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Serological evidence for avian H9N2 influenza virus infections among Romanian agriculture workers

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Summary In recent years, wild birds have introduced multiple highly pathogenic avian influenza (HPAI) H5N1 virus infections in Romanian poultry. In 2005 HPAI infections were widespread among domestic poultry and anecdotal reports suggested domestic pigs may also have been exposed. We sought to examine evidence for zoonotic influenza infections among Romanian agriculture workers. Between 2009 and 2010, 363 adult participants were enrolled in a cross-sectional, seroepidemiological study. Confined animal feeding operation (CAFO) swine workers in Tulcea and small, traditional backyard farmers in Cluj-Napoca were enrolled, as well as a non-animal exposed control group from Cluj-Napoca. Enrollment sera were examined for serological evidence of previous infection with 9 avian and 3 human influenza virus strains. Serologic assays showed no evidence of previous infection with 7 low pathogenic avian influenza viruses or with HPAI H5N1. However, 33 participants (9.1%) had elevated microneutralization antibody titers against avian-like A/Hong Kong/1073/1999(H9N2), 5 with titers $\geq$1:80 whom all reported exposure to poultry. Moderate poultry exposure was significantly associated with elevated titers after controlling for the subjects' age (adjusted OR = 3.6; 95% CI, 1.1—12.1). There was

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no evidence that previous infection with human H3N2 or H2N2 viruses were confounding the H9N2 seroreactivity. These data suggest that H9N2 virus may have circulated in Romanian poultry and occasionally infected man.

Introduction

Human infections with novel influenza viruses, such as highly pathogenic avian influenza (HPAI) H5N1 virus and the swine-like 2009 H1N1 pandemic influenza virus, are often only identified through clinical encounters. Little is known about subclinical human infections and risk factors for zoonotic transmission to man.

One of the newest members of the European Union (EU), Romania is economically behind many other EU nations. Approximately 45% of Romania's population of 22 million live in rural areas where small farms are a common form of subsistence [1]. HPAI H5N1 virus has been periodically detected through domestic bird die-offs in the southeastern part of Romania in 2005, 2006, 2007, and 2010 [2—4]. In 2005 and 2006 the infections in domestic poultry were widespread and caused much economic distress. Anecdotal reports suggested pigs may also have been exposed. Field studies documented that these viruses are being introduced through migrating birds that frequent the Danube Delta's flyways [5,6].

We sought to study agricultural workers in Romania for evidence of previous infection with avian influenza (AI) viruses.

Materials and methods

Study design

Cohort enrollment focused on two areas in Romania: lightly populated, Tulcea (population approximately 100,000), in the Danube Delta region, and densely populated Cluj-Napoca (population approximately 340,000) (Fig. 1). In Tulcea, we enrolled chiefly workers associated with large commercial swine CAFOs, while in Cluj, we enrolled farmers exposed to animals in their small traditional backyard farms. A total of five institutional review boards reviewed and approved the study.

Study personnel engaged village or business leaders who at an appointed time invited study subjects to areas where the study team explained the study and invited them to participate via an informed consent process. As we were targeting agricultural workers with intense and prolonged exposure to animals we chose to enroll persons $\geq$18 years of age. Consenting adult participants were interviewed by staff field workers who completed enrollment forms and collected sera. Animal exposure was classified as exposure to domestic poultry, wild birds, or pigs as part of daily activities for $\geq$5 cumulative h/wk. Age-group matched controls who did not meet the inclusion criteria for animal exposure were recruited from Babes-Bolyai University in Cluj-Napoca. Along with demographic information and medical history, community, household, and occupational animal exposures were assessed with the study's enrollment questionnaire. The questionnaire captured flock/herd size for various types of domestic poultry, wild birds, and other animals, well as years of exposure, such that animal exposure could be classified in an ordinal or continuous fashion (e.g. 1000 chicken-years or 1000 duck-years).

Laboratory methods

Whole blood specimens (10 mL red top tube) were transported at 10—15 °C to the field laboratory in Tulcea or to Babes-Bolyai University in Cluj-Napoca within 24 h after collection. Upon arrival, specimens were accessioned and blood tubes spun at 3000 $\times$ *g* for 15 min to separate serum. All collected serum was aliquoted and frozen at −80 °C. Frozen sera were transported on dry ice to the University of Florida for testing.

Influenza virus strains were selected by hemagglutinin (H) type for their best geographic and temporal proximity to the population (Table 1). Influenza virus strains were grown in fertilized eggs. The hemagglutination inhibition (HI) assay was employed as previously reported [7—12] to study human sera for antibodies against human and swine influenza viruses (SIVs). Sera were pre-treated with receptor destroying enzyme and hemabsorbed with either guinea pig or turkey erythrocytes. Titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.65% (guinea pig) or 0.50%

Figure 1 Map of the two study sites in Romania: Cluj-Napoca and Tulcea.

(turkey) solution of erythrocytes as previously established [13]. A microneutralization (MN) assay adapted from that reported by Rowe and previously reported [7,11,14—16] was used to detect antibodies to a large panel of avian and avian-like influenza viruses. Sera were first screened at a dilution of 1:10. Positive specimens were tittered in duplicate using 2-fold serial dilutions from 1:10 to 1:1280 in virus diluent [85.8% minimum essential medium (Invitrogen, Carlsbad, CA), 0.56% BSA, 25 mM HEPES buffer (Invitrogen), 100 mg/l streptomycin (Invitrogen), and 100,000 units/l penicillin (Invitrogen). Virus neutralization was performed by adding 100 $TCID_{50}$ of virus to the sera. The Reed Muench method was used to determine the $TCID_{50}/100\,\mu L$ [17]. MDCK cells in log phase growth were adjusted to 2.0×10^5 cells/mL with diluent. One hundred microliters of cell suspension were added to each well and the plate incubated at 37 °C with 5% CO_2 for 24 h. Plates were washed twice with PBS, fixed for 10 min with cold 80% acetone at room temperature. The ELISA endpoint 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. The back titer was run in duplicate and was only accepted when both replicates had matching results. For both the HI and MN assays, we followed our seroepidemiological laboratory standards used in these previous publications.

For avian and swine influenza virus infection modeling, we followed approaches we have used in previous publications [8—11,18]. Regarding avian

Table 1 Viruses used in serological studies.

Avian viruses[a]	Swine viruses[b]
A/Migratory duck/Hong Kong MPS180/2003(H4N6)	A/Swine/Lutol/3/2000(H1N1)
A/Nopi/Minnesota/2007/462960-2(H5N2)	A/Swine/Gent/7625/1999(H1N2)
A/Teal/Hong Kong/w312/1997(H6N1)	A/Swine/Flanders/1/1998(H3N2)
A/Water fowl/Hong Kong/Mpb127/2005(H7N7)	
A/Migratory duck/Hong Kong/MP2553/2004(H8N4)	**Human viruses**[b]
A/Hong Kong/1073/1999(H9N2)[c]	A/Brisbane/59/2007(H1N1)
A/Migratory duck/Hong Kong/MPD268/2007(H10N4)	A/New Caledonia/20/1999(H1N1)
A/Chicken/New Jersey/15906-9/1996(H11N1)	A/Mexico/4108/2009(H1N1)
A/Chicken/Romania/6059-1TS/2008(H5N1)	A/Brisbane/10/2007(H3N2)

[a] Virus studied with the microneutralization assay.
[b] Virus studied with the hemagglutination inhibition assay.
[c] Virus of avian origin but isolated from a human.

Table 2 Characteristics of study subjects upon enrollment, Romania, 2009. Unadjusted odds ratio for agriculture workers against control participants with binary logistic regression.

Variables	Total *N*	Agriculture workers *N* (%)	Controls *N* (%)	Unadjusted OR (95% CI)
Age (years)				
20—39	138	121 (38.8)	17 (33.3)	2.6 (1.3—5.4)
40—59	151	137 (43.9)	14 (27.5)	3.6 (1.7—7.7)
≥60	74	54 (17.3)	20 (39.2)	Reference
Gender				
Male	185	161 (51.6)	24 (47.1)	1.2 (0.7—2.2)
Female	178	151 (48.4)	27 (52.9)	Reference
Indoor water				
No	92	92 (29.5)	0 (0)	30.3 (5.4—infinity)[a]
Yes	271	220 (70.5)	51 (100)	Reference
Ever received vaccination for human influenza[b]				
No	204	188 (60.3)	16 (31.4)	3.5 (1.8—6.6)
Yes	153	118 (37.8)	35 (68.6)	Reference
Heart disease, hypertension, or stroke[b]				
No	277	250 (80.1)	27 (52.9)	3.7 (2.0—6.9)
Yes	84	60 (19.2)	24 (47.1)	Reference
Chronic breathing problems[b]				
No	321	280 (89.7)	41 (80.4)	2.4 (1.1—5.4)
Yes	38	28 (9.0)	10 (19.6)	Reference
Other chronic medical problems[b]				
No	322	282 (90.4)	40 (78.4)	2.8 (1.3—6.0)
Yes	39	28 (9.0)	11 (21.6)	Reference
Ever used tobacco products[b]				
No	195	168 (53.8)	27 (52.9)	1.1 (0.6—2.0)
Yes	155	132 (42.3)	23 (45.1)	Reference
Developed a respiratory illness in the last 12 months[b]				
No	217	202 (64.7)	15 (29.4)	4.5 (2.4—8.6)
Yes	144	108 (34.6)	36 (70.6)	Reference
Workplace pig exposure since 2003				
Yes	118	118 (37.8)	0 (0)	44.0 (7.9—infinity)[a]
No	245	194 (62.2)	51 (100)	Reference
Workplace poultry exposure since 2003				
Yes	29	29 (9.3)	0 (0)	7.4 (1.3—infinity)[a]
No	334	283 (90.7)	51 (100)	Reference
Non-workplace pig exposure since 2003				
Yes	165	162 (51.9)	3 (5.9)	17.2 (5.4—88.0)[a]
No	198	150 (48.1)	48 (94.1)	Reference
Non-workplace poultry exposure since 2003				
Yes	237	233 (74.7)	4 (7.8)	34.3 (12.0—135)[a]
No	126	79 (25.3)	47 (92.2)	Reference

[a] Fisher exact method used.
[b] Covariate has some missing data.

influenza virus (AIV) infections, as Buchy et al. [19] have previously documented that subclinical AIV infections may rapidly decline in antibody titer, and because we were searching for evidence of previous AIV infection which may have occurred years ago, we used a low threshold of antibody titer (≥1:10) as an indication of previous infection or outcome. In multivariate risk factor modeling for AIV infections, we controlled for potential confounding from antibodies against human viruses (e.g. H3N2 and H2N2 viruses) by creating binary covariates for such viruses (HI titer ≥1:40 counted as positive). In modeling for SIV infections, we chose a HI titer ≥1:40 as a positive outcome. Our previous SIV work indicated that a high titer is necessary to examine multiple risk factors for SIV infection, as SIV titers are heavily confounded with antibody against human influenza virus or vaccine [8—10,18].

Table 3 Serological activity against human, low pathogenic avian, and swine influenza viruses for subjects' enrollment sera. Unadjusted odds ratio with binary logistic regression for agriculture workers (poultry and/or swine exposed) against non-animal exposed control participants with binary logistic regression.

Virus strain	Total *N* (%)	Agricultural workers *n* (%)	Controls *n* (%)	Unadjusted OR (95% CI)
A/Brisbane/59/2007(H1N1)[a,b]				
Positive	39 (10.8)	9 (17.6)	30 (9.6)	0.5 (0.2–1.1)
Negative	321 (89.2)	42 (82.4)	279 (89.4)	Ref
A/New Caledonia/20/1999(H1N1)[a,b]				
Positive	42 (11.6)	5 (9.8)	37 (11.9)	1.2 (0.5–3.3)
Negative	320 (88.4)	46 (90.2)	274 (87.8)	Ref
A/Mexico/4108/2009(H1N1)[a,b]				
Positive	9 (2.6)	1 (2.1)	8 (2.6)	1.3 (0.2–10.3)[c]
Negative	338 (97.4)	46 (97.9)	292 (93.6)	Ref
A/Brisbane/10/2007(H3N2)[a]				
Positive	65 (17.9)	15 (29.4)	50 (16)	0.4 (0.2–0.9)
Negative	298 (82.1)	36 (70.6)	262 (84)	Ref
A/Hong Kong/1073/1999(H9N2)[d]				
Positive	33 (9.1)	2 (3.9)	31 (9.9)	2.7 (0.7–24.0)[c]
Negative	330 (90.9)	49 (96.1)	281 (90.1)	Ref
A/Swine/Lutol/3/2000(H1N1)[a,b]				
Positive	36 (11.1)	7 (13.7)	29 (9.3)	0.7 (0.3–1.8)
Negative	289 (88.9)	44 (86.3)	245 (78.5)	Ref
A/Swine/Gent/7625/1999(H1N2)[a]				
Positive	128 (35.3)	20 (39.2)	108 (34.6)	0.8 (0.4–1.5)
Negative	235 (64.7)	31 (60.8)	204 (65.4)	Ref
A/Swine/Flanders/1/1998(H3N2)[a,b]				
Positive	36 (9.9)	6 (11.8)	30 (9.6)	0.8 (0.3–2.0)
Negative	326 (90.1)	45 (88.2)	281 (90.1)	Ref

[a] Hemagglutination inhibition assay, negative = titer < 1:40, positive = titer ≥ 1:40.
[b] Covariate has some missing values.
[c] Fisher exact method used.
[d] Microneutralization assay, negative = titer < 1:10, positive = titer ≥ 1:10.

Statistical methods

Questionnaire data were manually entered twice in a relational database (Microsoft Inc., Redmond, WA, USA) and verified with structured query language. Questionnaire and laboratory data were later merged into a master dataset, using a unique study subject number.

Initially we examined risk factors for bivariate associations with MN and HI assay results for AIVs and SIVs using binary logistic regression and proportional odds modeling [20] and examining a number of potential risk factors. An exact conditional method was used for sparse data, and the score test was used to evaluate the proportional odds assumption. Covariates with p values <0.25 were considered for inclusion in multivariate models. Final multivariate models were designed using manual backwards elimination and included covariates with p values <0.05. Analyses were performed by using SAS v9.2 (SAS Institute, Inc., Cary, NC, USA).

Results

Participants

Between February 2009 and January 2010, field staff enrolled 363 participants all of who permitted sera and questionnaire data collections: 149 modern swine workers from Tulcea; 163 small traditional farmers from Cluj-Napoca; and 51 age-group matched controls (no animal exposure) from Babes-Bolyai University (Table 2). Following recruitment efforts, the participation percentages were estimated between 20 and 33%, depending on the enrollment site.

The participants had a mean age of 45.9 years and 51% were male. While all of the control subjects reported to have access to indoor water, 30% of the animal-exposed group reported no access to indoor plumbing. Several other demographic characteristics were significantly different between the exposed and unexposed populations (Table 2). Among the animal-exposed participants,

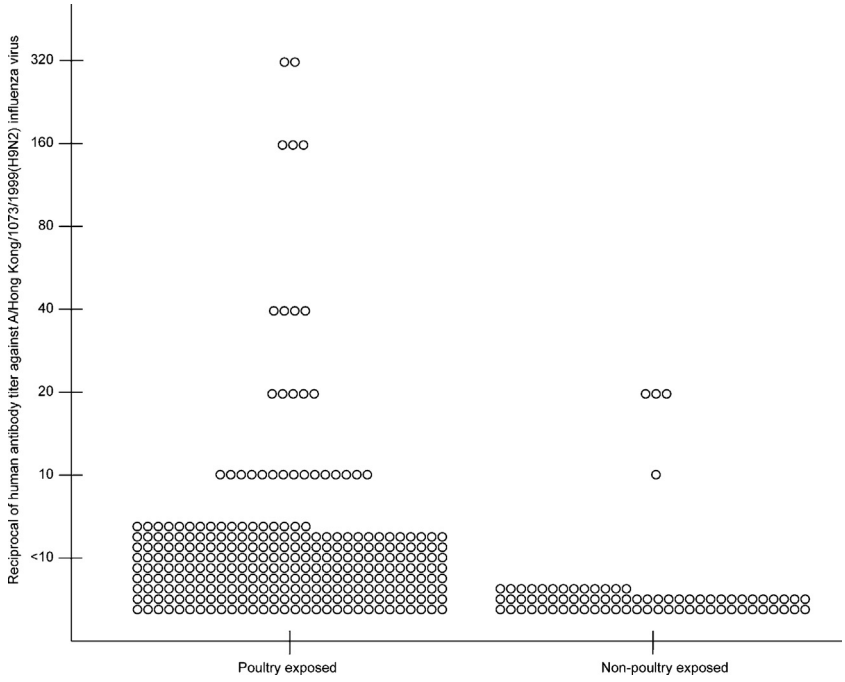

Figure 2 Distribution of microneutralization titers against avian-like A/Hong Kong/1073/1999(H9N2), by poultry exposure.

90% reported pig exposure and 84% reported poultry exposure, which were documented both by workplace and non-workplace (domestic/home) exposures (Table 2). The majority of the control population reported no pigs or poultry exposure; however, 3 (5.9%) and 4 (7.8%) of controls reported some exposure to pigs or poultry, respectively, since 2003, yet this exposure was below the 5 h/wk threshold for inclusion in the exposed group.

Serology

Enrollee serological assays showed some reactivity against all 4 human influenza viruses tested with the HI assay, although no statistically important differences between exposure groups were observed (Table 3). No serological reactivity was found with the MN assay against the 8 AIVs tested, including HPAI H5N1 virus. However, seropositivity was documented for the avian-like human influenza virus A/Hong Kong/1073/1999(H9N2), for which elevated antibody titers ($\geq$1:10) against this virus were detected in sera from 33 subjects (9.1%). Titers were as high as 1:320, with 5 persons having MN titers $\geq$1:80. All 5 subjects with titers $\geq$1:80 reported exposure to poultry (Fig. 2). When considering age as a continuous variable, younger participants were significantly more likely to have elevated H9N2 titers compared to older participants (OR = 0.97; 95% CI, 0.9—1.0). As we wondered if these H9N2 antibody findings might be confounded by elevated antibodies against human H3N2 virus, we included elevated antibody against this virus in a multivariate model and it was not found to be an important predictor (Table 4). To examine evidence for potential confounding with previous infection with H2N2 pandemic virus, we additionally stratified the subjects into two groups: born before or during 1968 (last circulation of H2N2 virus) and those born afterwards. Again there was no evidence of confounding due to antibodies against H2N2 viruses. Subjects born before or during 1968 were no more likely to have elevated antibody titers against the H9N2 virus (OR = 0.9, 95% CI, 0.4—1.8). There was also no significant difference between seropositive subjects residing in Cluj compared to those residing in Tulcea (OR = 1.1; 95% CI, 0.5—2.3). After adjusting for subjects' age, moderate exposure to poultry (301—900 poultry-years) was significantly associated with elevated titers against the avian-like H9N2 virus (adjusted OR = 3.6; 95% CI, 1.1—12.1) compared to non-poultry exposed subjects (Table 4).

Enrollment sera were also tested for antibodies against 3 SIVs (Table 1). With all groups combined, the seroprevalence of antibody titers $\geq$1:40 among enrollees was 9.9%, 11.1% and 35.3% (data not shown) for SwH3N2, SwH1N1, and SwH1N2, respectively. No risk factor associations could be found for the swine influenza virus serology.

Table 4 Risk factors for elevated antibodies against A/Hong Kong/1073/1999(H9N2), among adult participants, Romania, 2009.

Variables	Total *N*	*N* (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Age (years)				
	363	33 (100.0)	0.97 (0.9–1.0)[a]	0.97 (0.9–1.0)[b]
Poultry exposure				
>2130 poultry-years	72	7 (21.2)	2.0 (0.6–7.0)[b]	2.7 (0.7–10.4)[b]
901–2130 poultry-years	72	5 (15.2)	1.4 (0.4–5.3)[b]	1.6 (0.4–6.3)[b]
301–900 poultry-years	72	11 (33.3)	3.3 (1.0–10.9)[b]	3.6 (1.1–12.1)[b]
10–300 poultry-years	70	6 (18.2)	1.7 (0.5–6.3)[b]	1.7 (0.5–6.3)[b]
Unexposed	77	4 (12.1)	Ref	Ref
Birth year				
Born before 1968	332	19 (57.8)	0.9 (0.4–1.8)[a]	–
Born after 1968	142	14 (42.4)	Ref	–
Gender				
Female	178	18 (54.6)	1.3 (0.6–2.6)[a]	–
Male	185	15 (45.5)	Ref	–
Received an influenza vaccine since 2008[c]				
Yes	100	11 (33.3)	1.4 (0.6–2.9)[b]	–
No	263	22 (66.7)	Ref	–
A/Brisbane/10/2007(H3N2)[d]				
Positive	65	7 (21.2)	1.3 (0.5–3.0)[b]	–
Negative	298	26 (78.8)	Ref	–
Indoor water				
No	92	8 (24.2)	0.9 (0.4–2.2)[b]	–
Yes	271	25 (75.8)	Ref	–
Respiratory illness in the last 12 months[c]				
Yes	144	17 (51.5)	1.7 (0.8–3.4)[b]	–
No	217	26 (48.5)	Ref	–
Ever used tobacco products[c]				
Yes	155	15 (45.5)	1.1 (0.5–2.3)[a]	–
No	207	18 (54.6)	Ref	–
Chronic breathing problems[c]				
Yes	38	3 (9.1)	0.8 (0.2–2.9)[e]	–
No	324	30 (90.9)	Ref	–
Employed				
No	111	13 (39.4)	1.6 (0.8–3.3)[a]	–
Yes	252	20 (60.6)	Ref	–
Residence				
Cluj	214	20 (60.6)	1.1 (0.5–2.3)[a]	–
Tulcea	149	13 (39.4)	Ref	–

[a] Proportional odds model used with three antibody titer groups due to sparse data (<1:10, 1:10, >1:10).
[b] Binary logistic regression (negative = H9N2 titer < 1:10, positive = H9N2 titer ≥ 1:10).
[c] Covariate has missing data.
[d] H3N2 antibody titer: negative = titer < 1:40, positive = titer ≥ 1:40.
[e] Fisher's exact test used.

Discussion

Previous seroepidemiological studies of AIV infections among poultry workers in other countries have been mixed in their findings. Comparing these studies is challenging as serological assay protocols and reagents are not standardized and much depends upon matching any circulating virus with a similar virus in the assay. Serological assessment of antibodies against HPAI H5N1 virus have been numerous and recently reviewed [21]. Researchers in the Netherlands documented considerable human antibody response to HPAI H7N7 virus after a large H7N7 outbreak in poultry [22]. With respect to H9N2, we have previously reported serological evidence of H9N2 infections among agricultural workers in Thailand, Cambodia, and Nigeria [16,23,24]. In addition, a 2010 study in India found a 4–5% seroprevalence of antibodies against H9N2 AIV among poultry workers [25]. However, we failed to find

evidence of previous infection with H9N2 virus among poultry or wildlife workers in Peru [26], and the United States [7,8,15,27], as well among Mongolian herdsmen [under journal review]. Another epidemiological team failed to find evidence of H9N2 infections in a 2011 serosurvey of chicken growers in Lebanon [28].

In this study, 9.1% of the cohort had elevated antibody titers against the avian-like A/Hong Kong/1073/1999(H9N2) influenza virus. In 1999, Peiris et al. isolated this virus from a young girl in Hong Kong, and found it to be closely related to A/Quail/Hong Kong/G1/1997(H9N2) [29,30]. Data suggested that such H9N2 viruses contributed to the emergence of the H5N1 virus through genetic reassortment [31,32] and to the currently circulating H7N9 virus [33].

Moderate exposure to poultry in one's lifetime was significantly associated with H9N2 seropositivity in this study population. It was interesting to note that higher ordinal levels of poultry exposure were not associated with the outcome. Perhaps those with larger domestic farms implement stronger biosecurity measures or practice better personal protective equipment use. This positive association with moderate poultry exposure may be confounded by serological elevations due to antibody cross-reactivity; however, the high titers among poultry-exposed subjects suggest that some seroreactivity represented true infections.

Most of the poultry exposure among this study population was through domestic (i.e. household and community) exposures, rather than occupational exposures. Only 33 subjects cited occupational exposure to poultry, of which 9 reported only occupational poultry exposure without corresponding domestic exposure. Therefore, among this study population, it was difficult to examine associations between H9N2 seropositivity with specific occupational exposures.

Serological reactivity was detected for the 3 SIVs examined; however, little information was garnered from statistical analyses to understand risk factors for exposure as the elevations may reflect cross-reacting human influenza infections or vaccine receipt.

This study had some limitations. If the viruses we used to examine sera reactivity were antigenically different than those circulating in Romania, then our negative assays may have been misleading. The study is further limited in that H9N2 viruses have not yet been detected among poultry in Romania. However, avian influenza surveillance among poultry and wild birds is sparse in Romania. H9N2 carriage among migrating birds is highly biologically plausible as such viruses have been found in birds in Germany, Ireland, France, Pakistan, Israel, Iran, Dubai, The Netherlands, and Saudi Arabia [34,35]. In addition, due to the occupational setting of targeted enrollments, only adults at least 18 years of age were enrolled. Many human cases of AIV infections have been reported in children and therefore this study may have excluded a large subset of the at-risk population.

Conclusions

Although Romania has experienced multiple incursions of H5N1 HPAI, no evidence of previous human infections was found among 363 adults studied in two unique geographical areas in Romania. However, study data did reveal quite elevated antibodies against an avian-like H9N2 influenza virus among some of these same subjects and results did not seem to be confounded by antibodies against human influenza viruses. Further, study data suggest that moderate poultry exposure was associated with these elevated titers. As Romania has sparse surveillance for AIV in poultry and neighboring countries, particularly those across the Black Sea, have documented H9N2 infections in poultry, it seems biologically plausible that these seroepidemiological data indicate that H9N2 virus has somewhat recently circulated in Romanian poultry and occasionally infected man.

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Competing interests: None declared.

Ethical approval: A total of five institutional review boards (University of Iowa, University of Florida, Babes-Bolyai University, Center for Health Research in Cluj-Napoca, and Human Research Protection Office of the U.S. Army Medical Research and Materiel Command) reviewed and approved the study.

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