Avian Influenza A(H5N1) and A(H9N2) Seroprevalence and Risk Factors for Infection Among Egyptians: A Prospective, Controlled Seroepidemiological Study

Mokhtar R. Gomaa,[1,a] Ahmed S. Kayed,[1,a] Mona A. Elabd,[2] Dina Abu Zeid,[2] Shaimaa A. Zaki,[2] Amira S. El Rifay,[2] Lobna S. Sherif,[2] Pamela P. McKenzie,[3] Robert G. Webster,[3] Richard J. Webby,[3] Mohamed A. Ali,[1] and Ghazi Kayali[3]

[1]Center of Excellence for Influenza Viruses, and [2]Medical Research Division, National Research Center, Giza, Egypt; and [3]Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee

(See the editorial commentary by Morens and Taubenberger on pages 1364–6.)

Background. A(H5N1) and A(H9N2) avian influenza viruses are enzootic in Egyptian poultry, and most A (H5N1) human cases since 2009 have occurred in Egypt. Our understanding of the epidemiology of avian viruses in humans remains limited. Questions about the frequency of infection, the proportion of infections that are mild or subclinical, and the case-fatality rate remain largely unanswered.

Methods. We conducted a 3-year, prospective, controlled, seroepidemiological study that enrolled 750 poultry-exposed and 250 unexposed individuals in Egypt.

Results. At baseline, the seroprevalence of anti-A(H5N1) antibodies (titer, ≥80) among exposed individuals was 2% significantly higher than that among the controls (0%). Having chronic lung disease was a significant risk factor for infection. Antibodies against A(H9N2) were not detected at baseline when A(H9N2) was not circulating in poultry. At follow-up, A(H9N2) was detected in poultry, and consequently, the seroprevalence among exposed humans was between 5.6% and 7.5%. Vaccination of poultry, older age, and exposure to ducks were risk factors for A(H9N2) infection.

Conclusions. Results of this study indicate that the number of humans infected with avian influenza viruses is much larger than the number of reported confirmed cases. In an area where these viruses are enzootic in the poultry, human exposure to and infection with avian influenza becomes more common.

Keywords. influenza; avian; epidemiology; seroprevalence; cohort.

A clade 2.2 avian influenza A(H5N1) virus was detected first in Egyptian wild birds in 2005 and then in poultry in 2006. By 2008, the viruses were enzootic in Egyptian poultry [1, 2] and were detected by surveillance in all poultry production sectors (commercial and backyard farms, live bird markets, and abattoirs), in most poultry species, and in wild birds, throughout the year [3]. These enzootic viruses continued to evolve and were reported to undergo genetic and antigenic drift [4, 5]. In 2011, avian influenza A(H9N2) viruses were detected in Egyptian poultry [6, 7]. Surveillance data showed the A (H9N2) and A(H5N1) viruses to be cocirculating and frequently infecting the same avian hosts [8].

More than 15 years have passed since the first human cases of avian influenza A(H5N1) infection were reported, and the number continues to rise [9]. The World Health Organization (WHO) reports 650 confirmed cases, 386 (59%) of which were fatal [10]. In Egypt, most cases reported since 2009 have affected backyard poultry producers. The case-fatality rate in Egypt (36%) is approximately half the sum of case-fatality rates in all other affected countries. The epidemiological data show that most of the confirmed human A(H5N1) cases in Egypt before 2009 occurred in female patients

Received 11 July 2014; accepted 5 August 2014; electronically published 29 October 2014.

[a]M. R. G. and A. S. K. are co–first authors.

Correspondence: Ghazi Kayali, PhD, MPH, Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Pl, Memphis, TN 38105 (ghazi.kayali@stjude.org).

The Journal of Infectious Diseases® 2015;211:1399–407

DOI: 10.1093/infdis/jiu529

<18 years old. The case-fatality rate increased with age and with delayed hospitalization and was higher among females [11]. A (H9N2) viruses also cause human infection, but cases are sporadic, and no reported infection to date has been fatal [12, 13].

Our understanding of the epidemiology of A(H5N1) viruses in humans remains limited. Questions about the frequency of infection, the proportion of cases that are mild or subclinical, and the true case-fatality rate remain largely unknown [14, 15]. Several seroprevalence studies reported rates between 0% and 7% and identified risk factors [16, 17], but their findings were undermined by issues of study design and/or serological assays. Most studies were cross-sectional, lacked an unexposed control group and confirmatory assays, used a low threshold for positivity, or used H5 antigens not circulating in the study geographic area. The debate over the true seroprevalence of A(H5N1) virus recently intensified when a meta-analysis estimated it to be 1%–2% [16]. Other researchers countered that this rate suggests that millions of cases of A(H5N1) infection have been missed, with little evidence that these infections have occurred [18].

We set out to determine the extent of human infection due to these 2 avian influenza viruses in Egypt by determining the prevalence of antibodies to them, and we sought to identify the associated risk factors.

METHODS

Study Population

We conducted a 3-year controlled, prospective seroepidemiological study that enrolled 750 poultry-exposed subjects in 5 rural Egyptian sites and 250 unexposed control subjects in Cairo [19]. A convenience sample of 150 individuals was selected in each rural site in the Nile Delta region (the governorates of Sharkiya, Gharbiya, Qalyubiya, and Kafr El Sheikh) and in Upper Egypt (the governorate of Fayyoum). Subjects aged ≥2 years were recruited at their residences. At baseline (between November 2010 and July 2011), the study team completed the informed consent procedures and interviewed the subjects, using a tailored questionnaire that measured demographic, health, and exposure variables. A trained phlebotomist obtained a blood sample. Two follow-up visits were conducted at 1-year intervals, and blood samples were again obtained.

Laboratory Methods

Blood specimens were collected in vacuum tubes containing clotting agents. Clotted blood was kept on ice and delivered to the laboratory on the same day, where it was stored at 4°C. On the following day, serum was separated from cells by centrifugation for 5 minutes at 1000*g* and then aliquoted and frozen at −20°C until use.

The WHO microneutralization assay [20] was used to test sera for antibodies to A/duck/Egypt/M2583A/2010 (H5N1 clade 2.2.1) and A/quail/Lebanon/273/2010 (H9N2 G1-like) viruses. A(H5N1) virus isolated from a backyard flock in Egypt was selected as an assay antigen, as most reported cases in Egypt involved clade 2.2.1 viruses [21]; the pathogenicity of the assay virus was rendered low by reverse genetics as previously described [22]. The A(H9N2) virus used was a G1-like virus similar to those circulating in the Middle East. Equal volumes of serum and virus diluted to 100 median tissue culture infective doses per milliliter were incubated at 37°C for 1 hour. The mixture was added to a confluent layer of Madin-Darby canine kidney cells in 96-well tissue culture plates and incubated for 48 hours. Virus hemagglutination activity was then tested in 0.5% chicken red blood cells (RBCs). The absence of hemagglutination was considered a positive test result for antibodies to the virus. Sera were inactivated by heating at 56°C for 30 minutes and titrated out to determine the end point titer. All assay runs included positive control serum (chicken hyperimmune antiserum against the specific assay antigens), virus control wells, and cell control wells. A titer of ≥80 was considered positive.

Sera that tested positive for anti-A(H5N1) antibodies were further tested by a hemagglutination inhibition (HI) assay with 1% horse RBCs as previously described [23]. To determine the extent of antibody cross-reaction with human influenza viruses, an HI with 0.5% turkey RBCs was used to test for antibodies to 2009 pandemic A(H1N1) virus (A/California/04/09) and seasonal A(H3N2) virus (A/Brisbane/10/07). For HI assays, sera were mixed a ratio of 1:3 with receptor-destroying enzyme (RDE; Denka Seiken, Tokyo, Japan) and incubated at 37°C overnight. RDE was then inactivated at 56°C for 30 minutes. Sera were hemadsorbed by mixing 1 part packed RBCs to 19 parts serum and incubating at 4°C for 1 hour. The RBCs were then pelleted and the serum separated. Serum was incubated with an equal volume of a solution containing 4 hemagglutination units per 25 μL of the antigen at room temperature for 30 minutes. The RBC mixture was then added and incubated for 30 minutes at room temperature. Sera were considered positive for antibodies if hemagglutination was inhibited.

Statistical Analysis

The Pearson χ^2 test and the Fisher exact test were used to compare categorical variables. The Student *t* test was used to compare normally distributed continuous variables. The Mood median test for independent samples was used to compare the medians of continuous variables that were not normally distributed. Logistic regression was used for multivariate analysis. A P value of $< .05$ was used to indicate a significant difference. Analysis was performed by using PASW (SPSS) 18.0 software.

RESULTS

Demographic, Health, and Exposure Variables

Eighty-four percent of the study participants (841) were available for follow-up 1, of whom 93% (785) were available for

Table 1. Baseline Demographic and Health Variables Among Subjects With or Without Exposure to Poultry

Variable	Exposed Group (n = 750)	Unexposed Group (n = 250)	*P* Value[a]
Age, y			
Mean ± SD	28.7 ± 18.4	33.3 ± 16.7	<.001[b]
Median (range)	27 (2–79)	31 (2–76)	.031[c]
Age category, y			
<6	79 (10.5)	11 (4.4)	<.001
7–16	174 (23.2)	29 (11.6)	
17–50	386 (51.5)	164 (65.6)	
>51	111 (14.8)	46 (18.4)	
Sex			
Female	425 (56.7)	158 (63.2)	NS
Male	325 (43.3)	92 (36.8)	
Monthly cost of food, Egyptian pounds			
<500	141 (19.2)	40 (16.8)	.045
500–1000	352 (48.0)	99 (41.6)	
1001–1500	142 (19.4)	51 (21.4)	
>1500	98 (13.4)	48 (20.2)	
Indoor water source			
Yes	732 (98.0)	248 (99.2)	NS
No	15 (2.0)	2 (0.8)	
Marital status			
Single	325 (43.6)	98 (39.4)	NS
Married	383 (51.3)	130 (52.2)	
Other	38 (5.1)	21 (8.4)	
Children aged <5 y in household, no.			
0	387 (51.8)	192 (77.1)	<.001
1–3	341 (45.6)	57 (22.9)	
>4	19 (2.5)	0 (0.0)	
Occupational category			
Housewife	154 (20.6)	23 (9.3)	<.001
Student	192 (25.6)	39 (15.7)	
Unskilled worker	26 (3.5)	34 (13.7)	
Skilled worker	130 (17.4)	63 (25.4)	
Professional	38 (5.1)	27 (10.9)	
Unemployed	190 (25.4)	57 (23.0)	
Other	19 (2.5)	5 (2.0)	
Educational level			
Not educated	249 (33.5)	51 (20.4)	<.001
Elementary	184 (24.7)	43 (17.2)	
Intermediate	79 (10.6)	22 (8.8)	
Secondary	173 (23.3)	78 (31.2)	
College	59 (7.9)	56 (22.4)	
Chronic lung problems			
Yes	35 (4.7)	7 (2.8)	NS
No	709 (95.3)	242 (97.2)	
Cardiovascular problems			
Yes	47 (6.3)	35 (14.0)	<.001
No	701 (93.7)	215 (86.0)	
Other chronic problems			
Yes	39 (5.2)	21 (8.4)	NS
No	705 (94.8)	229 (91.6)	
Uses tobacco products			
Yes	88 (11.7)	44 (17.6)	.018
No	662 (88.3)	206 (82.4)	
Ever received influenza vaccine			
Yes	17 (2.3)	14 (5.6)	.008
No	732 (97.7)	235 (94.4)	
Had ILI within the preceding 12 mo			
Yes	354 (47.4)	66 (26.4)	<.001
No	393 (52.6)	184 (73.6)	
Household member had ILI			
Yes	356 (47.5)	81 (32.4)	<.001
No	393 (52.5)	169 (67.6)	

Table 1 continued.

Data are no. (%) of subjects, unless otherwise indicated. Missing responses were omitted.

Abbreviations: ILI, influenza-like illness; NS, not significant; SD, standard deviation.

[a] By the χ^2 test, unless otherwise indicated.

[b] By the Student *t* test.

[c] By the median test.

follow-up 2. Table 1 compares the distribution of baseline demographic and health indicator variables in the exposed and unexposed groups. Unexposed participants were significantly older than exposed participants, were more frequently skilled workers or professionals, spent more on food, were more educated, and had fewer children per household. The exposed group reported significantly fewer cardiovascular problems and less use of tobacco products. They reported having received influenza vaccine but also reported a greater incidence of influenza-like illness during the previous year.

Table 2 shows the distribution of exposure variables in the exposed group. The majority raised backyard poultry (98%) and were exposed to poultry daily (61%). More than half reported disease outbreaks in their poultry. More than 20% reported vaccinating their poultry (but could not accurately describe the types of vaccines used), of whom 18% reported personally administering vaccine. More than 93% were exposed to chickens, and more than 80% were exposed to ducks. Exposure to other poultry species was less frequently reported. A small percentage owned pet birds, cats, or dogs. None of the subjects reported consistent use and proper decontamination of personal protective equipment.

Serological Results

The prevalence of positive titers against the 2009 pandemic A (H1N1) and seasonal A(H3N2) viruses differed by group according to time point (Table 3). At baseline, the prevalence of antibodies to 2009 pandemic A(H1N1) was 26% in the exposed group, compared with 11% in the control group ($P < .001$). At the first follow-up, the prevalence dropped in the exposed group

Table 2. Distribution of Exposure Variables Among Subjects Exposed to Poultry

Variable, Category	Subjects, No. (%)
Exposure setting	
Backyard	731 (97.5)
Live bird market	3 (0.4)
Commercial farm	16 (2.1)
Days exposed per week	
≤2	221 (29.8)
3–6	66 (8.9)
7	455 (61.3)
Disease outbreaks in subject's poultry	
Yes	397 (52.9)
No	353 (47.1)
Vaccination of poultry	
Yes	164 (22.1)
No	577 (77.9)
Personally vaccinate poultry	
Yes	29 (17.8)
No	134 (82.2)
Own chickens	
Yes	699 (93.2)
No	51 (6.8)
Chickens in neighborhood	
Yes	109 (14.5)
No	641 (85.5)
Live chickens in patronized market	
Yes	85 (11.3)
No	665 (88.7)
Own ducks	
Yes	600 (80.0)
No	150 (20.0)
Ducks in neighborhood	
Yes	77 (10.3)
No	673 (89.7)
Live ducks in patronized market	
Yes	123 (16.4)
No	627 (83.6)
Own geese	
Yes	51 (7.5)
No	631 (92.5)
Own pigeons	
Yes	184 (24.5)
No	566 (75.5)
Own turkeys	
Yes	93 (12.4)
No	657 (87.6)
Own pet birds	
Yes	6 (0.8)
No	744 (99.2)
Own cats or dogs	
Yes	27 (3.6)
No	723 (96.4)

Table 3. Results of Serological Testing Among Subjects With or Without Exposure to Poultry

Antigen, Time Point, Result	Exposed Group, Subjects, No. (%) (n = 750)	Unexposed Group, Subjects, No. (%) (n = 250)	*P* Value
A(H1N1)			
Baseline			
Positive	185 (25.7)	25 (10.9)	<.001
Negative	536 (74.3)	204 (89.1)	
Follow-up 1			
Positive	87 (12.7)	24 (17.0)	NS
Negative	599 (87.3)	117 (83.0)	
Follow-up 2			
Positive	335 (51.5)	58 (55.8)	NS
Negative	316 (48.5)	46 (44.2)	
A(H3N2)			
Baseline			
Positive	252 (35.0)	74 (32.3)	NS
Negative	467 (65.0)	155 (67.7)	
Follow-up 1			
Positive	42 (6.1)	1 (0.7)	.008
Negative	644 (93.9)	140 (99.3)	
Follow-up 2			
Positive	202 (31.1)	41 (39.4)	NS
Negative	447 (68.9)	63 (60.6)	
A(H5N1)			
Baseline			
Positive	15 (2.1)	0 (0.0)	.028
Negative	693 (97.9)	224 (100.0)	
Follow-up 1			
Positive	3 (0.4)	0 (0.0)	NS
Negative	679 (99.6)	139 (100.0)	
Follow-up 2			
Positive	4 (0.6)	0 (0.0)	NS
Negative	645 (99.4)	104 (100.0)	
A(H9N2)			
Baseline			
Positive	8 (1.1)	0 (0.0)	NS
Negative	700 (98.9)	224 (100.0)	
Follow-up 1			
Positive	51 (7.5)	1 (0.7)	.003
Negative	631 (92.5)	138 (99.3)	
Follow-up 2			
Positive	38 (5.9)	1 (1.0)	.036
Negative	610 (94.1)	103 (99.0)	

Missing responses were omitted.

Abbreviation: NS, not significant.

and increased in the control group but did not differ significantly between the 2 groups. At follow-up 2, the prevalence increased to >50% in both groups and was not found to differ significantly. The prevalence of antibodies to A(H3N2) was similar at baseline and at follow-up 2 (approximately 30%),

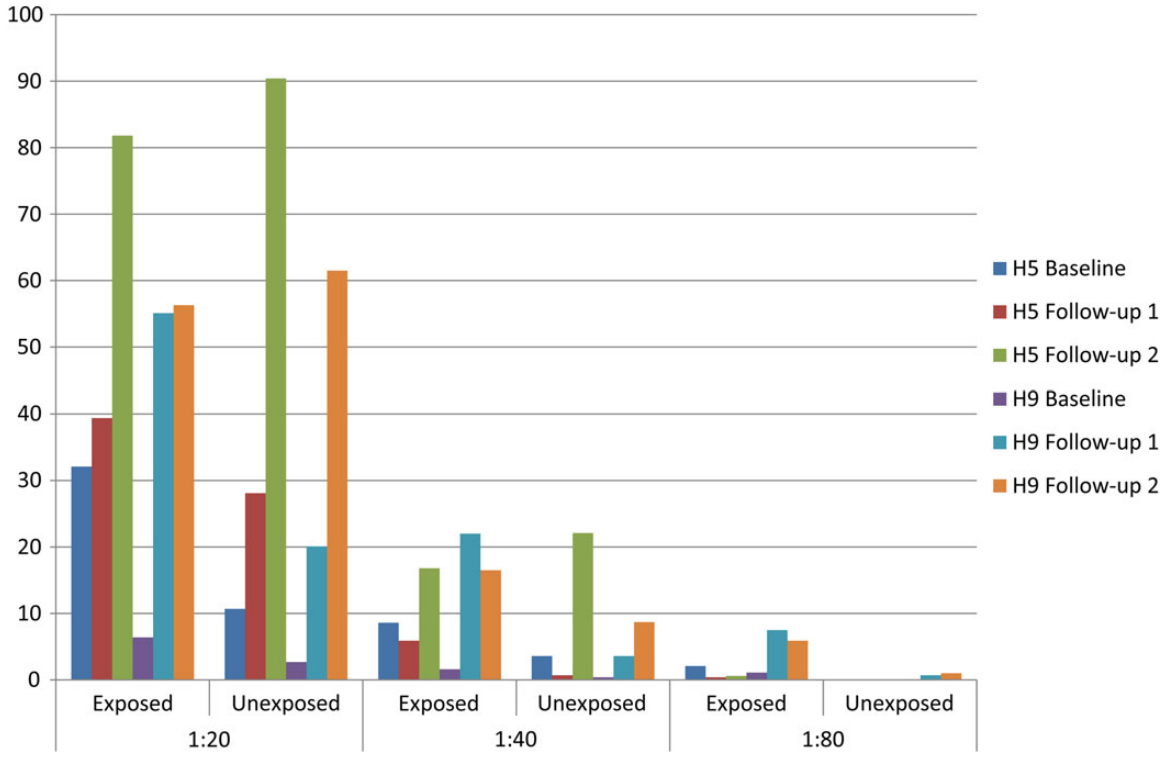

Figure 1. Titer categories at study time points among subjects with or without exposure to poultry.

while at follow-up 1 it was 6% in the exposed group and 0.7% in the unexposed group ($P = .028$).

A significantly higher prevalence of antibodies to A(H5N1) was detected in the exposed group at baseline (2.1% vs 0%; $P = .028$). The control group remained negative at both follow-up points, and the prevalence in the exposed group dropped to 0.4% and 0.6% a follow-up 1 and 2, respectively ($P > .05$). No significant difference was detected in the prevalence of antibodies to A(H9N2) at baseline. However, the prevalence in the exposed group at follow-up year 1 was 7.5%, compared with 0.7% in the unexposed group ($P = .003$). The same trend was observed at follow-up year 2, when the prevalence in the exposed and unexposed groups was 5.9% and 1%, respectively ($P = .036$). Importantly, at the low (negative) titers of 20 and 40, the prevalence of antibodies to both avian viruses was high in both groups, indicating high cross-reactivity with antibodies to 2009 pandemic A(H1N1) and A(H3N2) viruses. At the threshold positive titer of 80, none of the control subjects were seropositive for A(H5N1) virus, and only 1 subject was seropositive for A(H9N2) virus (Figure 1 and Table 3).

Characteristics of Subjects Seropositive at Baseline

We then focused on the 15 subjects positive for anti-A(H5N1) antibodies at baseline (Table 4). All were backyard poultry growers. Their ages were 9 to 50 years, and 10 were female. Each study site had at least 1 A(H5N1)-positive participant, but the Nile Delta sites (Gharbiya, Qalyubiya, and Kafr El Sheikh) had the majority of cases. Cases clustered by household: in Qalyubiya, 2 cases occurred in the same household, and another was in an adjacent household; in Gharbiya, 4 cases occurred in a single household, with a fifth case in the same neighborhood, while another 2 cases occurred in a single household in a different neighborhood; and in Kafr El Sheikh, 2 cases occurred in a single household, and another occurred in the same neighborhood. Within these clusters, positive subjects were not always blood relatives: 2 clusters included 2 siblings, and 1 cluster included a mother and her daughter. Only 1 of 15 subjects maintained a positive titer over the study duration; the others had negative titers at follow-ups 1 and 2. We also tested the 15 baseline sera by HI with horse RBCs. All sera but 1 had a detectable titer (7 had a titer of 80, and 7 had a titer of 40). Findings were similar in subjects with positive titers to A(H9N2) virus (Supplementary Tables 1 and 2). The age range was 6 to 65 years, and most subjects were female. Cases clustered by household and neighborhood, and many occurred in Fayyoum and in Nile Delta sites. Only 7 subjects tested positive at both follow-up points. Only 1 subject was seropositive for both A(H5N1) and A(H9N2) virus exposure (at follow-up year 1).

Risk Factors

Risk factors significantly associated with positive titers are shown in Table 5. In bivariate analysis, chronic lung disease, exposure to

Table 4. Characteristics of the 15 Participants Who Were H5 Positive at Baseline

Subject ID	Age, y	Sex	Governorate	Household ID	Baseline H5 Titer	Titer at Follow-up 1	Titer at Follow-up 2	Baseline HI Titer With Horse RBCs
10	31	M	Sharkiya	2	80	10	20	<10
282	38	F	Fayyoum	93	160	20	20	80
321	42	F	Qalyubiya	107	80	20	20	80
332	9	F	Qalyubiya	108	80	20	20	80
419	17	F	Qalyubiya	108	80	20	20	80
452	26	M	Gharbiya	118	80	20	ND	80
458	41	F	Gharbiya	118	160	ND	20	40
461	27	F	Gharbiya	118	80	40	10	40
464	9	M	Gharbiya	118	80	ND	ND	80
487	71	M	Gharbiya	121	80	ND	ND	40
531	29	F	Gharbiya	131	80	10	10	40
576	50	F	Gharbiya	131	80	20	20	40
606	14	F	Kafr El Sheikh	133	80	20	40	40
666	12	F	Kafr El Sheikh	140	80	10	40	80
669	9	M	Kafr El Sheikh	133	160	80	80	ND

Abbreviations: ID, identification number; ND, not done; RBC, red blood cell.

geese, and exposure to turkeys were associated with seropositivity against A(H5N1) virus. In multivariate analysis, only chronic lung disease remained significantly associated with A(H5N1) seropositivity. Older age, marital status, and cardiovascular disease were significantly associated with a positive antibody titer to A (H9N2) at follow-up year 1. However, in logistic regression, age 17–50 years was the only factor significantly associated with a positive titer to A(H9N2) virus. In bivariate analysis, older age (>51 years), being married, chronic heart disease, vaccination of poultry, and exposure to ducks were associated with positive antibody titers against A(H9N2) virus at follow-up 2. In logistic regression, only age >51 years, vaccination of poultry, and exposure to ducks were significant risk factors at follow-up 2.

DISCUSSION

A recent critical review of serological reports of A(H5N1) virus concluded that these studies often suffered from 2 major problems: (1) lack of a control group and (2) use of a titer with a low threshold for positivity or inconclusive results of laboratory assays [24]. Our study design was more stringent than those previously reported. By including a control unexposed group, we were able to exclude cross-reactivity caused by infection or immunization with other influenza viruses. The decision to define a positive antibody titer to avian influenza viruses as ≥80 not only met the WHO diagnostic criterion but, most importantly, avoided the cross-reactivity that can result from antibodies to A(H1N1) and A(H3N2) viruses. Cross-reactivity was evident in our study at titers of ≤40.

A microneutralization assay was our main serological test, and we confirmed A(H5N1)-positive serological findings by an HI assay with horse RBCs. Overall, antibody levels were relatively low (the highest titer was 160), consistent with the low immunogenicity of A(H5N1) viruses [25]. Further, most subjects who tested positive at baseline had a decrease in titer during follow-up, consistent with clinical trials of human A(H5N1) vaccines [26]. The antigens used in our study were contemporary isolates from Egypt, which reduced the likelihood of error from the use of viruses that differ from those in circulation. The seroprevalence of antibodies to A(H5N1) in the exposed group was <1% at the follow-up points, a rate not shown to be significantly different from that in the control group. A decreased number of confirmed cases was reported in Egypt during the same period (29 cases in 2010, 39 in 2011, 11 in 2012, and 4 in 2013) [10]. This decrease in seroprevalence and the number of cases remains unexplained. Two potential factors could be the cocirculation of A(H9N2) viruses and the continuous genetic drift of A(H5N1) viruses, which may have introduced changes that reduced its transmissibility to humans [4, 6].

We targeted backyard poultry growers in Egypt, a population that is continuously exposed to enzootic A(H5N1) virus and that comprises most of the world's recently reported cases. Previous serological studies were performed in populations likely to have limited, 1-time exposure. Continuous circulation of the virus in a population that uses little protective equipment suggests that more infections among humans are likely.

Our serological data credibly indicate that the prevalence of A(H5N1) infection is approximately 2% among Egyptians exposed to poultry. This prevalence demonstrates that the number of cases is greatly underreported and that the case-fatality rate is consequently greatly overestimated. The 2% prevalence amounts to a large number of people who might have been asymptomatically or mildly infected with A(H5N1) without

Table 5. Factors Associated With Positive Titers to A(H5N1) or A(H9N2) Influenza Viruses

Factor	Seropositive Subjects	Seronegative Subjects	*P* Value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
A(H5N1) at baseline					
Chronic lung problems					
Yes	5 (33.3)	30 (4.4)	<.001	**11.0 (3.5–34.5)**	**12.6 (3.8–41.7)**
No	10 (66.7)	659 (95.6)		Reference	Reference
Exposed to geese					
Yes	6 (40.0)	109 (15.7)	.012	**3.6 (1.2–10.2)**	3.1 (.9–10.4)
No	9 (60.0)	584 (84.3)		Reference	Reference
Exposed to turkeys					
Yes	5 (33.3)	81 (11.7)	.011	**3.8 (1.3–11.3)**	2.7 (.8–9.5)
No	10 (66.7)	612 (88.3)		Reference	Reference
A(H9N2) at follow-up 1					
Age, y					
Mean ± SD	43.2 ± 17.3	27.6 ± 18.0	<.001	. . .	. . .
Median	46	26	.031	. . .	. . .
Age category, y					
<6	1 (2.0)	69 (10.9)	<.001	Reference	Reference
7–16	4 (7.8)	160 (25.4)		1.7 (.2–15.7)	1.9 (.9–4.0)
17–50	28 (54.9)	319 (50.6)		6.0 (.8–45.3)	**5.2 (1.1–23.2)**
>51	18 (35.3)	83 (13.2)		**15.0 (1.9–115.0)**	8.5 (.9–85.5)
Marital status					
Single	9 (18.0)	285 (45.4)	.001	Reference	Reference
Married	36 (72.0)	313 (49.8)		**3.6 (1.7–7.7)**	1.0 (.3–3.0)
Other	5 (10.0)	30 (4.8)		**5.3 (1.7–16.8)**	1.4 (.3–5.8)
Heart problems					
Yes	9 (18.0)	35 (5.6)	.042	**3.7 (1.7–8.3)**	2.1 (.9–5.0)
No	41 (82.0)	595 (94.4)		Reference	Reference
A(H9N2) at follow-up 2					
Age, y					
Mean ± SD	45.4 ± 17.4	27.1 ± 18.0	<.001	. . .	. . .
Median	53	26	<.001	. . .	. . .
Age category, y					
<6	1 (2.6)	73 (12.0)	<.001	Reference	Reference
7–16	4 (10.5)	155 (25.4)		1.9 (.2–17.1)	7.5 (.5–120.2)
17–50	12 (31.6)	309 (50.7)		2.8 (.4–22.2)	4.2 (.6–29.2)
>51	21 (55.3)	73 (12.0)		**21.0 (2.8–160.2)**	**7.4 (3.1–17.4)**
Marital status					
Single	5 (13.2)	275 (45.4)	<.001	Reference	Reference
Married	27 (71.1)	305 (50.3)		**4.9 (1.8–11.7)**	1.1 (.4–3.2)
Other	6 (15.8)	26 (4.3)		**12.7 (3.6–44.4)**	3.7 (.5–29.5)
Heart problems					
Yes	6 (15.8)	33 (5.4)	.009	**3.3 (1.3–8.4)**	0.0 (.3–2.8)
No	32 (84.2)	575 (94.6)		Reference	Reference
Vaccinate poultry					
Yes	15 (39.5)	128 (21.3)	.009	**2.4 (1.2–4.8)**	**2.4 (1.1–5.0)**
No	23 (60.5)	473 (78.7)		Reference	Reference
Exposed to ducks					
Yes	36 (94.7)	484 (79.3)	.021	**4.7 (1.1–19.7)**	**5.7 (1.3–25.2)**
No	2 (5.3)	126 (20.7)		Reference	Reference

Data are no. (%) of subjects, unless otherwise indicated. Statistically significant values are in bold.

Abbreviations: CI, confidence interval; OR, odds ratio; SD, standard deviation.

being detected by the healthcare system. Only the few hundred cases who developed severe illness, whether due to underlying health conditions, infectious dose, or genetic predisposition, were eventually reported. Those cases represent the tip of the iceberg, and the 60% case-fatality rate applies only to this category. However, even the most accurate measurement of seroprevalence cannot indicate the true extent of human infection with A(H5N1) viruses, as we know too little about the factors that determine the timing and likelihood of seroconversion after exposure. Furthermore, given the finding that antibody titers drop over time, serological studies might miss individuals who were in fact infected but had a low antibody titer. The considerably lower case-fatality rate among Egyptian cases than reported in other countries suggests that different A(H5N1) strains may cause differential pathogenicity in humans. Hence, our results are applicable to the current situation in Egypt and do not necessarily apply to other regions, where different A(H5N1) viruses are circulating. As these viruses continue to undergo drift, it is important to continue monitoring their effects on exposed human and poultry populations. Our study showed evidence of infection with clade 2 A(H5N1) viruses, which are currently circulating in most affected countries [24]. Positive subjects clustered by household but were not always blood relatives, suggesting that infection was due to a common exposure, rather than to genetic predisposition. However, we cannot rule out the role of genetics in predisposition to avian influenza virus infection, as suggested by others [27, 28].

Serological evidence of human infection with A(H9N2) viruses has previously been reported in China, Romania, India, Vietnam, and Cambodia [29–35]. In those studies, the seroprevalence ranged between 1.2% and 9%. Not dissimilarly, in our study the seroprevalence of antibodies against A(H9N2) virus ranged from 5.9% to 7.5%. Serological evidence in our study occurred simultaneously with virus circulation in Egyptian poultry. No significant serological response was seen at baseline, when A(H9N2) virus had not yet been detected in poultry, but seropositive findings increased significantly when the virus was known to be infecting poultry. The antigen we used in our assay was a G1-like virus isolated in 2009 from quail in Lebanon, as A(H9N2) isolates from Egypt were not available at the start of the study. We continued to use this virus for follow-up testing. This virus was found to be genetically identical to the G1 viruses isolated later in Egypt [7].

Several risk factors for human infection with avian influenza viruses have been documented [36]. In our study, chronic lung problems were associated with elevated titers to A(H5N1) virus. Patients with chronic obstructive lung disease (COPD) are more susceptible to pulmonary infection and tend to have worse outcomes when infected with influenza virus [37]. A recent study found that having COPD is a risk factor for getting sick with avian influenza H7N9 virus infection in China [38]. Age, vaccination of poultry, and exposure to ducks were associated with elevated titers to A(H9N2) virus. Vaccination of poultry was a significant factor only at the time of second follow-up. A likely explanation is the similar timing of availability of a locally produced inactivated A(H9N2) vaccine, after A(H9N2) viruses became established in poultry; people using this vaccine may have been exposed to its antigens. The absence of an association between poultry vaccination and antibodies against A(H5N1) virus may be explained by the poor immunogenicity of H5 viruses. However, most subjects were unable to specify the type of vaccine used for their poultry. A(H9N2) viruses were detected in ducks in Egypt, but the prevalence was much lower than that among chickens [8]. However, duck A(H9N2) viruses in Egypt are not well studied, and their risk to humans is not well understood. Ducks have been previously associated with human infection with A(H5N1). In Cambodia, swimming in ponds possibly contaminated by ducks was a risk factor for having antibodies against A(H5N1) [39].

Our study had several limitations. Because we used convenience sampling, selection bias may have affected our results; however, poultry-raising practices in Egypt are generally homogenous. Misclassification bias may have affected the control group. A proportion of the controls could have been exposed to live poultry in markets or rooftop coops without revealing this fact to the study team, as such practices were banned as part of the Egyptian A(H5N1) control plan. Control subjects could also have traveled to rural areas to visit family. If some control subjects were misclassified, seroprevalence would have been underestimated.

Seroepidemiological studies are not designed to provide information about the incidence of disease in humans, the clinical course of infection, or the rate of secondary human-to-human infection. The answers to these questions will require large-scale prospective studies with close follow-up of subjects and their poultry. Such studies will allow us to verify infection by molecular or culture techniques, determine rates of transmission and seroconversion, and provide valuable immunological data.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, US Department of Health and Human Services (contract HHSN266200700005C); and by the American Lebanese Syrian Associated Charities.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Saad MD, Ahmed LS, Gamal-Eldein MA, et al. Possible avian influenza (H5N1) from migratory bird, Egypt. Emerg Infect Dis **2007**; 13:1120–1.
2. Meleigy M. Egypt battles with avian influenza. Lancet **2007**; 370:553–4.
3. Kayali G, El-Shesheny R, Kutkat MA, et al. Continuing threat of influenza (H5N1) virus circulation in Egypt. Emerg Infect Dis **2011**; 17: 2306–8.
4. El-Shesheny R, Kandeil A, Bagato O, et al. Molecular characterization of avian influenza H5N1 virus in Egypt and the emergence of a novel endemic sub-clade. J Gen Virol **2014**; 95:1444–63.
5. El-Shesheny R, Kayali G, Kandeil A, et al. Antigenic diversity and cross-reactivity of avian influenza H5N1 viruses in Egypt between 2006 and 2011. J Gen Virol **2012**; 93:2564–74.
6. Monne I, Hussein HA, Fusaro A, et al. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. Influenza Other Respi Viruses **2013**; 7:240–3.
7. Arafa AS, Hagag N, Erfan A, et al. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. Virus Genes **2012**; 45:283–94.
8. Kayali G, Kandeil A, El-Shesheny R, et al. Active surveillance for avian influenza virus, Egypt, 2010–2012. Emerg Infect Dis **2014**; 20:542–51.
9. WHO. H5N1 avian influenza: Timeline of major events. http://www.who.int/influenza/human_animal_interface/H5N1_avian_influenza_update150612N.pdf. Accessed May 2014.
10. WHO. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003–2014. http://www.who.int/influenza/human_animal_interface/EN_GIP_20140124CumulativeNumberH5N1cases.pdf?ua=1. Accessed May 2014.
11. Kayali G, Webby RJ, Ducatez MF, et al. The epidemiological and molecular aspects of influenza H5N1 viruses at the human-animal interface in Egypt. PLoS One **2011**; 6:e17730.
12. Butt KM, Smith GJ, Chen H, et al. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol **2005**; 43:5760–7.
13. Lin YP, Shaw M, Gregory V, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci U S A **2000**; 97:9654–8.
14. Li FC, Choi BC, Sly T, Pak AW. Finding the real case-fatality rate of H5N1 avian influenza. J Epidemiol Community Health **2008**; 62:555–9.
15. Yong E. Influenza: Five questions on H5N1. Nature **2012**; 486:456–8.
16. Wang TT, Parides MK, Palese P. Seroevidence for H5N1 influenza infections in humans: meta-analysis. Science **2012**; 335:1463.
17. Van Kerkhove MD, Mumford E, Mounts AW, et al. Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systematic review. PLoS One **2011**; 6:e14582.
18. Van Kerkhove MD, Riley S, Lipsitch M, et al. Comment on "Seroevidence for H5N1 influenza infections in humans: meta-analysis". Science **2012**; 336:1506; author reply.
19. Kayali G, Webby RJ, Xiong X, Sherif LS, El-Ghafar EA, Ali MA. Prospective study of avian influenza transmission to humans in Egypt. BMC Public Health **2010**; 10:685.
20. WHO. Serological diagnosis of influenza by microneutralization assay. http://www.who.int/influenza/gisrs_laboratory/2010_12_06_serological_diagnosis_of_influenza_by_microneutralization_assay.pdf. Accessed May 2014.
21. Younan M, Poh MK, Elassal E, et al. Microevolution of highly pathogenic avian influenza A(H5N1) viruses isolated from humans, Egypt, 2007–2011. Emerg Infect Dis **2013**; 19:43–50.
22. Webby RJ, Perez DR, Coleman JS, et al. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet **2004**; 363:1099–103.
23. Kayali G, Setterquist SF, Capuano AW, Myers KP, Gill JS, Gray GC. Testing human sera for antibodies against avian influenza viruses: horse RBC hemagglutination inhibition vs. microneutralization assays. J Clin Virol **2008**; 43:73–8.
24. Toner ES, Adalja AA, Nuzzo JB, Inglesby TV, Henderson DA, Burke DS. Assessment of serosurveys for H5N1. Clin Infect Dis **2013**; 56:1206–12.
25. Clegg CH, Roque R, Van Hoeven N, et al. Adjuvant solution for pandemic influenza vaccine production. Proc Natl Acad Sci U S A **2012**; 109:17585–90.
26. Nolan TM, Richmond PC, Skeljo MV, et al. Phase I and II randomised trials of the safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in healthy adults. Vaccine **2008**; 26:4160–7.
27. Juno J, Fowke KR, Keynan Y. Immunogenetic factors associated with severe respiratory illness caused by zoonotic H1N1 and H5N1 influenza viruses. Clin Dev Immunol **2012**; 2012:797180.
28. Horby P, Sudoyo H, Viprakasit V, et al. What is the evidence of a role for host genetics in susceptibility to influenza A/H5N1? Epidemiol Infect **2010**; 138:1550–8.
29. Zhou P, Zhu W, Gu H, et al. Avian influenza H9N2 seroprevalence among swine farm residents in China. J Med Virol **2014**; 86:597–600.
30. Coman A, Maftei DN, Krueger WS, et al. Serological evidence for avian H9N2 influenza virus infections among Romanian agriculture workers. J Infect Public Health **2013**; 6:438–47.
31. Huang R, Wang AR, Liu ZH, et al. Seroprevalence of avian influenza H9N2 among poultry workers in Shandong Province, China. Eur J Clin Microbiol Infect Dis **2013**; 32:1347–51.
32. Blair PJ, Putnam SD, Krueger WS, et al. Evidence for avian H9N2 influenza virus infections among rural villagers in Cambodia. J Infect Public Health **2013**; 6:69–79.
33. Uyeki TM, Nguyen DC, Rowe T, et al. Seroprevalence of antibodies to avian influenza A (H5) and A (H9) viruses among market poultry workers, Hanoi, Vietnam, 2001. PLoS One **2012**; 7:e43948.
34. Pawar SD, Tandale BV, Raut CG, et al. Avian influenza H9N2 seroprevalence among poultry workers in Pune, India, 2010. PLoS One **2012**; 7: e36374.
35. Wang M, Fu CX, Zheng BJ. Antibodies against H5 and H9 avian influenza among poultry workers in China. N Engl J Med **2009**; 360:2583–4.
36. Van Kerkhove MD. Brief literature review for the WHO global influenza research agenda–highly pathogenic avian influenza H5N1 risk in humans. Influenza Other Respir Viruses **2013**; 7(suppl 2):26–33.
37. Sung LC, Chen CI, Fang YA, et al. Influenza vaccination reduces hospitalization for acute coronary syndrome in elderly patients with chronic obstructive pulmonary disease: A population-based cohort study. Vaccine **2014**; 32:3843–9.
38. Liu B, Havers F, Chen E, et al. Risk Factors for Influenza A(H7N9) Disease-China, 2013. Clin Infect Dis **2014**; 59:787–94.
39. Vong S, Ly S, Van Kerkhove MD, et al. Risk factors associated with subclinical human infection with avian influenza A (H5N1) virus–Cambodia, 2006. J Infect Dis **2009**; 199:1744–52.