MAJOR ARTICLE

Infection Due to 3 Avian Influenza Subtypes in United States Veterinarians

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Background. Pandemic influenza virus strains originate in avian species. We examined veterinarians in the United States for evidence of previous avian influenza virus infection.

Methods. We performed a controlled, cross-sectional seroprevalence study among 42 veterinarians and 66 healthy control subjects using serum samples collected from 2002 through 2004. Serum samples were tested using a microneutralization assay against 9 influenza A virus strains.

Results. Using multivariable logistic regression modeling, veterinarians exposed to birds demonstrated statistically significant elevated titers against the H5, H6, and H7 avian influenza virus isolates, compared with control subjects.

Conclusions. These data suggest that occupational exposure to avian species may increase veterinarians' risk of avian influenza virus infection. Veterinarians should be considered for priority access to vaccines and antiviral drugs in pandemic planning.

Influenza A viruses are known to infect a wide variety of animals, including humans, pigs, birds, horses, and sea mammals. The primary reservoir of influenza A virus is aquatic waterfowl, and birds are the source of all influenza viruses in other species [1]. It was previously believed that adaptation in an intermediate host was necessary for avian influenza strains to acquire the ability to infect humans. However, recent experience with the H5N1 virus has clearly demonstrated that the virus may jump directly from birds to humans without an intermediate host.

Since 1997, there have been 285 cases and 170 fatalities in humans caused by H5N1 virus [2]. Most infected individuals have had direct contact with sick poultry through exposures such as butchering or culling infected poultry [3] or through preparing poultry for consumption [4]. More recently, cases of human illness due to avian H9N2 [5], H7N2 [6], H7N7 [7], and H7N3 viruses [8] have been reported.

A serosurvey conducted in China in 1992 suggests frequent human infection from avian influenza strains [9]. Little is known about the seroprevalence of avian influenza among humans in the United States. In this pilot study, we sought to estimate the seroprevalence of antibodies against avian influenza viruses in veterinarians with exposure to birds and to determine risk factors for infection.

SUBJECTS AND METHODS

Study subjects. Veterinarians attending a conference of the Iowa Veterinary Medical Association in the spring of 2004 were invited to enroll in the study. Control subjects were comprised of volunteers associated with the University of Iowa (Iowa City) who were enrolled in the study during the spring of 2006. Study participants completed occupational risk factor questionnaires and were only permitted to participate if they had no immunocompromising conditions, were >18 years of age, and were not pregnant. The study was conducted after institutional review board approval and with signed informed consent.

Laboratory methods. Virus stocks were grown in the allantoic cavities of 10-day old embryonated hens' eggs. Infected eggs were incubated for 3 days at 37°C

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and were chilled at 4°C overnight. Allantoic and amniotic fluids were harvested and clarified by centrifugation (600 *g* for 10 min). A hemagglutination assay was performed on the fluids from individual eggs; fluids with positive results were then pooled, aliquotted, and frozen at −80°C. Serum specimens were collected in serum separator tubes, allowed to clot at room temperature, centrifuged for 10 min at 1100–1300 *g*, aliquotted, and frozen at −80°C on the same day. Madin-Darby canine kidney cells used for the microneutralization assays were derived from the London lineage obtained from the Centers for Disease Control and Prevention and maintained in 5% Dulbecco minimum essential media containing 5% fetal bovine serum (Invitrogen/Gibco).

A microneutralization assay, adapted from that of Rowe et al. [10], was used to detect antibodies to avian strains H4–H12 (table 1). Because prevalence was expected to be low in the control group, serum samples were first screened at a dilution of 1:10, with 2-fold serial dilutions from 1:10 to 1:1280 run on all positive samples and tested in duplicate. Anticipating more reactivity, 2-fold serial dilutions from 1:10 to 1:1280 in duplicate were run on all veterinarian serum samples. Serial one-half–log dilutions of virus (1:100) in Modified Eagle's Medium with Earle's salts and L-glutamine, 7.5% bovine serum albumin, Hepes, and penicillin-streptomycin solution were performed in 96-well, flat-bottom cell culture plates (Falcon 35–3072). Madin-Darby canine kidney cells in log phase growth were adjusted to 2.0×10^5 cells/mL with diluent. One hundred microliters of cells were added to each well, and the plate was incubated at 37°C with 5% CO_2 for 24 h. Plates were washed twice with PBS, fixed with cold 80% acetone, and incubated at room temperature for 10 min. ELISA was performed to detect the viral nucleoprotein. The end point titer was expressed as the reciprocal of the highest dilution of serum with optical density (OD) less than *X*, where $X =$ [(average OD of virus control wells) + (average OD of cell control wells)]/2. Test cells with an OD >2 times the cell control OD mean were considered to be positive for virus growth. Virus suspension was calculated by the Reed Muench method to determine the $TCID_{50}$/100 μL. The back titer was run in duplicate and was only accepted when both replicates had matching results.

Serum samples were also tested using a hemagglutination inhibition (HI) assay against 3 isolates of recently circulating human influenza A virus (table 1). The Centers for Disease Control and Prevention HI serological protocol was followed. Antigen strains were grown in embryonated chicken eggs. Serum samples were pretreated with receptor-destroying enzyme (1 part serum to 3 parts enzyme) from *Vibrio cholerae* overnight and then were hemadsorbed with guinea pig blood. HI titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.5% solution of guinea pig erythrocytes.

HI assays with horse erythrocytes were performed on all serum samples with positive microneutralization assay results and on a subset of serum samples with negative results for subtypes H5, H6, and H7 [11]. Serum samples were treated with receptor-destroying enzyme, heat inactivated at 56°C for 30 min, and hemadsorbed with horse erythrocytes. Two-fold serial dilution series of serum were incubated with virus at 8 hemagglutinin U per 50 μL with 1% horse erythrocytes in 0.5% bovine serum albumin in phosphate buffered saline for 1 h at room temperature in V-bottom plates.

Statistical methods. Geometric mean HI titers were calculated for each virus strain and were compared by risk factor using the Wilcoxon rank-sum test with normal approximation. Only strains that demonstrated a significant difference between veterinarians and control subjects were further analyzed.

Exact logistic regression was then used to screen risk factors for their association with the outcome, with HI titers ⩾1:10

Table 1. Influenza viruses and antisera used for serological assays.

Influenza virus, subtype	Antigen	Antisera
Avian		
H4	A/Duck/Czechoslovakia/1/56 (H4N8)	A/Duck/Shantou/461/00
H5	A/Chucker/Minnesota/14591-7/98 (H5N2)	A/Goose/Hong Kong/437/99 and A/Tern/South Africa/61
H6	A/Turkey/Massachusetts/65 (H6N2)	A/Turkey/Massachussets/65
H7	A/Turkey/Virginia/4529/02 (H7N2)	A/Fowl Plague Virus/Rostock/34
H8	A/Turkey/Ontario/68 (H8N5)	A/Turkey/Ontario/68
H9	A/Turkey/Minnesota/38391-6/95 (H9N2)	A/Turkey/Minnesota/38391-6/95
H10	A/Chicken/Germany/49 (H10N7)	A/Chicken/Germany/49
H11	A/Duck/Memphis/546/76 (H11N9)	A/Duck/Hong Kong/M603/98
H12	A/Duck/Alberta/60/76 (H12N5)	A/Duck/Alberta/60/76
Human		
H1	A/New Caledonia/20/99 (H1N1)	…
H3	A/Panama/2007/99 (H3N2) and A/Nanchang/933/95 (H3N2)	…

considered to be positive. Covariates with bivariate P values <.25 were considered for inclusion in the multivariable logistic regression models. Final multivariable models included screened risk factors using a saturated model with manual backwards elimination.

RESULTS

We enrolled a total of 75 veterinarians and 77 control subjects. Veterinarians who had no exposure to birds were excluded from the analysis. Of the 42 remaining veterinarians, 32 worked with live chickens, 21 worked with live ducks, 18 worked with live turkeys, 12 worked with live geese, and 7 worked with live quail. Eleven of the control subjects reported exposure to birds and were excluded from the analysis, leaving 66 control subjects. We had insufficient serum samples for 1 veterinarian and were not able to include samples from that veterinarian in all analyses.

Demographic characteristics of participants are presented in table 2. Veterinarians were more likely to be male, but there were no other significant differences between the groups. Geometric mean antibody titers were elevated for the avian strains H5, H6, H7, and H9 in veterinarians and differed from the geometric mean antibody titers in control subjects by the Wilcoxon rank-sum test with normal approximation (table 3).

We examined a number of possible risk factors, including age, chronic medical conditions, race and ethnicity, medication use, military service, children in the home, and smoking (data not shown). Potential occupational risk factors included working with birds known to be infected with influenza, number of years of exposure to birds, and the use of protective personal equipment, such as gloves and masks.

Possible confounding caused by serologic cross-reactivity between avian and human viral strains was assessed by evaluating associations with receiving human influenza vaccine in the past 3 years, receiving the swine influenza vaccine in 1976, or having titers ⩾40 to human antibody strains H1N1 and H3N2. None of these possible confounders were found to be significantly associated with elevated titers against any of the viral strains. A power analysis (exact method) of the exposure variable data indicated that we had >90% probability to detect an important association (OR, ⩾10) with avian strains H5, H6, or H7 serologic assays.

After adjusting for possible confounders, multivariable logistic regression revealed that veterinarians had elevated ORs for 3 of the avian influenza strains (table 4). Although the geometric mean antibody titer for avian H9 was significantly higher in veterinarians, there was no significant difference in seropositivity between veterinarians and control subjects with logistic regression. Veterinarians had much greater adjusted ORs than did control subjects for being seropositive for avian H5 (adjusted OR, 16.7; 95% CI, 2.1–∞), avian H6 (adjusted OR, 12.2; 95% CI, 2.0–138.2), and avian H7 (adjusted OR, 17.7; 95% CI, 2.3–∞). Veterinarians who reported having examined birds known to be infected with influenza presented an increasing trend of being seropositive, compared with veterinarians without this exposure and with control subjects. No other risk factors showed a statistically significant association with elevated antibody titers. Because data were sparse for certain potential risk factors (e.g., few subjects answered "yes" to chronic medical conditions and smoking), the power to detect statistically significant associations of these variables with the avian influenza virus serologic assays was <.8. In general, horse erythrocyte HI titers were higher than those for microneutralization assay, with agreement (± 1 titer) of 65% for H5, 85% for H6, and 100% for H7.

Table 2. Demographic characteristics of the study population.

Variable	Control subjects (n = 66)	Veterinarians (n = 42)
Age		
18–41 years	31 (47.0)	13 (31)
42–51 years	17 (25.8)	15 (35.7)
52–77 years	18 (27.3)	14 (33.3)
Median years	44	48
Sex		
Female	45 (68.2)	14 (33.3)
Male	21 (31.8)	28 (66.7)
Race		
Asian	2 (3.0)	0 (0)
White	64 (97.0)	42 (100)
Ethnicity		
Not Hispanic	60 (98.4)	42 (100)
Hispanic	1 (1.6)	0 (0)
Smoked a total of ⩾5 packs of tobacco products in the past year		
No	62 (93.9)	42 (100)
Yes	4 (6.1)	0 (0)

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

DISCUSSION

This study provides evidence that veterinarians are at increased risk for infection due to avian influenza virus. To our knowledge, this is the first investigation of seroprevalence of a wide variety of avian influenza subtypes in occupationally exposed veterinarians in the United States.

Distribution of hemagglutinin subtypes in birds varies geographically, temporally, and by species. However, the H6 subtype appears to be commonly found in birds, whereas H5 and H7 are less common [12–14]. Why these veterinarians have elevated risk for antibodies to subtypes not frequently found in birds is unknown. Different subtypes are likely to vary in their ability to infect and produce an antibody response in

Table 3. Geometric mean and distribution of antibody titers against avian influenza viruses.

Influenza virus, titer	Control subjects (n = 66)	Veterinarians (n = 42)
Avian H4		
<1:10	66 (100)	40 (97.6)
1:10	0 (0)	1 (2.4)
Geometric mean titer	5.0	5.1
Avian H5[a]		
<1:10	66 (100)	36 (87.8)
1:10	0 (0)	4 (9.8)
1:20	0 (0)	1 (2.4)
Geometric mean titer	5.0	5.5
Avian H6[a]		
<1:10	64 (97)	32 (76.2)
1:10	0 (0)	9 (21.4)
1:20	2 (3)	1 (2.4)
Geometric mean titer	5.2	6.0
Avian H7[a]		
<1:10	66 (100)	35 (85.4)
1:10	0 (0)	6 (14.6)
Geometric mean titer	5.0	5.5
Avian H8		
<1:10	66 (100)	42 (100)
Geometric mean titer	5.0	5.0
Avian H9[a]		
<1:10	65 (98.5)	34 (89.5)
1:10	1 (1.5)	3 (7.9)
1:20	0 (0)	1 (2.6)
Geometric mean titer	5.1	5.5
Avian H10		
<1:10	66 (100)	41 (97.6)
1:10	0 (0)	1 (2.4)
Geometric mean titer	5.0	5.1
Avian H11		
<1:10	66 (100)	40 (97.6)
1:10	0 (0)	0 (0)
1:20	0 (0)	0 (0)
1:40	0 (0)	1 (2.4)
Geometric mean titer	5.0	5.3
Avian H12		
<1:10	65 (98.5)	39 (97.5)
1:10	0 (0)	0 (0)
1:20	1 (1.5)	0 (0)
1:40	0 (0)	0 (0)
1:80	0 (0)	1 (2.5)
Geometric mean titer	5.1	5.4

NOTE. Data are no. (%) of subjects, unless otherwise specified.

[a] Differed from that of control subjects by the Wilcoxon rank sum analysis.

humans. In addition, surveillance data for avian species are limited, and it is possible that outbreaks of these subtypes have occurred but have gone unnoticed.

The possibility of cross-reaction between subtypes must also be considered. However, the lack of an association between seropositivity and having received a human influenza vaccine suggests that there is no cross-reactivity of the avian H5, H6, and H7 serotypes against human H1 and H3 subtypes. We were unable to assess the degree of cross-reactivity of avian subtypes against each other in this small study.

Table 4. ORs for a positive serological response against avian influenza virus by microneutralization assay using exact logistic regression.

Variable	Avian H5				Avian H6				Avian H7			
	No. of subjects	No. (%) of subjects with titers ⩾1:10	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	No. of subjects	No. (%) of subjects with titers ⩾1:10	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	No. of subjects	No. (%) of subjects with titers ⩾1:10	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Age, continuous	107	5 (4.7)	1 (0.9–1.1)	…	108	12 (11.1)	1 (0.9–1)	0.96 (0.9–1.0)	107	6 (5.6)	1 (0.9–1.1)	…
Exposure												
Avian-exposed veterinarians	41	5 (12.2)	11.9 (1.6–∞)	16.7 (2.1–∞)	42	10 (23.8)	9.8 (1.9–97.1)	12.2 (2–138.2)	41	6 (14.6)	14.8 (2.1-∞)	17.7 (2.3-∞)
Control subjects	66	0 (0.0)	Reference	Reference	66	2 (3)	Reference	Reference	66	0 (0.0)	Reference	Reference
Reported having received a flu shot within the last 3 years												
Yes	76	4 (5.3)	1.7 (0.2–84.8)	4.3 (0.4–231.5)	77	6 (7.8)	0.4 (0.1–1.5)	1.1 (0.2–5.3)	76	4 (5.3)	0.8 (0.1–9.4)	2.1 (0.3–25.8)
No	31	1 (3.2)	Reference	Reference	31	6 (19.4)	Reference	Reference	31	2 (6.5)	Reference	Reference

NOTE. Titers >1:10 were considered to be positive. Final multivariable models were designed after screening risk factors for associations with the outcomes ($P < .25$), using a saturated model that included all potential risk factors of important bivariate predictors and manual backwards elimination.

Pandemic influenza viruses must have the ability to spread efficiently from person to person. When different influenza subtypes coexist in a single host, recombination of genetic material can occur, creating a novel virus with new characteristics that may increase infectivity and transmissibility [15]. Increasing vaccination rates among veterinarians with human influenza vaccine would decrease opportunities for reassortment events and should be considered.

There are several limitations of this study. The study design did not allow for the evaluation of an association of seropositivity with clinical symptoms. The nature, frequency, and severity of clinical illness caused by infection due to avian influenza remains unknown. The use of gloves and masks in this population was inconsistent and infrequent, making it unlikely that we would be able to detect any protective effect of personal protective equipment, even if it were present.

A better understanding of interspecies transmission of avian influenza is a crucial component in efforts to minimize the effects of the next pandemic. Humans with frequent and close contact to infected birds may be among the first to be infected and, by spreading the illness to their families and communities, may serve as a bridging population to the general population [16]. Early detection and intervention will be an important component of pandemic preparedness. Knowledge of prevalence rates and risk factors for zoonotic influenza transmission are fundamental in developing pandemic plans. These study data suggest that avian-exposed veterinarians may be at increased risk of zoonotic influenza infection. We posit that they should be considered for inclusion on priority access lists for pandemic vaccines and antiviral drugs.

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