowl and shorebirds (10), and in some cases, viruses shared between waterfowl and gulls may be genetically identical (19). The high prevalence of antibodies to Group 1 IAV and their overrepresentation in reported isolates from gulls (Fig. 2) may reflect some degree of gull adaptation, as occurs with H13 and H16. Evidence for a potential gull lineage of H11 viruses was recently reported based on phylogenetic characterization of viruses isolated from gulls in Georgia (11).

The inconsistency observed between HA subtype diversity, as indicated by neutralizing antibodies in gulls and virus isolation from shorebirds, may reflect host-related variation in gull *vs.* shorebird susceptibility; it may also relate to viral source. At present, the sources of IAV that infect shorebirds annually at Delaware Bay are not understood, but the multiple IAV subtypes annually present at this site probably represent viruses that migrate with shorebirds and viruses that are transmitted to shorebirds by gulls or ducks during spring migration or after arrival at Delaware Bay (3,15). The high prevalence of H11 antibodies in gulls and the occurrence of these viruses in shorebirds during most years at this site are consistent with a pattern of local interspecies transmission that could reflect either a gull or shorebird source. Isolates of H11 IAV from wintering ruddy turnstones in Brazil are most related genetically to isolates from ruddy turnstones from Delaware Bay (4); wintering areas in South America also are utilized by laughing gulls. In contrast, the frequent occurrence of H12 in shorebirds with limited evidence of infection and spillover in gulls is consistent with a pattern of local transmission that is more restricted within shorebird populations. The H12 viruses have been reported from laughing gulls at Delaware Bay during surveillance from 1986–2014 but represent only 3% of the observed HA diversity. Also, the H12 IAVs are not well represented in isolates reported from gulls in the IRD, where they represent only 2 of 234 (1%) isolates (16). Variation in the ability of IAV derived from shorebirds to

replicate in ducks has been previously demonstrated (8). Based on results from our study, host differences may also exist between gulls and shorebirds.

Serologic data, especially related to IAV, are difficult to interpret due to potential cross-reactions between subtypes, lack of comparative data between different tests, and limited information related to host immune response. This is especially true with serologic data derived from wild birds such as gulls, which are long lived and can be infected numerous times with different IAV subtypes during their lifetime. It is possible that the high prevalence of antibodies to the Group 1 HAs was exaggerated by cross-reactive antibodies between related HAs. Although we cannot discount this possibility, the low antibody prevalence to other Group 1 HAs (H2 and H8) suggests that these effects were minimal. With regard to test format, it is likely that the low prevalence of H13 and H16 antibodies as compared to H1, H5, H6, and H11 reflected differences in HI and MN test sensitivity rather than true differences. Although HI antibody prevalence estimates for H13 and H16 viruses were low compared to neutralizing antibodies of H1–H12, H14, and H15, the H13 and H16 HA subtypes are the most common viruses isolated from gulls worldwide (1) and locally at Delaware Bay (10,17). It also is unknown how long an antibody response for a given subtype is detectable by either MN or HI. Without knowledge on the duration of the detectable immune response, it is difficult to directly compare serologic and virus isolation data. The potential long-term persistence of antibodies and the relatively short duration of IAV shedding may explain some of the inconsistency observed in this study, such as the detection of antibodies to H1 with few virus isolates from gulls or cohabiting shorebirds during the sampling period. Although additional work is needed to improve our ability to interpret serologic data from gulls and other wild birds, supporting subtype-specific serologic data can provide a unique perspective to better define IAV subtype diversity present in avian species or taxonomic groups and to identify potential species interactions that may facilitate IAV maintenance within the wild bird reservoir.

ACKNOWLEDGMENTS

We thank Larry Niles, Amanda Dey, Benjamin Wilcox, Morgan Slusher, and the many volunteers who captured the gull and shorebirds tested in this study. This work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract HHSN272201400006C. The funding agencies were not involved in the design, implementation, or publishing of this study, and the research presented herein represents the opinions of the authors but not necessarily the opinions of the funding agencies.

Abbreviations:

CPE	cytopathic effect
HI	hemagglutination inhibition
IAV	influenza A virus
IRD	Influenza Research Database
MEM	minimal essential media
MDCK	Madin Darby canine kidney cells

MN	microneutralization
NP	nucleoprotein
TCID₅₀	median tissue culture infective doses

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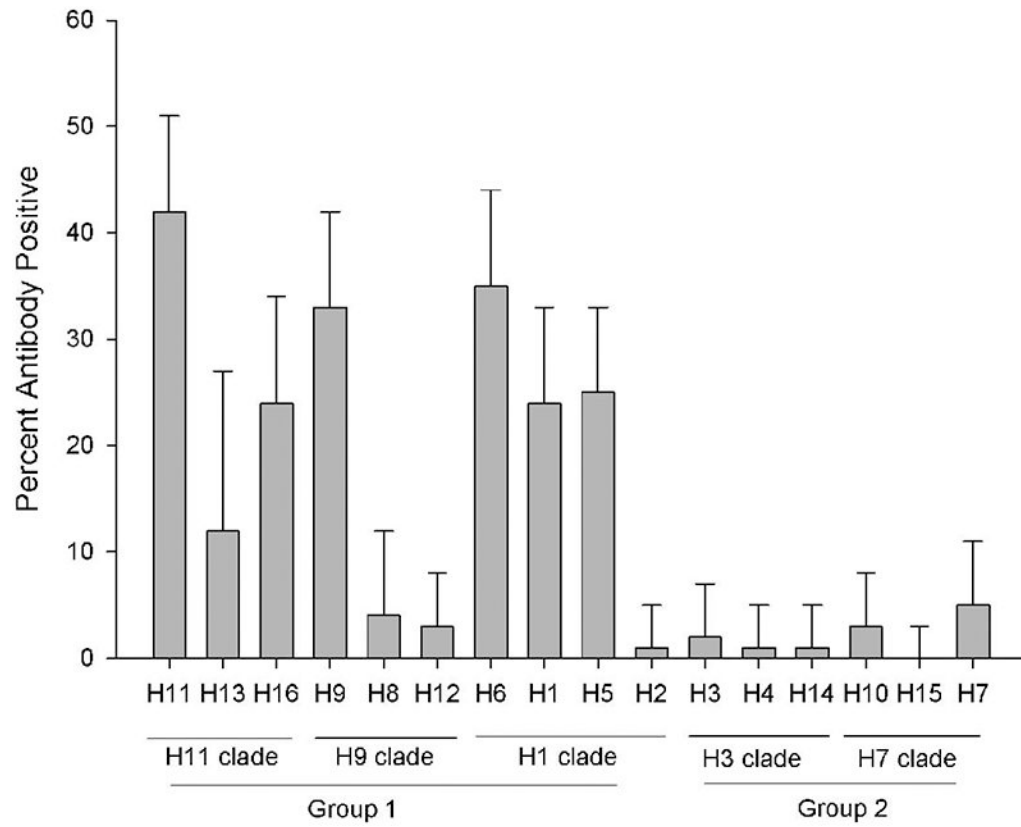


Fig. 1. Hemagglutinin-specific antibody prevalence in laughing gulls as determined by MN (H1–12, 14, and 15) and HI (H13 and 16). Subtypes as arranged based on phylogenetic relatedness. Error bars represent 95% confidence limits.

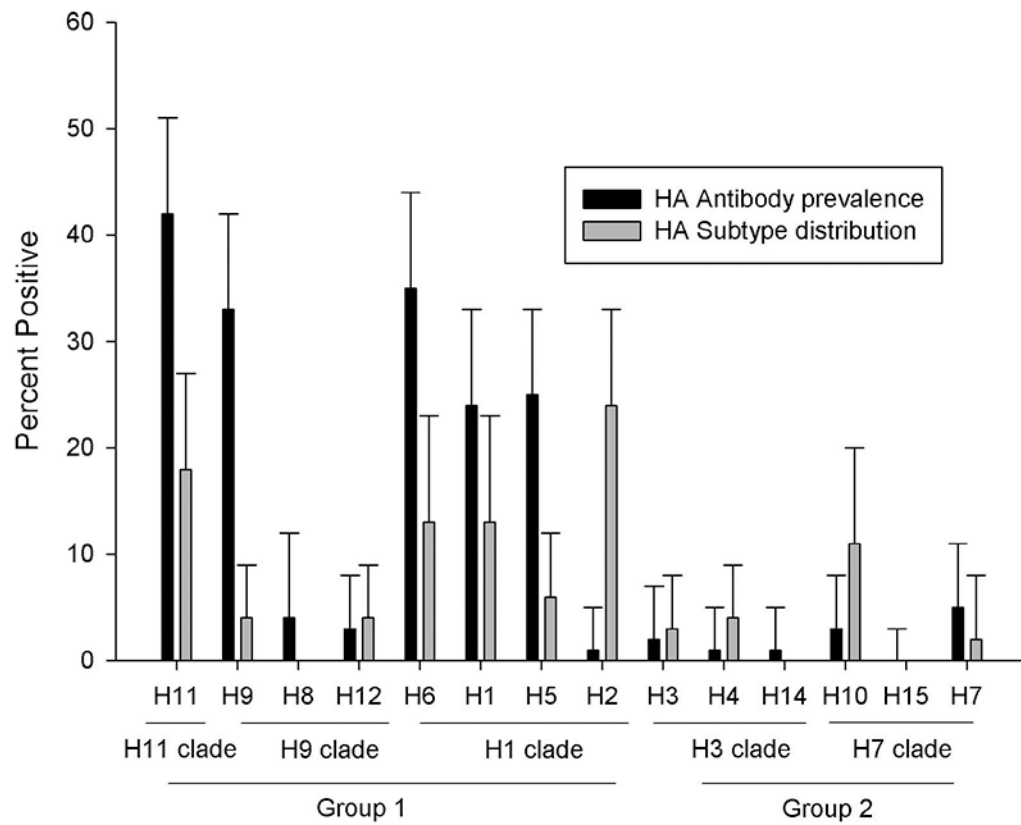


Fig. 2. Antibody prevalence observed in laughing gulls (based on 104 and 82 samples tested by MN and HI, respectively) and HA subtype diversity represented in 234 reported IAV isolations from Laridae worldwide as included in the IRD (16). Error bars represent 95% confidence limits.

Table 1.

Prevalence of antibodies to influenza A viruses as detected by bELISA in laughing gulls sampled at Delaware Bay, New Jersey, U. S. A.

Year	Number tested	No. positive	% positive
2010	32	23	72
2011	28	7	25
2012	14	9	64
2013	80	54	68
2014	45	28	62
Total	199	121	61

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Table 2.

Subtype-specific antibodies detected from laughing gulls sampled at Delaware Bay, NJ as determined by microneutralization (MN) and hemagglutination inhibition (HI) tests.

Year	MN											HI				
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H14	H15	H16	
2010	1/15 (6) ^A	0/15 ^B	0/15	0/15	4/15 (23)	3/15 ^C (17)	0/15	0/15	5/15 (29)	1/15 (6)	5/15 (29)	0/15	1/15	0/15	NT	NT
2011	1/5 (20)	0/5	0/5	0/5	1/5 (20)	1/5 (20)	0/5	0/5	<i>1/5 (20)</i>	0/5	1/5 (20)	0/5	0/5	0/5	NT	NT
2012	2/9 (22)	1/9 (11)	0/9	0/9	1/9 (11)	1/9 (11)	0/9	0/9	2/9 (40)	0/9	2 (40)	0/9	0/9	0/9	2/9 (22)	4/9 (44)
2013	19/49 (39)	0/49	2/49 (4)	1/49 (4)	14/49 (29)	22/49 (45)	3/49 (6)	3/49 (4)	27/49 (55)	0/49	27/49 (55)	3/49 (6)	0/49	0/49	3/47 (6)	9/47 (18)
2014	2/26 (8)	0/26	0/26	0/26	6/26 (23)	9/26 (35)	2/26 (4)	0/26	9/26 (35)	2/26 (4)	9/26 (35)	0/26	0/26	0/26	10/26 (38)	7/26 (27)
Total (%)	25/104 (24)	1/104 (1)	2/104 (2)	1/104 (1)	26/104 (25)	36/104 (35)	5/104 (5)	4/104 (4)	35/104 (33)	3/104 (3)	44/104 (42)	3/104 (3)	1/104 (1)	0/104 (0)	15/82 (12)	20/82 (24)

^ANumber positive/number tested (% positive). All tested sera also tested positive on bELISA for antibodies to type A influenza virus.

^BItalicized data indicate the presence of that subtype in avian influenza virus isolates from shorebirds, primarily ruddy turnstones, sampled at the same time.

^CBoldface italicized data represent the predominant HA subtype detected that year in shorebirds.