MAJOR ARTICLE

Risk Factors Associated with Subclinical Human Infection with Avian Influenza A (H5N1) Virus—Cambodia, 2006

Sirenda Vong,[1] Sowath Ly,[1] Maria D. Van Kerkhove,[1] Jenna Achenbach,[4] Davun Holl,[2] Philippe Buchy,[1] San Sorn,[2] Heng Seng,[3] Timothy M. Uyeki,[4] Touch Sok,[3] and Jacqueline M. Katz[4]

[1]Institut Pasteur in Cambodia, [2]Ministry of Agriculture, Forestry and Fisheries, and [3]Ministry of Health, Phnom Penh, Cambodia; and [4]Centers for Disease Control and Prevention, Atlanta, Georgia

(See the article by Zhou et al. on pages 1726–34, the article by Tiensin et al. on pages 1735–43, and the editorial commentary by Briand and Fukuda on pages 1717–9.)

Background. We conducted investigations in 2 villages in Cambodia where outbreaks of influenza H5N1 occurred among humans and poultry to determine the frequency of and risk factors for H5N1 virus transmission.

Methods. During May 2006, ~7 weeks after outbreaks of influenza H5N1 among poultry occurred, villagers living near households of 2 patients with influenza H5N1 were interviewed about potential H5N1 exposures and had blood samples obtained for H5N1 serological testing by microneutralization assay. A seropositive result was defined as an influenza H5N1 neutralizing antibody titer of ⩾1:80, with confirmation by Western blot assay. A case-control study was conducted to identify risk factors for influenza H5N1 virus infection. Control subjects, who had seronegative results of tests, were matched with H5N1-seropositive persons by village residence, households with an influenza H5N1–infected poultry flock, sex, and age.

Results. Seven (1.0%) of 674 villagers tested seropositive for influenza H5N1 antibodies and did not report severe illness; 6 (85.7%) were male. The 7 H5N1-seropositive persons, all of whom were aged ⩽18 years, were younger than participants who tested seronegative for H5N1 antibodies (median age, 12.0 years vs. 27.4 years; $P = .03$) and were more likely than were the 24 control subjects to report bathing or swimming in household ponds (71.4% vs. 20.8%; matched odds ratio, 11.3; $P = .03$).

Conclusions. Avian-to-human transmission of influenza H5N1 virus remains low, despite extensive poultry contact. Exposure to a potentially contaminated environment was a risk factor for human infection.

The recent global spread of highly pathogenic avian influenza A (H5N1) viruses among poultry is unparalleled [1, 2]. In Cambodia, outbreaks of H5N1 among poultry that have been associated with high mortality have been widespread, and 8 human H5N1 cases have been detected since January 2005 [2, 3]. Despite the worldwide spread of influenza H5N1 and repeated and intense exposure, evidence suggests that H5N1 viruses may not be easily transmitted from infected poultry to humans [4–8]. As influenza H5N1 viruses continue to circulate and evolve among poultry, poultry-to-human transmission of H5N1 viruses could increase. Therefore, the extent of asymptomatic and clinically mild illness among humans that is caused by circulating H5N1 virus strains should be monitored.

In Cambodia, a 3-year-old girl (the fifth confirmed case) from a village in Kampong Speu Province and a 12-year-old boy (the sixth confirmed case) from a village in Prey Veng Province died of H5N1 virus infection on 23 March and 5 April 2006, respectively. These 2 cases were not epidemiologically linked [3]. As part of H5N1 outbreak investigation activities, we conducted a sero-epidemiological investigation among persons living in the affected villages a few weeks after the children's fatal

Received 27 August 2008; accepted 28 October 2008; electronically published 5 May 2009.

Potential conflicts of interest: none reported.

Financial support: Institut Pasteur in Cambodia, Food and Agriculture Organization of the United Nations, and Centers for Disease Control and Prevention.

Presented in part: Bangkok International Conference on Avian Influenza 2008: Integration from Knowledge to Control, Bangkok, Thailand, 23–25 January 2008.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Reprints or correspondence: Dr. Sirenda Vong, Institut Pasteur in Cambodia, 5 Blvd. Monivong, POB 983, Phnom Penh, Cambodia (svong@pasteur-kh.org).

The Journal of Infectious Diseases 2009; 199:1744–52

0022-1899/2009/19912-0006$15.00
DOI: 10.1086/599208

illnesses. We sought to determine the frequency of H5N1 virus transmission from poultry to humans and to explore potential risk factors for H5N1 virus infection.

PARTICIPANTS AND METHODS

Retrospective poultry mortality survey. Within 1 day after notification of confirmed human influenza H5N1 cases in a village of Kampong Speu Province (village 1) and in a village of Prey Veng Province (village 2), we surveyed all households located within a 1-km radius of those of the 2 patients with influenza H5N1, and we collected information about animal illness suggestive of H5N1 in each household by interviewing the poultry owner with use of a standardized questionnaire. We also collected serum samples and tracheal and cloacal swab samples from 10 randomly selected live, healthy-appearing ducks from each household where such birds remained for H5N1 testing. We did not attempt to collect samples from healthy chickens that likely were not infected because of the high fatality rate among H5N1-infected chickens.

Sero-epidemiological investigation. We conducted a sero-epidemiological investigation during 8–11 May 2006, ~7 weeks after confirmation of the first human H5N1 case. Four investigation teams with 3 members each visited all households within a 1-km radius of the households of the 2 patients with influenza H5N1 and interviewed residents and parents of children aged <12 years with use of a standard questionnaire to collect demographic data and information about basic poultry exposures (21 standardized closed-ended questions) from 29 January through 17 April. The investigators also obtained blood specimens for H5N1 serological testing [4]. The exposure period marked the beginning of the Chinese New Year (29 January) and the end of the Khmer New Year (17 April). Repeated home visits were made to interview absent household members.

Matched case-control study. During 15–16 February 2007, after the initial serological test results were available, 2 teams of 5 persons (3 interviewers, 1 phlebotomist, and 1 supervisor) reinterviewed the H5N1-seropositive persons (i.e., participants whose samples were seropositive for influenza H5N1 antibodies) and matched control subjects by administering an in-depth questionnaire. Control subjects were randomly selected from the pool of available participants who had seronegative samples and were matched 4:1 with H5N1-seropositive persons by village residence, households with an H5N1 virus–infected poultry flock, sex, and age (±3 years). The in-depth questionnaires were administered to collect information from before the occurrence of the H5N1 cases in 2006 on basic hygiene, general health (e.g., chronic medical conditions, smoking, and drinking habits), and usual frequency of direct and indirect contact with poultry. Participants were asked about direct contact with poultry through food preparation, handling or caring for poultry, and in the case of children, playing with domestic and/or wild poultry populations. The questionnaires also included inquiries about indirect contact with poultry in the immediate environment around the home and village through house types, water sources, ponds, and rice fields. All interviewers were blinded to the case-control status of the participant. H5N1-seropositive persons were informed in private of their serological test results by the study supervisor and were given a special informed consent form requesting permission to collect a second blood sample. After providing consent, H5N1-seropositive persons had blood samples obtained before each interview without any interviewers present. Subsequently, the interviewers obtained signed informed consent for participation in the case-control study with use of the in-depth questionnaire from each participant or parent or guardian of children aged ≤16 years. The study was approved by the Cambodian National Ethics Committee as part of routine sero-epidemiological investigations in response to H5N1 case notification.

Laboratory methods. Tracheal and cloacal swab specimens were collected from ducks, placed directly into sterile tubes containing viral transportation medium tubes, and transported cold (4°C–8°C) each day to the National Animal Health Laboratory, Ministry of Agriculture, Forestry and Fisheries in Phnom Penh, where they were inoculated into embryonated hen's eggs. Allantoic fluid was tested by hemagglutination using chicken red blood cells for influenza virus detection, and then H5 virus was identified by hemaglutinination-inhibition (HI) assay using the A/Chicken/Scotland/59(H5N1) strain and A/Chicken/Scotland/59(H5N1) anti-serum (both provided by the World Animal Health Reference Laboratory). Any positive specimen was to be sent to the Institut Pasteur in Cambodia for subtyping by real-time reverse-transcriptase polymerase chain reaction (PCR) [3]. Serological testing of duck serum samples was performed using an HI assay with antigen and serum samples, as described above. A poultry flock was defined as H5N1 virus infected when ≥1 swab specimen tested positive for H5N1 by real-time reverse-transcriptase PCR or when ≥1 serum specimen tested positive for antibody to H5N1 by HI assay (titer, ≥1:16).

Human serological testing. A blood specimen (5 mL) was obtained from participants and was transported cold (4°C–8°C) to the Virology Laboratory at the Institut Pasteur in Cambodia. Serum was separated from blood samples, aliquoted, and frozen at −80°C. Serum samples were shipped frozen on dry ice to the Influenza Division laboratories at the US Centers for Disease Control and Prevention (Atlanta, GA) for serological analyses. All serum samples were tested by an H5N1 virus–specific microneutralization assay and a modified HI assay using 1% horse red blood cells (with use of the methods described elsewhere [9, 10]) and with A/Vietnam/JP/14/2005 (H5N1) virus as antigen (a clade 1 virus that shared high hemagglutinin amino acid sequence identity with H5N1 viruses circulating in Cambodia during 2006) [9, 10]. Serum samples that had titers ≥1:80 in duplicate microneutralization assays were considered to be positive

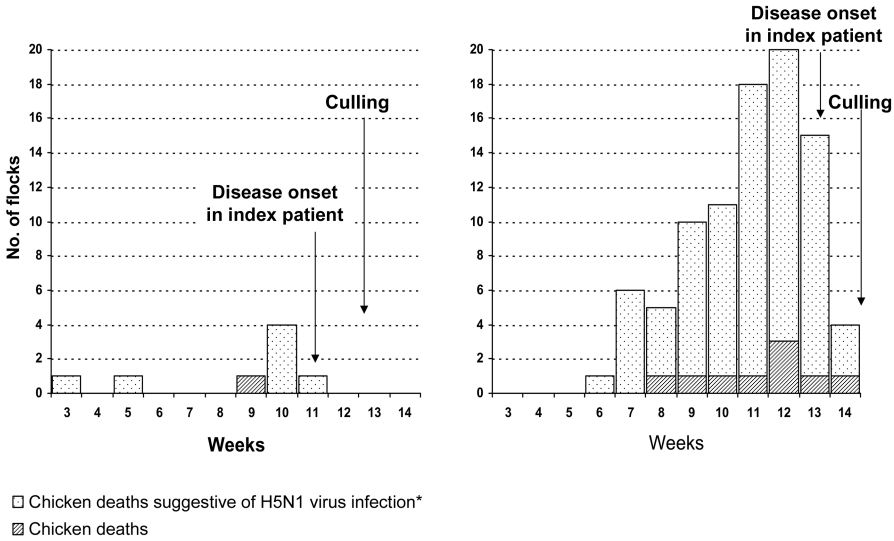

Figure 1. Number of flocks with chicken deaths in 2 villages of Prey Veng ($n = 90$; *right*) and Kampong Speu ($n = 8$; *left*) Provinces, Cambodia, January–April 2006. *Defined as sudden death (<2 days) and 100% chicken fatality.

for anti-H5 neutralizing antibody [11]. Serum samples that tested positive in the microneutralization assay were also tested by Western blot assay using a clade 1 recombinant HA antigen (A/Vietnam/1203/2004; Protein Sciences), as described elsewhere [8]. In accordance with World Health Organization guidelines, serum samples that tested positive by both microneutralization and Western blot assays were considered to be positive for anti-H5 antibodies [12]. All of the individuals whose samples tested negative for anti-H5 antibodies had titers <1:10 in the modified HI assay. The distribution of microneutralization titers among individuals with seronegative samples was as follows: 95% had a titer ⩽1:20, 4% had a titer of 1:40, and 1% had a titer of 1:80–1:160 but had negative results of confirmatory serological assays.

Statistical analysis. Stata, version 9 (StataCorp), was used for all statistical analyses. All tests were 2-tailed; statistical significance was set at $P < .05$, without correction for the number of statistical tests performed. We compared proportions with use of Fisher's exact test, mean values with use of Student's *t* test, and median values with use of the Kruskal Wallis test. In univariate analysis, maximum likelihood estimates for the matched odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using a conditional logistic regression model and the Wald χ^2 test.

RESULTS

Outbreaks of influenza H5N1 among poultry in the villages. Among 193 households within a 1-km radius of the households of the 2 patients with influenza H5N1, residents of 169 households (87.6%) in the 2 villages were home at the time of the survey, and all agreed to participate; village 1 accounted for 29.6% of the participants. Among interviewed households, poultry ownership was high and flock sizes were small, with no differences between the 2 villages before H5N1 virus detection; chickens were raised in 151 households (89.4%) (median household flock size, 15 chickens; range,

Table 1. Characteristics of 674 participants in 2 villages of Kampong Speu and Prey Veng Provinces, Cambodia, May 2006.

Characteristic	Participants ($n = 674$)
Province	
Kampong Speu	248 (36.8)
Prey Veng	426 (63.2)
Male sex	291 (43.2)
Age	
Median, years (range)	21.5 years (4 months–89 years)
Mean, years	27.4
0–4 years	53 (7.9)
5–19 years	269 (39.9)
20– 39 years	162 (24.0)
40– 59 years	114 (16.9)
>59 years	76 (11.3)
Occupation[a]	
Farmer	344 (57.5)
Student	238 (39.8)
Other	16 (2.7)
Family size[b]	
Median, no. of persons per family (range)	5 (1–11)
Median, no. of persons aged <19 years per family (range)	2 (0–5)

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

[a] Data are for 598 participants.

[b] Data are for 161 participants.

Table 2. Characteristics of 7 participants infected with influenza H5N1 virus, Kampong Speu and Prey Veng provinces, Cambodia, 2006.

	H5N1-infected participant						
Variable	1	2	3	4	5	6	7
Demographic characteristic							
Sex	M	F	M	M	M	M	M
Age, years	4	4	10	12	16	18	16
Province	Prey Veng	Prey Veng	Prey Veng	Prey Veng	Prey Veng	Prey Veng	Kampong Speu
Worked in rice field(s)	Yes	No	Yes	Yes	Yes	Yes	Yes
General characteristic							
Lived in household with a pond	Yes	Yes	Yes	Yes	Yes	Yes	No
Swam in duck pond	Never	Sometimes	Sometimes	Sometimes	Sometimes	Never	Frequently
Pond was household's only water source	Yes	Yes	Yes	Yes	Yes	Yes	No
Free-ranging birds	Yes	Yes	Yes	No	Yes	Yes	Yes
Poultry handling							
Touched live poultry with bare hands	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Touched sick poultry with bare hands	Yes	No	No	Yes	Yes	No	No
Fed poultry	Yes	Yes	Yes	Yes	Yes	No	Yes
Gatheredand/or placed poultry in cages	Yes	Yes	Yes	Yes	Yes	No	Yes
Removed and/or cleaned poultry feces	No	No	Yes	Yes	Yes	Yes	Yes
Gatheredand/or washed away feathers	No	No	Yes	Yes	Yes	Yes	Yes
Touched and/or collected eggs	Yes	Yes	Yes	Yes	Yes	No	Yes
Gathered dead birds for food	No	No	Yes	Yes	No	No	Yes
Ate poultry bought from market	No	No	No	No	No	No	Yes
Ate raw poultry blood	No	No	No	No	No	No	No
Poultry preparation practices							
Helped prepare poultry for food	No	No	No	Yes	Yes	Yes	Yes
Slaughtered and/or bled poultry	No	No	No	No	Yes	Yes	Yes
Removed and/or washed internal organs from poultry	No	No	No	Yes	Yes	Yes	No
Cut and/or washed poultry meat	No	No	No	No	Yes	Yes	No
Defeathered sick poultry	No	No	Yes	Yes	Yes	No	No
Protection when handling poultry and/or poultry products							
Wore plastic bags over hands	No	No	No	No	No	No	No
Wore gloves	No	No	No	No	No	No	No
Wore mask	No	No	No	No	No	No	No
Contact with patient with confirmed case within 1 week before illness onset							
Played with patient	Yes	Yes	No	No	Yes	No	No
Prepared food with patient	No	No	No	No	No	No	No
Went to school with patient	No	No	No	Yes	Yes	No	No
Swam in pond with patient	No	No	No	No	No	No	No
Visited patient during illness	Yes	No	No	No	No	No	No
Family related to patient	Yes	Yes	No	No	Yes	No	Yes
Basic hygiene							
Washed hands after touching poultry	Sometimes	Rarely	Rarely	Rarely	Sometimes	Sometimes	Rarely
Washed hands before eating	Sometimes	Rarely	Rarely	Rarely	Rarely	Sometimes	Sometimes
Wore shoes	Sometimes	Never	Sometimes	Always	Always	Always	Sometimes
General health issues							
Asthma	Yes	No	No	No	No	No	No
Chronic bronchitis	No	Yes	No	No	No	No	No
Heart disease	No	No	No	No	No	No	No
Diabetes	No	No	No	No	No	No	No
Smoking habit	No	No	No	No	No	Yes	No
Fever	No	Yes	No	No	No	No	No
Cough	No	Yes	No	No	No	No	No
Shortness of breath	No	No	No	No	No	No	No
Other symptoms	No	No	No	No	No	No	No

Table 3. Univariate analysis of factors associated with influenza H5N1 infection in 31 humans with use of conditional logistic regression, Cambodia, 2006.

Variable	No. (%) of participants		Matched OR (95% CI)	P^a
	H5N1-seropositive persons (n= 7)	Control subjects (n = 24)		
Education				
Ability to read	4 (57.1)	10 (41.7)	1.9 (0.32–10.70)	.67
Ability to write	4 (57.1)	9 (37.5)	2.2 (0.38–12.95)	.41
Occupation: worked in rice fields	6 (85.7)	20 (83.3)	1.2 (0.11–13.41)	.88
House characteristic				
Floor of house: wood or bamboo	7 (100.0)	24 (100.0)	. . .	
Built on stilts	6 (85.7)	20 (83.3)	0.3 (0.01–5.27)	.38
Floor under house: soil	100.0	100.0	. . .	
Fence sealent to animals	4 (57.1)	14 (82.3)	0.3 (0.04–2.25)	.33
Pond in house yard	6 (86.7)	23 (95.8)	0.4 (0.04–2.93)	.40
Duck pond in house yard	5 (71.4)	21 (87.5)	. . .	
Swim and/or bathe in ponds	5 (71.4)	5 (20.8)	**11.3 (1.25–102.18)**	**.03**
Water source in house	0 (0)	0 (0)	. . .	
Water source in house yard (not inside house)				
Open water well	0 (0)	1 (4.2)	. . .	
Pond	6 (85.7)	10 (41.7)	**6.8 (0.68–66.43)**	**.08**
Water well with pump	5 (71.4)	11 (45.8)	4.0 (0.27–169.71)	.24
Water tap	0 (0)	0 (0)	. . .	
Caring for poultry				
Touched live poultry with bare hands	7 (100.0)	16 (66.7)	. . .	
Touched sick and/or dead poultry with bare hands	3 (42.9)	12 (50.0)	0.6 (0.08–4.51)	.61
Fed poultry	6 (85.7)	22 (91.7)	0.6 (0.01–19.46)	.64
Gathered poultry and placed in cages and/or poultry areas	6 (85.7)	10 (41.7)	**5.8 (0.98–34.12)**	**.05**
Removed and/or cleaned feces from cages and/or poultry areas	5 (71.4)	8 (33.3)	**5.0 (0.69–36.33)**	**.09**
Touched and/or collected eggs	6 (85.7)	17 (70.8)	2.5 (0.23–26.02)	.44
Gathered dead birds for food	3 (42.9)	16 (66.7)	0.4 (0.06–2.24)	.26
Poultry preparation practices				
Helped prepare poultry for food	4 (57.1)	13 (54.2)	1.1 (0.20–6.34)	.89
Slaughtered and/or bled poultry	3 (42.9)	7 (29.2)	2.5 (0.31–10.8)	.45
Removed internal organs from poultry	3 (42.9)	8 (33.3)	1.5 (0.26–8.68)	.64
Cut and/or washed internal organs	3 (42.9)	8 (33.3)	1.5 (0.26–8.68)	.64
Cut and/or washed poultry meat	2 (28.6)	4 (16.7)	2.0 (0.27–14.9)	.49
Defeathered poultry that died of illness	3 (42.9)	6 (25.0)	3.1 (0.28–34.87)	.35
Used personal protective equipment when handling poultry or poultry products				
Plastic bags over hands	0 (0)	1 (4.2)	. . .	
Gloves	0 (0)	1 (4.2)	. . .	
Mask (cotton mask)	0 (0)	2 (8.4)	. . .	
Contact with patient with confirmed influenza H5N1				
Played with patient	3 (42.9)	7 (29.2)	1.8 (0.31–10.79)	.50
Went to school with patient	2 (28.6)	6 (25.0)	1.2 (0.16–8.39)	.88
Swam in pond with patient	0	2 (12.5)	. . .	
Visited patient during illness	1 (14.3)	4 (16.7)	0.8 (0.07–9.31)	.88
Cared for patient during illness	0	1 (4.2)	. . .	
Family related to patient	4 (57.1)	15 (62.5)	0.8 (0.14–4.56)	.80
Basic hygiene				
Washed hands with soap before eating	3 (42.8)	17 (70.8)	0.2 (0.02–2.09)	.19
Washed hands with soap after handling poultry	2 (28.6)	11 (45.8)	0.4 (0.03–3.90)	.40
General health				
Smoked	1 (14.3)	1 (4.2)	3.8 (0.19–77.52)	.34
Asthma	1 (14.3)	1 (4.2)	3.8 (0.19–77.52)	.34
Chronic bronchitis	1 (14.3)	0 (0)	. . .	

NOTE. CI, confidence interval; OR, odds ratio.

[a] By Wald χ^2 test.

Table 4. Serum antibody response to influenza H5N1 virus over time, sero-epidemiological investigation in 2 villages of Cambodia, May 2006 and February 2007

Patient	Age, years	Sex	Test 1[a]		Test 2: MicroNT 1–2 months after exposure; MicroNT 10–11 months after exposure[b]
			MicroNT	Horse HI	
1	12	M	80	40	80; 20
2	16	M	320	160	320; 40
3	10	M	1280	640	1280; 320
4	4	F	320	⩾1280	640; 20
5	4	M	1280	320	640; 160
6	18	M	640	640	640; 80
7	16	M	160	80	160; 20

NOTE. Serum samples from 7 H5N1-seropositive persons that tested positive in the microneutralization assay (MicroNT) also tested positive at a dilution of 1:100 in an H5-specific Western blot assay. MicroNT was performed twice (tests 1 and 2), in May 2006 (1–2 months after exposure) and in February 2007 (10–11 months after exposure). Assays were performed using A/Vietnam/JP14/2005 (H5N1) virus. Horse HI, hemaglutination-inhibition assay.

[a] Performed 1–2 months after exposure, in May 2006.

[b] Results for samples collected 1–2 months after exposure that were tested at a second time point and for samples collected 10–11 months after exposure.

1–100 chickens), and ducks were raised in 80 households (47.3%; median household flock size, 7 ducks; range, 1–40 ducks). Seventy-seven households (45.6%) reared both chickens and ducks. From 15 January (week 3) through 5 April (week 14) 2006, 104 households (68.8%) reported chicken deaths. Although the 2 villages were >200 km apart and had no apparent links, the outbreaks occurred during approximately the same period and peaked during weeks 10–12, approximately 2–3 weeks before the 2 human H5N1 cases were identified. However, chicken die-offs during the previous 3 months were reported more frequently in village 2 than in village 1 (affecting 90 [83.3%] of 108 chicken flocks vs. 8 [18.6%] of 43 chicken flocks; $P < .001$) (figure 1). Mortality among household chicken flocks was higher in village 2 than in village 1 (median, 68% [range, 7.0%–100%] vs. 39.5% [range, 2.0%–87.0%]; $P < .001$). Sudden death among chickens (<2 days) and 100% chicken fatality, suggestive of outbreaks of H5N1, affected 88 (89.8%) of the 98 flocks that experienced any mortality; there were no differences between the villages (90.0% vs. 87.5%; $P = .67$) [13]. Thirty-six households (45.0%) also reported duck die-offs; the median mortality among household duck flocks was 58.5% (range, 4%–100%). Of the samples collected from 217 ducks of 51 flocks, none were positive for H5 antibody by egg's inoculation; however, 140 duck serum samples (64.5%) from 35 households (68.6%) tested positive for H5 antibody by HI assay.

Sero-epidemiological investigation. A total of 674 villagers from 162 households were recruited; there were no refusals to participate in the sero-epidemiological study. The median age of the participants was 21.5 years (range, 4 months–89 years), and 43.6% were male (table 1). The 2 village study populations did not differ with regard to age, sex, or occupation (data not shown). During the study period, most participants reported repeated direct and close poultry contact, including feeding or touching poultry (73.3%), collecting poultry feces for manure (50.9%), plucking feathers of sick poultry (31.1%), or collecting sick and/or dead poultry with bare hands (36.8%). In contrast, few persons (0.8%) purchased live or freshly killed poultry from a market.

Risk factors for influenza H5N1 virus infection. Of the 674 participants, 7 (1.0% [95% CI, 0.8%–3.8%]), including 6 from village 2, tested positive for H5N1-neutralizing antibodies. Six (85.7%) of these participants were male, and the median age was 12 years (range, 4–18 years), which was significantly younger than that of the overall study population ($P = .04$). Only H5N1-infected participant 2, a 4-year-old girl with a medical history of chronic bronchitis, had a reported febrile illness with cough during the study period, and none were hospitalized or presented for medical attention (table 2). All 7 H5N1-seropositive persons reported close contact with poultry or poultry products, lived in different households, and were not members of the households of the earlier identified 2 patients with H5N1. None of them were identified as close contacts of 1 of the earlier identified patients with influenza H5N1during the outbreak investigations. However, 3 H5N1-seropositive persons (ages, 4, 16, and 16 years) reported that they were blood relatives of the earlier identified patient with H5N1 from village 2 who died. All H5N1-seropositive persons lived in wooden houses on stilts with well or pond water as the only water source for the family. H5N1-infected participant 6 stayed only 5 days in village 2 during the study period (from 30 March through 3 April 2006) and did not report any contact with pond water but reported preparing poultry to eat and cleaning and removing poultry feces (table 2).

For the case-control study, 24 control subjects were enrolled and matched to the 7 H5N1-seropositive persons; 4 control subjects each were matched to 3 H5N1-seropositive persons, and 3

control subjects each were matched to the 4 remaining H5N1-seropositive persons. H5N1-seropositive persons were more likely than control subjects to report bathing or swimming in household ponds (71.4% vs. 20.8%; matched OR, 11.3 [95% CI, 1.3– 102.2]; $P = .03$, by Wald χ^2 test) (table 3). Gathering poultry and placing poultry in cages was associated with H5N1 virus infection ($P = .05$). There was a trend toward statistical significance for environmental contamination; H5N1-seropositive persons' households had ponds as the only water source, whereas control subjects' households did not (85.7% vs. 41.7%; $P = .08$). In addition, H5N1-seropositive persons were more likely than control subjects to have cleaned and/or removed feces from poultry cages ($P = .09$). No differences were observed between H5N1-seropositive persons and control subjects with regard to basic hygiene, poultry food preparation practices, or contact with a patient with confirmed influenza H5N1. The small number of H5N1-seropositive persons precluded multivariable analyses.

Neutralizing antibody response to influenza H5N1 virus infection. The initial H5N1 neutralizing antibody titers in the 7 H5N1-seropositive persons were measured >8 weeks after the peak of the poultry outbreaks in both village. It is assumed that exposures to H5N1 virus–infected poultry occurred before April 2006 in both villages, after which exposure was presumably eliminated because of poultry culling and environmental disinfection activities. At that time, the median H5N1-neutralizing antibody titer was 1:320 (range, 1:80–1:1280). The median HI titer was also 1:320 (1:40 to >1:1280). All serum samples that tested positive for H5N1-neutralizing antibody also had positive results at a dilution of 1:100 for reactivity with recombinant H5 HA in a Western blot assay (table 4). Serum samples collected from the 7 H5N1-seropositive persons during the case-control study 10 months after the initial testing of samples demonstrated a 4- to 32-fold reduction in H5N1-neutralizing antibody titers, compared with the earlier samples. At this later time point, only 3 individuals had H5N1-neutralizing antibody titers ⩾1:80; these 3 individuals had the highest initial H5N1-neutralizing antibody titers (1:640–1:1280). Similar reductions in serum HI titers were also detected in all 7 H5N1-seropositive persons at this later time point (data not shown).

DISCUSSION

Our findings suggest that H5N1 virus transmission from poultry to humans was rare in Cambodia during 2006, despite overwhelming evidence of H5N1 virus circulation among poultry flocks during the study period [4–8, 14]. We detected 7 influenza H5N1 virus infections through a sero-epidemiological investigation in 2 villages, in addition to 2 fatal cases that were detected through surveillance. Our findings suggest that the H5N1 case-fatality rate of 29% (2 of 9 H5N1-seropositive persons) in the populations of the 2 Cambodian villages living within a 1-km radius of the 2 patients with influenza H5N1 who died was substantially lower than the current 63% case-fatality rate worldwide, in which the denominator includes severe and fatal cases detected through surveillance as of September 2008; however, the difference is not statistically significant ($P = .16$) [2]. Our finding of 1% seroprevalence differs slightly from the H5N1 seroprevalence found in previous studies that identified either no evidence or limited evidence of asymptomatic infection or mild disease [4–6, 8]. However, previous studies were underpowered to detect such low H5N1 seroprevalence. Because of our negative findings in a previous sero-epidemiological survey of rural villagers exposed to sick or dead poultry, these results warrant further ongoing sero-epidemiological investigations to assess additional subclinical or mild H5N1 virus infections among humans exposed to influenza H5N1 viruses worldwide, especially as H5N1 viruses continue to evolve [4].

Our results also indicate that swimming or bathing in household ponds could be a risk factor for influenza H5N1 virus infection. These small ponds are common and usually serve as a water source for backyard animals and gardening. Ducks usually have access to these ponds and may deposit large amounts of feces in ponds in which children commonly bathe and play. In addition to the human case investigations, we also collected environmental specimens in areas presumably contaminated with H5N1 virus to determine potential risks for bird-to-human transmission. H5N1 viral RNA was detected in 27 of 77 environmental specimens, including pond water plants in the H5N1-associated households and their surroundings [15]. Although we were not able to isolate H5N1 viruses from these environmental specimens, experimental studies have shown that avian influenza A viruses can remain detectable in water and wet feces for up to 4–6 days at 37°C [16, 17]. Avian-to-human transmission of H5N1 virus is thought to occur through aerosolized virus inhalation into the respiratory tract or by inoculation of the nose, mouth, or conjunctival mucosa through self-transfer from contaminated hands, because H5N1 viruses replicate primarily in the human respiratory tract [18, 19]. However, there is evidence to suggest that H5N1 viruses can penetrate via the gastrointestinal tract, suggesting that ingestion of contaminated food (e.g., ingestion of H5N1 virus–infected duck blood or uncooked or undercooked poultry or poultry products) or ingestion of water contaminated with H5N1 virus–infected feces could be potential infectious sources [20–23]. In contrast, we found that some exposures identified in other studies as risk factors for H5N1 disease (i.e., slaughtering, defeathering, butchering, and preparing poultry for cooking, particularly when combined with poor hand hygiene) were not prevalent among our H5N1-seropositive persons and, thus, were not significant risk factors for H5N1 virus infection in our study [24, 25]. However, preparation of poultry for cooking may have been the source of infection in H5N1-infected participant 6.

In these 2 villages, 7 of the 9 H5N1-seropositive persons who were infected with influenza H5N1 were male, and all were aged ⩽18 years. The explanation for these H5N1-associated demographic characteristics is not well understood. It is possible that differences in intensity or frequency of possible H5N1 exposures could account for the age and sex pattern that we observed. Previous assessments of poultry-handling practices in rural Cambodia revealed that young boys were exposed to potentially contaminated environments more frequently than were young girls and adults, because (1) boys aged <10 years participate in outdoor activities (e.g., swimming in ponds and playing with poultry) more frequently than do girls and (2) adults and older children handle and clean poultry and areas where poultry are kept more often than do younger children [26]. Alternatively, it has been suggested that adults may have some protection against H5N1 virus, because they have a higher likelihood of preexisting immunity to human influenza A viruses [27, 28].

Similar to the patterns observed in Hong Kong during 1997 and in Vietnam, Indonesia, and Turkey during 2005–2006, most of the H5N1 virus–infected children detected in our investigations presented with either mild disease or were asymptomatic, and none sought medical attention. Moreover, of the 6 H5N1-seropositive persons who lived in village 2, 3 were blood relatives of one of the patients with H5N1 who were initially detected [29, 30]. It is conceivable that the severity of the disease, which has been associated with an overwhelming inflammatory response, could be mediated by intrinsic immunological susceptibility [31]. In addition, inherited biologic factors, such as α2,3 sialic acid virus receptor phenotype variations, may also explain why fatal illness occurred in 2 persons and subclinical infection occurred in 7 others, whereas many other residents of these 2 villages reported similar exposures but did not have serological evidence of H5N1 virus infection [32, 33].

The H5N1-neutralizing antibody titers detected in serum samples collected from the 7 H5N1-seropositive persons in May 2006 were comparable to those detected in samples from patients with confirmed H5N1 cases reported to the World Health Organization (authors' unpublished data) [10]. Recently, the World Health Organization reported an asymptomatic H5N1 virus infection in a 33-year-old man from Pakistan. The infection was diagnosed on the basis of results of H5 real-time reverse-transcriptase PCR, and an antibody titer of 1:320 was detected in a serum specimen collected 9 days after symptom onset [34]. These findings are consistent with the conclusion that the 7 cases identified in our study were indeed a result of asymptomatic H5N1 virus infection rather than transient mucosal exposure to noninfectious antigen. H5N1-neutralizing antibody titers had decreased substantially in all H5N1-seropositive persons' serum samples collected ~10 months after exposure to H5N1 virus. These findings have implications for retrospective sero-epidemiological studies seeking evidence of human H5N1 virus infection in general, because they suggest that such studies should be conducted within a few months, if possible, after an H5N1 outbreak among poultry for optimal serological detection of human infection. Because there are few data available on the longevity of human serum antibody responses to H5N1 viruses, even in virologically confirmed cases, we cannot conclude whether the decrease in antibody titer is a general property of the human H5N1 antibody response or a consequence of the presumably reduced viral replication in persons with subclinical H5N1 viral infection.

Our study has several limitations. First, because the H5N1-seropositive persons and control subjects were asked about practices that occurred ~1 year earlier, recall errors could have occurred, which would have resulted in misclassification of reported exposures. However, recall errors may have been minimized by using a referent study period (Chinese and Khmer New Year) that was recognized easily by participants. Furthermore, awareness of the 2 H5N1 fatal cases, poultry culling activities, and media attention may have helped all participants to remember pertinent exposures during the study period. Because of the small number of H5N1-seropositive persons, the study may have been underpowered to detect significant differences for some potential risk factors. Despite matching procedures, the small sample size also limited the number of risk factors that we could assess as potential confounding factors. In addition, some H5N1-seropositive persons were blood related to 1 of the initial 2 patients who died, which may have emphasized some lifestyle in common that could be unrelated to the infection. Although the independence between H5N1-seropositive persons is difficult to assess, it was notable that none of these relatives lived in the same household. However, the overall pattern of the results suggests that our findings support an association between direct or indirect poultry contact and H5N1 virus infection.

Taken together, our results underscore the risk of indirect transmission of H5N1 virus infection from poultry to humans through exposure to a contaminated environment, as well as the risk of direct transmission from poultry to humans, particularly among younger children in rural Cambodia. However, despite reports of potential transmission from contaminated water, observational and analytical studies have not identified exposure to a contaminated environment as an established risk factor for influenza H5N1 [25, 35]. Additional investigations are required to confirm the importance of this transmission route. Nevertheless, basic hygiene education, strict separation between human and backyard poultry areas in Cambodian households, restriction of access to potentially contaminated water, and limiting of interactions between poultry and children should be recommended.

Acknowledgments

We thank the staff of the Virology and Epidemiology Units, Institut Pasteur in Cambodia, and the staff of the National Veterinary Research Institute, Cambodia Ministry of Agriculture.

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