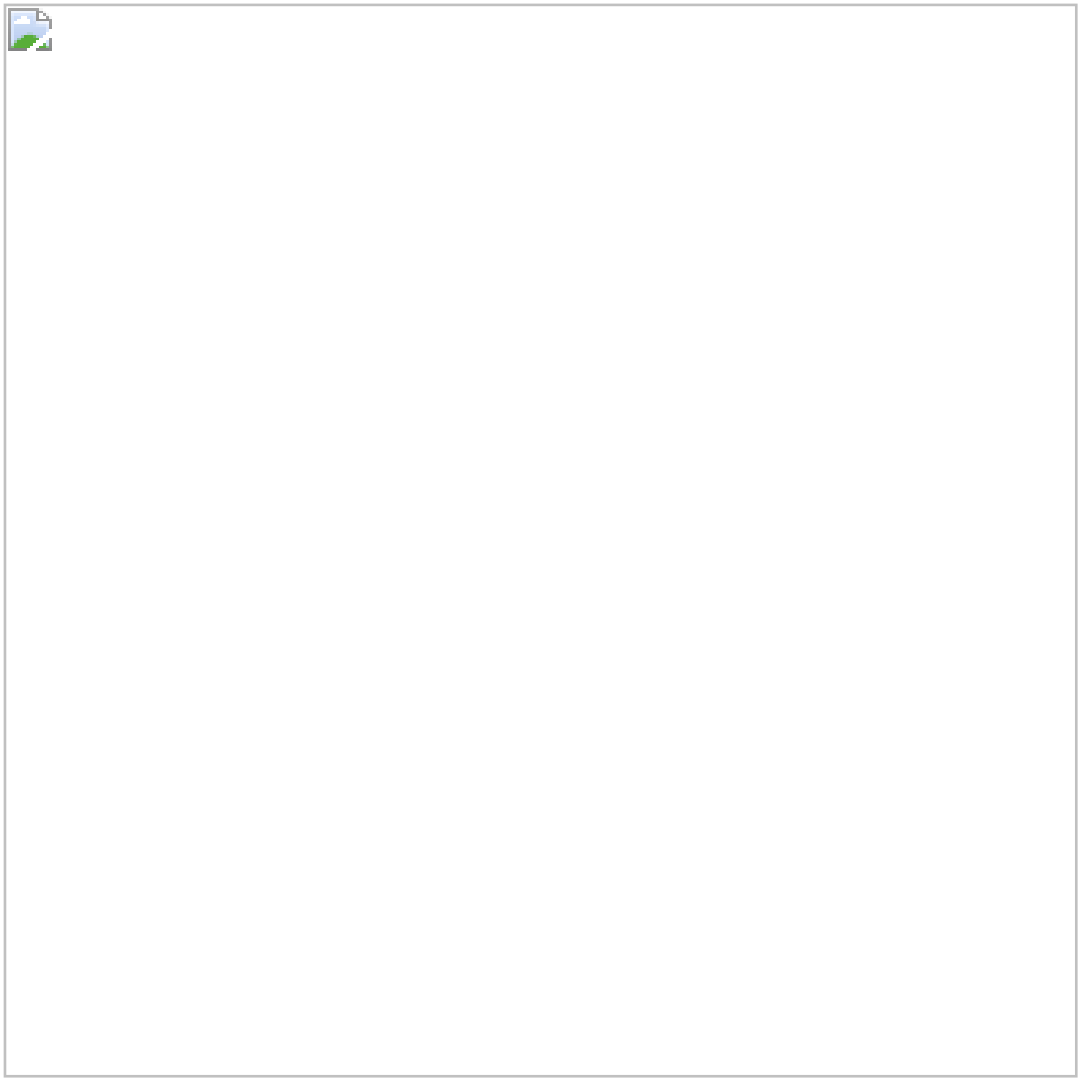

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Source: Journal of Wildlife Diseases, 31(2) : 179-185

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-31.2.179

Journal of Wildlife Diseases, 31(2), 1995, pp. 179–185

INFLUENZA A VIRUSES ISOLATED FROM WATERFOWL IN TWO WILDLIFE MANAGEMENT AREAS OF PENNSYLVANIA

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ABSTRACT: A survey was conducted at two wildlife management areas of Pennsylvania (USA) to evaluate an antigen capture enzyme-linked immunosorbent assay (AC-ELISA) for the detection of avian influenza viruses (AIV) in cloacal swabs from waterfowl and to determine the influenza A virus subtypes and the distribution of these viruses among waterfowl. We collected 330 cloacal swabs from hunter-killed waterfowl in the fall of 1990 and from cage-captured waterfowl in the summer of 1991. Thirty-one hemagglutinating agents were isolated by chicken embryo inoculation (CEI) of which 27 were influenza A viruses and four Newcastle disease viruses (NDV). The prevalence of AIV infection was 8.2%. Compared to CEI, AC-ELISA was only 15% sensitive and 61% specific. Based on the distribution of AIV by species of waterfowl, mallards (*Anas platyrhynchos*) and American wigeons (*Anas americana*) were at equal risk of AIV infection even though most of the AIV isolates came from mallards. Although significant crude effects of sampling site and season on AIV recovery could be established, juvenile age was identified as the primary risk factor of AIV recovery. Twelve AIV subtypes were identified by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests. The most prevalent subytpes were H4N8 and H6N8. We concluded that AC-ELISA was not useful for the detection of AIV in cloacal swabs from waterfowl and that CEI, HI, and NI tests remain as the method of choice for AIV screening in waterfowl. Based on the results AIV infected preferentially the young which represent the high risk group in waterfowl populations. The results from the AIV subtyping in our waterfowl survey are consistent with the results from numerous longitudinal studies of waterfowl in North America.

Key words: Orthomyxoviridae, influenza A viruses, avian influenza viruses, waterfowl survey, enzyme-linked immunosorbent assay, ELISA.

INTRODUCTION

Influenza A viruses, representing the 14 hemagglutinin and the nine neuraminidase subtypes in almost all possible combinations, have been isolated from waterfowl and shorebirds, the natural reservoir of these viruses (Hinshaw et al., 1980; Kawaoka et al., 1988, 1990). Waterfowl may play an important role in the generation, transmission, and spread of avian influenza (AI) (Hinshaw et al., 1979; Karunakaran et al., 1983; Webster et al., 1992). Information on avian influenza viruses (AIV) subtypes circulating in waterfowl can help identify AIV potentially pathogenic for poultry. The most commonly used laboratory method for AIV screening in free-living birds is chicken embryo inoculation (CEI) (Burnet, 1936; Hirst, 1941) accompanied by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests for virus subtyping (Aymard-Henry et al., 1973; Pearson and Senne, 1986). Our objectives were to compare an antigen capture enzyme-linked immunosorbent assay (AC-ELISA) with CEI for the detection of influenza A viruses in cloacal swabs from waterfowl, and to determine the influenza A virus subtypes circulating in waterfowl and the distribution of these viruses in avian populations at two wildlife management areas of Pennsylvania (USA).

MATERIALS AND METHODS

Two areas in Pennsylvania were selected for this study: Pymatuning Wildlife Management Area (41°37′N, 80°30′W) and Middle Creek Wildlife Management Area (40°16′N, 76°15′W). Pymatuning, located in the northwestern corner of Pennsylvania at the Pennsylvania-Ohio (USA) border, is the largest (10,121 ha) inland impoundment in the state; 6,883 ha are in water and 3,239 ha occur on land. Pymatuning provides habitat for an estimated 25,000 to 35,000 non-resident geese and ducks during the fall and spring migrations.

Middle Creek is located in southeastern Pennsylvania and consists of 2,024 ha. Middle Creek has a 162-ha shallow water lake which includes several nesting islands, and 28 ha of water impounded in a series of potholes, ponds, and dikes.

These areas provide resting, loafing, feeding, and nesting areas for resident and migratory species.

We collected 330 cloacal swabs in the study; 94 cloacal swabs were collected in Pymatuning and 45 in Middle Creek from hunter-killed waterfowl during the hunting season in the fall of 1990. We also collected 120 cloacal swabs in Pymatuning and 71 in Middle Creek from birds cage-captured for population census during the summer of 1991.

Every cloacal swab was collected with sterile cotton applicators and placed in 2 ml of phosphate buffered saline (PBS) supplemented with 10,000 units/ml of penicillin (Sigma Chemical Company, Saint Louis, Missouri, USA), and 10 mg/ml of streptomycin (Sigma Chemical Company). Swabs were stored at −70 C until tested. At the time of assay, swabs were thawed, the cotton applicator removed, and fluid centrifuged at 1,500 rpm for 15 min. The supernatant was sonicated three times for 10 sec each at 120 watts in a Tekmar sonic disrupter (Tekmar® Company, Cincinnati, Ohio).

Virus isolation was done by CEI (Burnet, 1936). Two-hundred μl of swab supernatant were inoculated into the allantoic sac of two 10-day-old chicken embryos. Embryos were incubated at 37 C for 48 hr and allantoic fluid was evaluated by hemagglutination (HA) test for the presence of HA activity (Hirst, 1941). Allantoic fluid with hemagglutinating activity was tested by HI and NI tests for specificity to influenza A virus and for virus subtype, respectively (Aymard-Henry et al., 1973; Pearson and Senne, 1986). Influenza A virus subtyping was done by the National Veterinary Services Laboratories, Ames, Iowa (USA).

Four-and-a-half μg/ml of chromatographically purified goat immunoglobulin G (IgG) antibody to whole influenza A/USSR/90/77 (H1N1) and A/Victoria/1/75 (H3N2) virion (Virostat, Portland, Maine, USA) diluted in coating buffer (0.1 M PBS pH 8.8, ELISAmate®, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA) were allowed to attach to 96-well flat-bottom polystyrene microplates (Immunolon®, Dynatech Laboratories, Inc, Chantilly, Virginia, USA) for 2 hr at 4 C. After washing the plates with a solution of 0.5 M NaCl and 0.05% Tween-20 (Sigma Chemical Company), 100 μl of either cloacal swab or allantoic fluid were added and allowed to incubate for 4 hr at 37 C. The plates were washed and 100 μl of a 1:1,000 dilution of mouse monoclonal antibody to influenza A nucleoprotein (NP) in diluent-blocking buffer (0.1 M PBS pH 7.5, 0.05% Tween-20, 1% bovine serum albumin, ELISAmate®, Kirkegaard and Perry Laboratories, Inc.) were added. The plates were incubated for 4 hr at 4 C and again washed. One-hundred μl containing 0.5 μl/ml of biotinylated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc.) diluted in diluent-blocking buffer was added and allowed to incubate for 1 hr at 18 to 20 C. After washing, 100 μl containing 0.5 μg/ml of horse radish peroxidase labeled streptavidin (Kirkegaard and Perry Laboratories, Inc.) diluted in diluent blocking buffer were added. The plates were incubated for 1 hr at 18 to 20 C and washed again. One-hundred μl of 2,2'-Azino-bis (ethyl-benzthiazoline-6-sulfonic acid) (Kirkegaard and Perry Laboratories, Inc.) were added and the absorbance recorded at dual wavelength of 405 and 630 nm on a Microplate Reader EL-311 (Bio-Tek Instruments, Inc., Winooski, Vermont, USA).

A set of low positive, medium positive, and high positive samples containing 5.8×10^5, 4.6×10^6, and 3.7×10^7 embryo infectious dose $(EID)_{50}$ of Ty/Ont (H5N9), respectively, were included in every plate. Positive controls were prepared in diluent-blocking buffer. Negative controls consisted of diluent buffer only and also were included in every plate. Plates were read when the optical density (OD) of high positive control wells reached approximately 1.0. Controls were run in triplicates and case samples in duplicates. The OD cut-off point was the sum of the mean OD of three observations of the negative control plus three standard deviations (SD). A sample with mean absorbance equal to or less than the OD cut-off point was considered negative. A sample with mean absorbance higher than the OD cut-off point was considered positive.

The AC-ELISA results were compared to the results from CEI for the detection of AIV using the Bayes's analysis (Brown, 1981). Prevalence of infection (%), sensitivity (%), specificity (%), and likelihood ratios were calculated using a 2 × 2 contingency table (Brown, 1981) and the post-test probabilities of AC-ELISA test results were calculated using the nomogram for Bayes's theorem (Fagan, 1975). The crude effect of species, age, sex, sampling site, and season on the frequency of AIV recovery was tested by chi-square analysis (SAS Institute Inc., Cary, North Carolina, USA). Crude effect refers to the probability that changes in the frequency of virus recovery were due to the effect of, i.e., species and not to random variation. A P value of ≤ 0.05 was considered statistically significant.

The crude prevalence odds ratios for the effect of age, sampling site, and season on the probability of AIV recovery were calculated for 2 × 2 contingency tables according to the method of Cornfield (1951). These ratios measure the strength of association between a risk factor and AIV revocery. The Mantel-Haenszel (1959)

TABLE 1. Frequency of avian influenza viruses (AIV) recovery from waterfowl by species, sampling site, and season in Pennsylvania.

Species of waterfowl	Middle Creek		Pymatuning		Total by species
	Fall 1990	Summer 1991	Fall 1990	Summer 1991	
American wigeon	0/2[a]	0/0	2/17	0/0	2/19 (11)[a]
Mallard	0/26	2/69	4/29	19/116	25/240 (10)
Others[b]	0/17	0/2	0/48	0/4	0/71 (0)
Total by site and season	0/45 (0)	2/71 (2.8)	6/94 (6.4)	19/120 (16)[c]	27/330 (8.2)

[a] Number infected/number sampled (prevalence of AIV recovery in percent).

[b] Others include wood duck (n = 25), gadwall (n = 10), green-winged teal (n = 10), black duck (n = 9), Canada goose (n = 5), northern pintail (n = 4), blue-winged teal (n = 2), hooded merganser (n = 2), northern shoveler (n = 2), and ring-necked duck (n = 2).

[c] Significant ($P < 0.05$) crude effect of sampling site and season on AIV recovery.

method of estimating summary odds ratios was used to estimate the strength of association between age and AIV recovery adjusting for the effect of sampling site.

RESULTS

Based on CEI, we found AIV in 27 (8.2%) of 330 waterfowl sampled (Table 1). To evaluate the AC-ELISA as a screening test for AIV in cloacal swabs from waterfowl, the AC-ELISA was compared with CEI. From the 330 samples collected, 31 hemagglutinating agents were recovered by CEI; 27 were AIV and four were Newcastle Disease Virus (NDV). The AC-ELISA accurately detected only four of 27 true AIV positives, and differentiated 183 of 299 true negatives. The number of false negatives and false positives was 23 and 118, respectively. Compared to CEI, AC-ELISA was only 15% sensitive and 61% specific. The negative and positive likelihood ratios were 1.39 and 0.39, respectively. According to the nomogram for Bayes's theorem, the post-test probability of a positive AC-ELISA result was only 3% and the one of a negative AC-ELISA result was 13%. Thus, AC-ELISA was not useful as a screening test for AIV in cloacal swabs from waterfowl.

Twelve species of waterfowl were sampled in this survey: American wigeon (*Anas americana*), American black duck (*Anas rubripes*), blue-winged teal (*Anas discors*), Canada goose (*Branta canadiensis*), gadwall (*Anas strepera*), green-winged teal (*Anas crecca*), hooded merganser (*Lophodytes cucullatus*), mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta*), northern shoveler (*Anas clypeata*), ring-necked duck (*Aythya collaris*), and wood duck (*Aix sponsa*) (Table 1). The diversity of species in the sample was greater in the fall of 1990 than in the summer of 1991 in both Pymatuning and Middle Creek. Mallards were the most abundant species in both the fall of 1990 and the summer of 1991. In this survey, influenza A viruses were recovered from two species of waterfowl American wigeon (*A. americana*) and mallard (*A. platyrhynchos*). Based on a chi-square analysis, sampling site (P = 0.002) and season (P = 0.03) had a significant crude effect on the frequency of AIV recovery. On the contrary, species did not have a significant effect on the frequency of AIV recovery, based on a chi-square analysis.

The waterfowl sampled included 105 juvenile, 97 adult, and 128 birds of unknown age (Table 2). The sampled population included 143 females, 179 males, and eight birds of unknown sex. The unknown-age and unknown-sex categories included birds whose age or sex were not recorded or could not be determined at the time of sampling. Based on a chi-square analysis, there was a significant ($P < 0.001$) crude effect of age on the frequency of AIV recovery. On the contrary, there was

TABLE 2. Frequency of avian influenza viruses (AIV) recovery from waterfowl by age and by sex in Pennsylvania, 1990 and 1991.

	Females	Males	Unknown	Total by age
Juvenile	8/47[a]	11/58	0/0	19/105 (18)[a,b]
Adult	1/46	1/51	0/0	2/97 (2.0)
Unknown	1/50	4/70	1/8	6/128 (4.7)
Total by sex	10/143 (7.0)	16/179 (8.9)	1/8 (12.5)	27/330 (8.2)

[a] Number infected/number sampled (prevalence of AIV recovery in percent).
[b] Significant ($P < 0.05$) effect of age on AIV recovery.

no significant crude effect of sex on the frequency of AIV recovery with a chi-square analysis.

The adult, Middle Creek, and fall of 1990 categories were used as the control risk factors for age, site, and season, respectively. Based on the crude prevalence odds ratios for age, AIV was 10.5 times more likely to be recovered from juvenile than adult birds (Table 3). On the other hand, AIV was 2.34 times more likely to be recovered from waterfowl of unknown age than adults. In terms of sampling site, AIV was 7.59 times more likely to be recovered from waterfowl sampled at Pymatuning than at Middle Creek. Similarly, AIV was 2.74 times more likely to be recovered from birds sampled in the summer of 1991 than birds sampled in the fall of 1990 (Table 3). Based on the test statistics of the prevalence odds ratios, there was a significant ($P < 0.005$) effect of juvenile age, Pymatuning sampling site, and summer of 1991 sampling season on the probability of AIV recovery. Based on the summary odds ratio, AIV was 6.35 times more likely to be recovered from juvenile than adult birds when the confounding effect of sampling site was adjusted. A chi-square analysis indicated that this association was significant ($P < 0.025$).

We observed eight combinations of HA and NA in the 27 hemagglutinating allantoic fluids analyzed by HI and NI test. The AIV subtypes included one H2N3, two H4N2, one H4N6, six H4N8, 13 H6N8, and one H10N7. Two isolates had unidentifiable HA and one had unidentifiable NA. The prevalent AIV subtypes circulating in waterfowl were H4N8 and H6N8; the former more predominant in the fall of 1990, the latter more prevalent in the summer of 1991. In addition to the AIV, four NDV isolates were recovered.

DISCUSSION

Based on our results, we conclude that AC-ELISA was of little usefulness compared to CEI for the screening of AIV in waterfowl populations. Several factors may account for the low sensitivity and fair specificity of AC-ELISA. First, CEI requires infectious particles to amplify the virus, whereas, AC-ELISA detects virus antigen and does not amplify the infectious virus (Hietala et al., 1988). Second, the detection limit of AC-ELISA was several orders of magnitude higher for AIV in cloacal swabs than in tracheal swabs and tissue homogenate (C. P. Alfonso, B. S. Cowen, H. Van Campen, unpubl.). In addition, virus detection was significantly increased by sonication; thus, aggregation of virus particles in cloacal swab samples may mask epitopes recognized by the antibodies in AC-ELISA. Taking into account that the samples from waterfowl in this study were sonicated, the virus concentration in cloacal swabs may be under the detection limit of the test. Inhibitory or proteolytic factors in avian cloacal swabs also may inhibit virus binding to the coating antibody or may degrade the reagents in the assay. Finally, the fairly low specificity of AC-ELISA for AIV in cloacal swabs from waterfowl may be due to the high nonspecific binding which led to undesirable high background absorbance in the test.

Reports on prevalence of AIV infection

TABLE 3. Crude prevalence odds ratios for the effect of age, site, and season on the probability of avian influenza viruses (AIV) recovery.

Risk factor	Category	Total number	Infected	Non-infected	Odds ratio	Variance	95% confidence interval	
							Lower	Upper
Age	Juvenile	105	19	86	10.5*	0.6	2.4	46
	Unknown	128	6	122	2.3	0.7	0.5	12
	Adult	97	2	95	Control	—	—	—
Site	Pymatuning	214	25	189	7.6*	0.6	1.8	33
	Middle Creek	116	2	114	Control	—	—	—
Season	Summer 1991	191	21	170	2.7*	0.2	1.1	7
	Fall 1990	139	6	133	Control	—	—	—

* Significant ($P < 0.05$) effect on the probability of AIV recovery.

in waterfowl range from 0.6% to 26% (Hinshaw et al., 1980; Turek et al., 1983; Nettles et al., 1985; Otsuki et al., 1987; Slemons et al., 1991). The overall prevalence of AIV infection (8.2%) in this study was within the range of similar studies in the USA and other countries.

Influenza A viruses most frequently have been isolated from mallard ducks (Hinshaw et al., 1980; Kocan et al., 1980; Deibel et al., 1985; Hinshaw et al., 1986) but can be isolated also from other species of waterfowl (Nettles et al., 1985; Stallknecht et al., 1990; Graves, 1992). In our study, AIV were isolated from only two species, mallard and American wigeon. Even though 25 of the 27 virus isolations came from mallards, mallards and American wigeons were equally at risk for AIV infection. Unlike Deibel et al. (1985), we did not find a significant effect of species on AIV recovery. In order to quantitatively demonstrate or discard a particular species effect on AIV recovery, sufficient number of birds per species must be sampled. Our findings are in agreement with those of Hinshaw et al. (1986) and Slemons et al. (1991) and may be evidence that the importance of mallards as a reservoir of AIV is related to the abundance of this species in North America and not to a particular property of mallards to harbor the virus.

We identified at least three confounders or biases in this study. The first confounder was the low number of birds sampled per species other than mallards. The second confounder was the large number of birds of unknown age in the sample population. The third confounder was the effect of sampling site on virus recovery. To adjust for these confounders, species with sample size less than 10 and birds of unknown age were excluded when analyzing the frequency of AIV recovery. Since age was the risk factor of primary importance, the data were stratified according to the confounding effect of sampling site to determine the unbiased effect of age on AIV recovery (Mantel and Haenszel, 1959).

Our findings are evidence that even though significant crude effects of site and season on AIV recovery could be established, these associations were biased and that juvenile age was the primary risk factor for AIV recovery. This age effect is in agreement with previous findings that AIV preferentially infects the young which represent the high risk group in waterfowl populations (Hinshaw et al., 1980; Slemons et al., 1991). Similar to other waterfowl surveys (Deibel et al., 1985; Slemons et al., 1991), a significant effect of sex on the frequency of AIV recovery was not found in our study.

Another likely confounder was the differences in sampling methods (hunter-killed in the fall 1990 versus cage-captured birds in the summer 1991) which could have influenced the frequency of AIV recovery. During the controlled hunting seasons in Pennsylvania, hunters are required to present their kill to the administration

center before leaving the premises. Since changes in carcass temperatures were not recorded, this bias may not be completely eliminated from the study. However, postmortem intervals may have not had a detrimental effect on virus recovery since all birds were sampled at the administration center within 2 to 3 hr after hunting.

Several AIV subtypes were found in the waterfowl sampled. The most common subtypes were H4N8 and H6N8. Influenza A viruses change from year to year and subtypes of all possible HA and NA combinations can be isolated (Deibel et al., 1985; Kawaoka et al., 1990; Slemons et al., 1991). In longitudinal studies of wild ducks in Canada from 1976 to 1990, hemagglutinin H4, and H6, and neuraminidase N8 were found consistently whereas hemagglutinin H2 and H10 and neuraminidase N3, N2, N6, and N7 were found sporadically (Webster et al., 1992). Hemagglutinin H5 and H7 were found rarely (Hinshaw et al., 1986; Webster et al., 1992). The results in our waterfowl survey are consistent with the results of other surveys.

In sum, the CEI, HI, and NI tests remain as the methods of choice for AIV screening in waterfowl. The perpetuation of influenza A viruses in free-living birds is evidence that these avian populations are the source of viruses for poultry and mammals. Waterfowl population surveillance has been, and continues to be, an important component of AIV research.

ACKNOWLEDGMENTS

We thank the employees of the Pennsylvania Game Commission of the Bureau of Wildlife Management who collaborated in the waterfowl sampling. We thank Dr. Virginia Hinshaw, Departments of Pathobiological Sciences and Medical Microbiology, Univeristy of Wisconsin–Madison, Madison, Wisconsin 53706, USA, for kindly supplying the monoclonal antibody to influenza A virus nucleoprotein. We also thank Dr. Brundaban Panigrahi, National Veterinary Services Laboratory, Ames, Iowa 50010, USA, for the virus typing and subtyping. This work was supported by the Pennsylvania Department of Agriculture contract No. ME449025.

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Received for publication 19 November 1993.