MAJOR ARTICLE

Evidence for Subclinical Avian Influenza Virus Infections Among Rural Thai Villagers

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Background. Regions of Thailand reported sporadic outbreaks of A/H5N1 highly pathogenic avian influenza (HPAI) among poultry between 2004 and 2008. Kamphaeng Phet Province, in north-central Thailand had over 50 HPAI poultry outbreaks in 2004 alone, and 1 confirmed and 2 likely other human HPAI infections between 2004 and 2006.

Methods. In 2008, we enrolled a cohort of 800 rural Thai adults living in 8 sites within Kamphaeng Phet Province in a prospective study of zoonotic influenza transmission. We studied participants' sera with serologic assays against 16 avian, 2 swine, and 8 human influenza viruses.

Results. Among participants (mean age 49.6 years and 58% female) 65% reported lifetime poultry exposure of at least 30 consecutive minutes. Enrollees had elevated antibodies by microneutralization assay against 3 avian viruses: A/Hong Kong/1073/1999(H9N2), A/Thailand/676/2005(H5N1), and A/Thailand/384/2006(H5N1). Bivariate risk factor modeling demonstrated that male gender, lack of an indoor water source, and tobacco use were associated with elevated titers against avian H9N2 virus. Multivariate modeling suggested that increasing age, lack of an indoor water source, and chronic breathing problems were associated with infection with 1 or both HPAI H5N1 strains. Poultry exposure was not associated with positive serologic findings.

Conclusions. These data suggest that people in rural central Thailand may have experienced subclinical avian influenza infections as a result of yet unidentified environmental exposures. Lack of an indoor water source may play a role in transmission.

Highly pathogenic avian influenza (HPAI) infections have been particularly problematic in Asia. Thailand detected its first HPAI poultry outbreaks during 2003 and its first human cases in 2004 [1] (Figure 1). Detections continued through 2006 when intensive bird and human surveillance, poultry culling, poultry vaccination programs, and several other interventions seem to have stopped transmission [1–7]. In concert with the poultry epizootics, 25 human HPAI cases occurred during the period 2004–2006, with a 68% case-fatality rate [8].

In most areas of the world, influenza surveillance is conducted in urban areas at the best medical facilities [9, 10]. People living in rural settings or people with mild influenza infections who do not seek medical care may be missed in such surveillance.

In this work we sought to prospectively study adults with poultry exposure living in rural central Thailand for evidence of avian influenza virus infections. This report details our methods of enrolling the study cohort and presents our findings from enrollment questionnaire data and the serological investigation of enrollment sera.

METHODS

Study Location

Kamphaeng Phet Province was chosen as a study site because of its location within the region of the country most affected by an outbreak of HPAI from 2004 to 2005. Kamphaeng Phet is located ~300 kilometers

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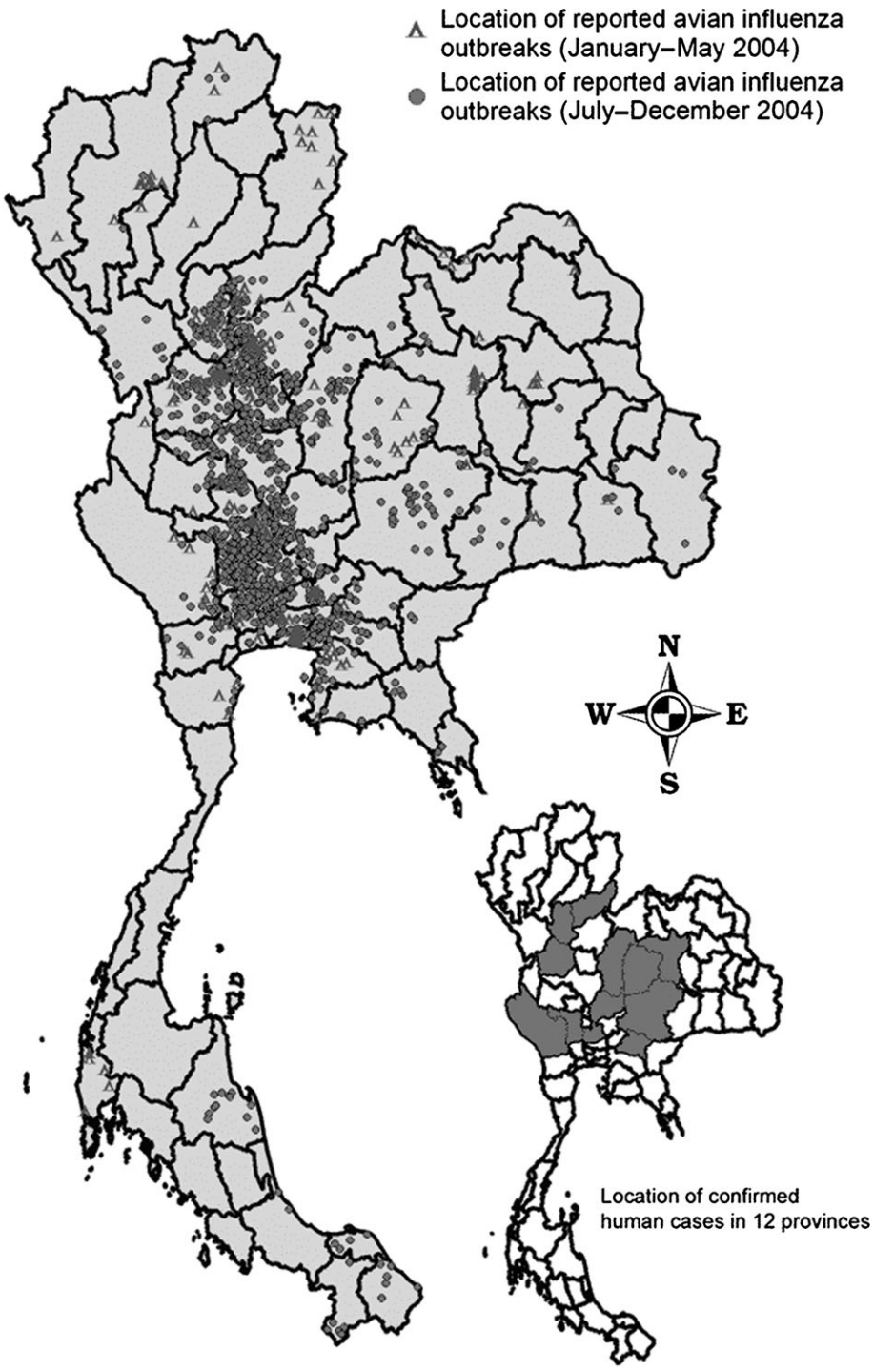

Figure 1. Locations of highly pathogenic avian influenza H5N1 detection among poultry in Thailand, 2004. Source: Tiensin T, Chaitaweesub P, Songserm T, Chaisingh A, Hoonsuwan W, Buranathai C, Parakamawongsa T, Premashthira S, Amonsin A, Gilbert M, Nielen M, Stegeman A. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg Infect Dis. 2005; 11:1664–72.

north of Bangkok in north-central Thailand, and is the site of the Kamphaeng Phet-AFRIMS Virology Research Unit (KAVRU), a Virology Department field station of the Armed Forces Research Institute of Medical Sciences (AFRIMS). In 2000, the population of Kamphaeng Phet was nearly 700 000. Villages located in the districts of Mueng and Phran Kratai of Kamphaeng Phet were chosen as study enrollment sites. Many laboratory-confirmed H5N1 infections in poultry have occurred within the various subdistricts of Mueng District.

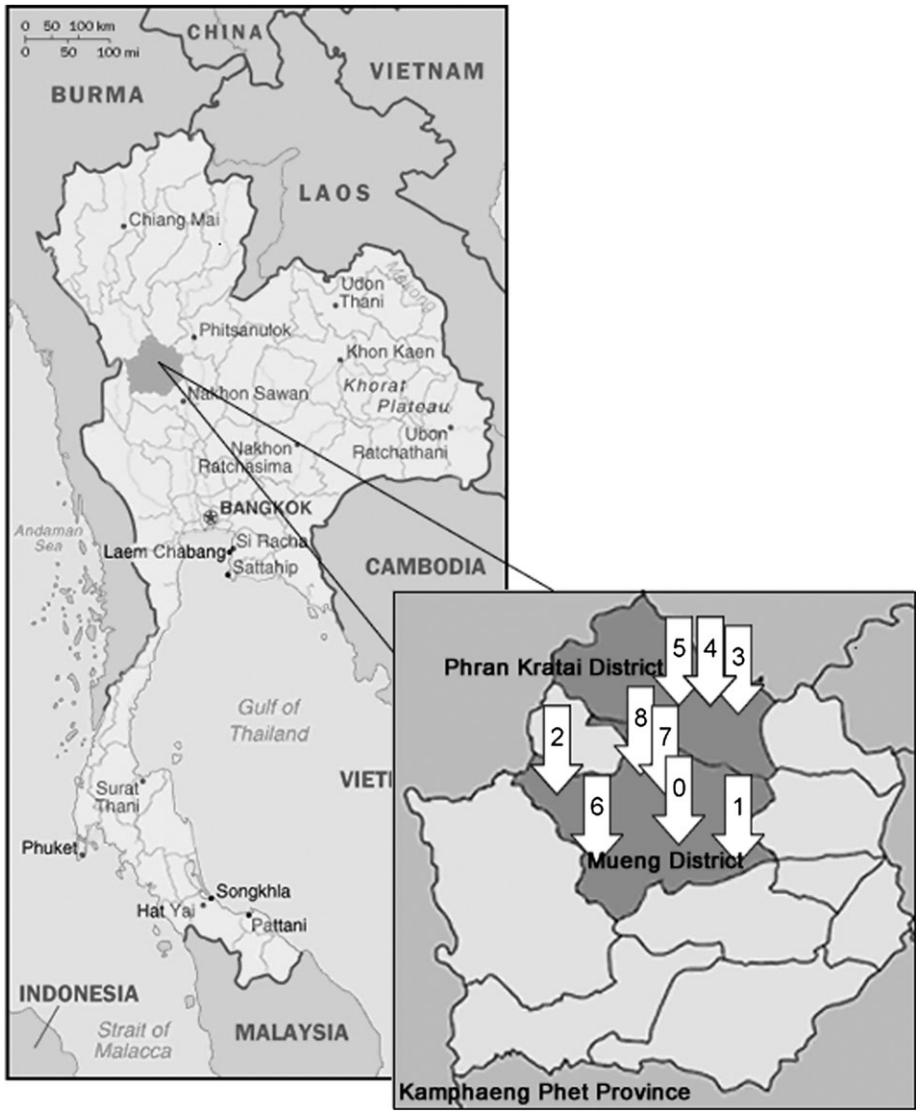

Figure 2. Map showing the location of Kamphaeng Phet-AFRIMS Virology Research Unit (KAVRU), our study field laboratory and the 8 enrollment sites: 0 = KAVRU Laboratory, 1 = Village #9 Thep Nakhon District, 2 = Villages #4 and #10 Na Bo Kham District, 3 = Village #3 Khui Ban Ong District, 4 = Village #7 Khui Ban Ong District, 5 = Village #8 Khui Ban Ong District, 6 = Village #12 Tha Khun Ram District, 7 = Village #6 Nong Pling District, 8 = Village #11 Nong Pling District. Figure created From 3 Figures: https://www.cia.gov/library/publications/the-world-factbook/graphics/maps/large/th-map.gif, http://upload.wikimedia.org/wikipedia/commons/thumb/e/e0/Thailand_Kamphaeng_Phet_locator_map.svg, and http://upload.wikimedia.org/wikipedia/commons/9/9f/Amphoe_6201.png, accessed 6 September 2011.

Study Participants

A total of 6 institutional review boards reviewed and approved the study. Prior to enrollment, village assessments were made through meetings with rural village leaders, and local Ministry of Public Health and Ministry of Agriculture and Cooperatives professionals. Over an approximate 6-month period, assessments were made for intense and diverse poultry exposures among 22 villages within a 30-minute drive of the KAVRU Field Laboratory. The villages were evaluated for their previous detections of A/H5N1 HPAI among domestic poultry, human population size, number of homes with poultry and swine exposure, and the variety of poultry exposures. We selected 8 sites (a total of 11 villages) within Mueng and Phran Kratai districts (Figure 2). Most villagers had small flocks of domestic poultry (chickens, ducks, quail). Some residents raised and trained fighting cocks.

Enrollment

Adults living in the study villages were recruited and trained as study field workers. Houses in the 8 sites were mapped and numbered. Using a systematic selection sampling approach with a random-number generated start, study field workers met with adults $\geq$ 20 years of age (age of legal consent) in the selected households to explain the study. Participants were

Table 1. Influenza Viruses Used in Serological Studies

Avian viruses	Swine viruses
A/Duck/Alberta/35/76(H1N1)	A/Swine/Wisconsin/238/97(H1N1)[a] (Classical North American H1N1 strain)
A/Env/Hong Kong/MPU3156/2005(H2N2)	
A/Duck/Czech Republic/1/56(H4N6)	A/Swine/Minnesota/593/99(H3N2)[a] (North American lineage strain)
A/Migratory duck/Hong Kong/MPS180/2003(H4N6)	
A/Chukkar/Minnesota/14591-7/98(H5N2)	
A/Teal/Hong Kong/w312/97(H6N1)	
A/Turkey/Massachusetts/3740/65(H6N2)	Human viruses
A/Turkey/Virginia/4529/2002(H7N2)	A/New Caledonia/20/99(H1N1)[a]
A/Env/Hong Kong/MPB127/2005(H7N7)	A/Brisbane/59/2007(H1N1)[a]
A/Turkey/Ontario/6118/68(H8N4)	A/Panama/2007/99(H3N2)[a]
A/Migratory duck/Hong Kong/MP2553/2004(H8N4)	A/Brisbane/10/2007(H3N2)[a]
A/Turkey/Minnesota/38391(H9N2)	A/Thailand/384/2006(H5N1)[b,c]
A/Migratory duck/Hong Kong/MPD268/2007(H10N4)	A/Thailand/676/2005(H5N1)[b,c]
A/Chicken/Germany/49(H10N7)	A/Hong Kong/1073/99(H9N2)[b]
A/Duck/Memphis/546/74(H11N9)	A/Mexico/4108/2009(pandemic H1N1)[a]
A/Duck/Alberta/60/76(H12N5)	

Unless otherwise indicated, serologic study was performed using the microneutralization technique.

[a] Virus studied with hemagglutination inhibition assay.

[b] Virus of avian origin.

[c] Highly pathogenic virus, Clade1.

required to reside in the household $\geq$ 20 days each month and to have no known immunosuppressive conditions. Participants were informed of the prospective nature of the study which involved annual serum specimen collections, active surveillance for influenza-like-illness (ILI) through weekly home visits by study field workers to assess for illness, and if they experienced an influenza virus infection, they and their family members would be asked to cooperate with additional studies for influenza. Potential enrollees were assigned a number and through a random-number generator, 1 adult was selected from each household. Selected participants were then enrolled after informed consent was obtained. Field staff administered an enrollment questionnaire through face-to-face interviews, collected a venous blood sample, provided training for the use of a digital thermometer, and provided written and oral instructions to the participants to contact study staff should they develop an ILI. ILI was defined as an acute onset of a respiratory illness with a measured temperature $\geq$ 38°C and a sore throat or cough for $\geq$ 4 hours. Participants were provided with snacks, vitamins, or personal items worth $<$ 5 US dollars in support for time lost during enrollment and subsequent study encounters.

Laboratory Methods

Blood specimens were transported at room temperature and respiratory swabs transported at 4°C to a study laboratory at KAVRU between 30 and 180 minutes following collection. Serum was separated and stored at −80°C.

Serological studies were performed at the University of Iowa's Center for Emerging Infectious Diseases, at the Thailand National Institute of Health, Nonthabauri, Thailand, and at the University of Florida's Global Pathogens Laboratory. Serological tests included hemagglutination inhibition (HI) assays to study human sera for antibodies against human and swine influenza viruses and microneutralization (MN) assays to study human sera for antibodies against viruses of avian origin. Avian influenza virus strains were selected by H type for their geographic and temporal proximity to the population (Table 1).

We used a previously described HI assay [11] to test for serum antibodies against 4 previously prevalent human, 2 swine, and the 2009 pandemic H1N1 influenza A viruses (Table 1). Influenza virus strains were grown in fertilized eggs. Sera were pretreated 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% (turkey) solution of erythrocytes as previously established [12].

A MN assay adapted from that reported by Rowe [13–15] was used to detect antibodies to a large panel of avian and avian-like viruses (Table 1). These viruses were also grown in fertilized eggs. Sera were first screened at a dilution of 1:10. Positive specimens were titrated in duplicate by examining 2-fold serial dilutions from 1:10 to 1:1280 in virus diluent (85.8% minimum essential medium [Invitrogen], 0.56% bovine serum albumin [BSA], 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer [Invitrogen], 100 mg/L streptomycin [Invitrogen], and 100 000 units/L penicillin [Invitrogen]). Virus neutralization was performed by adding 100 median tissue culture

infective dose ($TCID_{50}$) of virus to the sera. The Reed–Muench method was used to determine the $TCID_{50}$/100μL [16]. Madin–Darby canine kidney cells (MDCK) cells in log phase growth were adjusted to 2.0×10^5 cells/mL with diluent. We added 100 μL of cell suspension to each well and the plate incubated at 37°C with 5% CO_2 for 24 hours. Plates were washed twice with phosphate-buffered saline (PBS), fixed for 10 minutes with cold 80% acetone at room temperature. The enzyme-linked immunosorbent assay (ELISA) endpoint titer was expressed as the reciprocal of the highest dilution of serum with optical density (OD) $<$ X, where X = [(average OD of virus control wells) + (average OD of cell control wells)]/2. The back titration was run in duplicate and was only accepted when both replicates had matching results.

Statistical Methods

Questionnaire data were manually entered twice in a relational database designed in Microsoft Access, and verified for data-entry problems and questionnaire-administration inconsistencies with structured query language. Questionnaire data and laboratory data were merged using unique participant identifiers.

Our study outcomes were serological evidence of previous infection with avian influenza viruses by the MN assay run on enrollment sera. Because of a low prevalence of elevated antibodies against the various avian influenza viruses and our inability to determine in this cross-sectional analyses when such an infection might have occurred, we chose a low threshold of antibody titer ($\geq$ 1:10) as evidence of previous infection with an avian influenza strain. Because we know that cross-reactions from previous infection with human viruses might confound avian influenza virus serology, we sought to control such potential confounding by adding human influenza virus reactivity covariates to the multivariate models when the bivariate analyses suggested they were important outcome predictors. As done previously [15, 17–19], a HI titer $\geq$ 1:40 was accepted as evidence of human or swine influenza virus infection or human influenza vaccination.

Initially we examined risk factors for bivariate associations with MN assay results using binary logistic regression and proportional odds modeling [20]. 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 $<$ .25 were considered for inclusion in multivariate models. Final multivariate models were designed using manual backward elimination. Analyses were performed with SAS v9.2 (SAS Institute).

RESULTS

Between April and October 2008, field staff enrolled a total of 800 participants (100 from each of 8 sites) (Figure 2). Adults in 3% of selected homes declined to participate. The median age of the 800 participants was 49.6 years. Participants were more often female (57.6%) and frequently had no indoor plumbing (32.1%); few reported ever receiving a human influenza vaccine (1.6%). Most participants reported taking a medication during the past 30 days (78.8%) and about half reported having had a respiratory illness during the last 12 months (53.4%). A substantial percentage (20.4%) reported that they had a history of heart disease, hypertension, or stroke. Self-reported poultry exposure, defined as ever being within 1 meter of live poultry for 30 consecutive minutes, was prevalent among the participants (65.4%), although only 11.4% of participants reported ever being exposed to swine (Table 2).

Serological activity against low-pathogenic avian influenza (LPAI) viruses was sparse, with the exception of A/Env/Hong Kong/MPU3156/2005(H2N2) and A/Hong Kong/1073/1999(H9N2), for which 322 participants (40.2%) and 38 participants (4.7%) had elevated titers, respectively. All 322 respondents who had elevated titers for A/Env/Hong Kong/MPU3156/2005(H2N2) were born before 1968, suggesting the serologic activity represented cross-reaction from a human pandemic H2N2 virus infection that ceased to circulate in 1968. In bivariate logistic analysis, 2 covariates were weakly associated with an increased risk for elevated titer for A/Hong Kong/1073/1999(H9N2) (Table 3), but with various multivariate logistic models, only tobacco use remained a significant risk factor (unadjusted odds ratio [OR] = 2.3; 95% confidence interval [CI], 1.2–4.5). No strong associations of elevated antibodies to human influenza viruses were detected to suggest cross-reaction from human influenza infection. Other LPAIs with low serologic activity included an H5N2 (1 positive), H6N1 (2 positives), H7N7 (1 positive), a second H9N2 (1 positive), an H10N4 (1 positive), and an H12N5 (1 positive) (Table 1).

Only 1 respondent had an elevated titer for A/Swine/Wisconsin/238/97(H1N1), but 245 participants (30.6%) had an elevated titer for A/Swine/Minnesota/593/99(H3N2). However, as only 91 respondents reported any swine exposure, this seroreactivity is likely a reflection of cross-reactivity due to human virus infection. Multivariate logistic modeling validated this suggestion in that risk factors for elevated antibody to swH3N2 included age group, elevated titer to human A/Brisbane/59/2007(H1N1) (adjusted OR = 2.4; 95% CI, 1.0–5.7), and to human A/Panama/59/2007/99(H3N2) (adjusted OR = 9.3; 95% CI, 6.5–13.4) (data not shown).

More interesting was the serological activity against the 2 HPAI H5N1 viruses detected in this cohort. We found that 45 participants (5.6%) had elevated antibody titers against A/Thailand/676/2005(H5N1) and 28 participants (3.5%) had elevated titers against A/Thailand/384/2006(H5N1). Adjusting for potential confounders, evidence of infection with these 2 viruses was not statistically associated with self-reported poultry

Table 2. Demographic Characteristics and Poultry/Swine Exposure Upon Enrollment, Adult Participants, Kamphaeng Phet Province, Thailand, 2008

Exposure variables	(n = 800) N (%)
Age group	
20–39 y	189 (23.6)
40–59 y	427 (53.4)
≥60 y	184 (23.0)
Gender	
Male	339 (42.4)
Female	461 (57.6)
Indoor water	
Yes	543 (67.9)
No	257 (32.1)
Ever received vaccination for human influenza[a]	
Yes	13 (1.6)
No	781 (97.6)
Heart disease, hypertension, or stroke	
Yes	163 (20.4)
No	637 (79.6)
Chronic breathing problems	
Yes	37 (4.6)
No	763 (95.4)
Other chronic medical problems	
Yes	52 (6.5)
No	748 (93.5)
Ever used tobacco products	
Yes	268 (33.5)
No	532 (66.5)
Developed a respiratory illness in the preceding 12 mo[a]	
Yes	427 (53.4)
No	371 (46.4)
Any poultry exposure	
Yes	523 (65.4)
No	277 (34.6)
Any poultry exposure type[b]	
Chickens	494 (61.8)
Fighting cocks	14 (1.8)
Ducks	86 (10.8)
None	277 (34.6)
Poultry exposure 2003 or after	
Yes	476 (59.5)
No	324 (40.5)
Any swine exposure	
Yes	91 (11.4)
No	709 (88.6)

[a] Missing values omitted.

[b] Participants may be included in multiple categories.

exposure (Table 4). Not having an indoor water source was a significant risk factor for exposure to either A/Thailand/676/2005 or A/Thailand/384/2006 HPAI H5N1 virus even after controlling for confounding variables (adjusted OR = 3.2; 95% CI, 1.7–6.1 and adjusted OR = 3.1; 95% CI, 1.4–6.7, respectively). Respondents over age 60 were far more likely to have elevated titers than respondents 20–39 years for both A/Thailand/676/2005(H5N1) (adjusted OR = 31.2; 95% CI, 5.0–infinity) and A/Thailand/384/2006(H5N1) (adjusted OR = 8.2; 95% CI, 1.9–75.2). Elevated titer for A/New Caledonia/20/99(H1N1) was also significant for the 2005 HPAI strain (adjusted OR = 4.2; 95% CI, 1.4–12.9) and a history of chronic breathing problems was significant for the 2006 HPAI strain (adjusted OR = 4.0; 95% CI, 1.3–11.7).

DISCUSSION

Study data suggest that a number of cohort members had previously been infected with either low-pathogenic avian H9N2 or highly pathogenic avian H5N1 influenza viruses. These serological findings were not associated with poultry exposure or with recent clinical disease.

In 1999, Peiris et al isolated the A/Hong Kong/1073/1999(H9N2) influenza virus from a young girl in Hong Kong. They found the virus to be closely related to a quail H9N2 virus isolated previously in 1997 [21, 22]. Molecular characterization suggested viral reassortment had occurred, as both H9 viruses shared the 6 internal genes with that of the novel H5N1 HPAI [23, 24]. Between 1998 and 1999, 10 additional human H9N2 virus infections occurred in China [25], and since the early 2000s, H9N2 subtype viruses have been frequently detected in poultry across Asia [26].

It has been suggested that the A/Hong Kong/1073/1999(H9N2) virus possesses a unique combination of 3 amino acids, also found in the hemagglutinin gene of human H3 viruses, that gave it human virus-like receptor specificity, similar to that of human H3N2 epidemic strains [21, 24, 27]. In addition, Peiris et al discovered evidence of the interspecies transmission of H9N2 AIVs to pigs in China and their cocirculation with human H3N2 influenza viruses (A/Sydney/5/97-like and Sydney/97-like viruses) in pigs, providing an opportunity for further genetic reassortment [28]. The characteristics of internal genes similar to H5N1 HPAI viruses, surface glycoproteins with a broader host range (including humans), and the ability to infect birds, pigs, and humans suggest the pandemic potential of these H9N2 AI viruses.

Our multivariate modeling data suggest that older adults with a damaged respiratory tract from years of smoking may be more susceptible to A/Hong Kong/1073/1999(H9N2) infections. If lack of an indoor water source is a risk factor, perhaps older generations were more exposed to outdoor water sources in the distant past. Although we cannot completely rule out cross-reactivity, seroreactivity against human H1 or H3 influenza viruses was not suggested by the multivariate models.

Table 3. Risk Factors for Elevated Antibodies Against A/Hong Kong/1073/1999(H9N2) Among Adult Participants, Kamphaeng Phet Province, Thailand, 2008

Variables	Total N	A/Hong Kong/1073/1999(H9N2)	
		N (%)	Unadjusted OR (95% CI)
Age, y[a,b]			
≥60	184	10 (26.3)	27 (0.7–11.8)
40–59	427	24 (63.2)	27 (0.9–11.0)
20–39	189	4 (10.5)	Reference
Gender[c]			
Male	339	22 (57.9)	1.9 (1.00–3.8)
Female	461	16 (42.1)	Reference
Poultry exposure[a]			
Yes	523	28 (73.7)	1.5 (0.7–3.2)
No	277	10 (26.3)	Reference
Swine exposure[a,b]			
No	709	34 (89.5)	1.1 (0.4–4.3)
Yes	91	4 (10.5)	Reference
A/Brisbane/59/2007(H1N1)[a,b,d,e]			
Negative	760	36 (94.7)	1.6 (0.2–66.7)
Positive	33	1 (2.6)	Reference
A/New Caledonia/20/99(H1N1)[a,b,d,e]			
Negative	765	36 (94.7)	1.5 (0.2–62.2)
Positive	32	1 (2.6)	Reference
A/Panama/2007/99(H3N2)[c,d,e]			
Positive	250	14 (36.8)	1.3 (0.7–2.5)
Negative	549	24 (63.2)	Reference
A/Brisbane/10/2007(H3N2)[a,d,e]			
Negative	258	14 (36.5)	1.3 (0.7–2.6)
Positive	539	23 (60.5)	Reference
Indoor water[c]			
No	257	20 (52.6)	2.0 (1.02–3.8)[f]
Yes	543	18 (47.4)	Reference
Developed a respiratory illness in the last 12 mo[c,e]			
Yes	427	22 (57.9)	1.2 (0.6–2.3)
No	371	16 (42.1)	Reference
Ever used tobacco products[c]			
Yes	268	20 (52.6)	2.3 (1.2–4.5)[f]
No	532	18 (47.4)	Reference
Chronic breathing problems[a,b]			
Yes	37	4 (10.5)	2.6 (0.6–7.9)
No	763	34 (89.5)	Reference

[a] Binary logistic regression (Negative = titer < 1:10, Positive = titer ≥ 1:10).

[b] Exact conditional logistic regression method used.

[c] Proportional odds model used with 2 highest antibody titer levels grouped because of sparse data.

[d] Negative = titer < 1:40, Positive = titer ≥ 1:40.

[e] These covariates have missing data.

[f] Statistically significant data ($P < .05$).

Serological results for the 2 H5N1 HPAI viruses suggest that these viruses were once present in the study villages. A/Thailand/676/2005(H5N1) was isolated in 2005 from a 5-year-old boy in Thailand who died 12 days after illness onset [29]. Researchers have previously demonstrated that this virus gained the ability to bind to the human-type sialic acid receptor (α2,6Gal) in the human respiratory tract [29]. Approximately 6% of the cohort had elevated antibody titers against this virus; however, poultry exposure was not significantly associated with seropositivity. Because access to an indoor water source resulted in a reduced odds ratio of having an elevated A/Thailand/676/2005(H5N1) titer, perhaps infected poultry were shedding infectious virus

Table 4. Risk Factors for Elevated Antibodies Against A/Thailand/676/2005(H5N1) and A/Thailand/384/2006(H5N1), Using Proportional Odds and Binary Logistic Modeling, Among Adult Participants, Kamphaeng Phet Province, Thailand, 2008

		A/Thailand/676/2005(H5N1)		A/Thailand/384/2006(H5N1)	
Variables	Total N	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Age, years					
≥60	184	32.1 (5.2-infinity)[a,b,c]	31.2 (5.0–infinity)[a,b,c]	8.9 (2.0–39.3)[a,b,c]	8.2 (1.9–75.2)[a,b,c]
40–59	427	7.8 (1.2–327.5)[a,b,c]	8.4 (1.3–354.8)[a,b,c]	2.2 (0.5–10.3)[a,b]	2.3 (0.5–22.1)[a,b]
20–39	189	Reference	Reference	Reference	Reference
Gender					
Male	339	1.9 (1.1–3.6)[c,d]	—	2.2 (0.99–4.7)[d]	—
Female	461	Reference	—	Reference	—
Any poultry exposure					
Yes	523	1.9 (0.9–3.9)[a]	—	3.3 (1.1–13.1)[a,b,c]	—
No	277	Reference	—	Reference	—
A/Brisbane/59/2007(H1N1)[e,f]					
Positive	33	2.4 (0.6–7.4)[a,b]	—	2.9 (0.5–10.5)[a,b]	—
Negative	760	Reference	—	Reference	—
A/New Caledonia/20/99(H1N1)[e,f]					
Positive	32	3.5 (1.3–9.4)[c,d]	4.2 (1.4–12.9)[a,b,c]	1.9 (0.2–8.2)[a,b]	—
Negative	765	Reference	Reference	Reference	—
A/Panama/2007/99(H3N2)[e,f]					
Positive	250	1.4 (0.7–2.5)[a]	—	1.9 (0.9–4.2)[d]	—
Negative	549	Reference	—	Reference	—
A/Brisbane/10/2007(H3N2)[e,f]					
Positive	539	1.7 (0.8–3.6)[d]	—	1.8 (0.7–4.5)[d]	—
Negative	258	Reference	—	Reference	—
Indoor water					
No	257	3.1 (1.7–5.7)[c,d]	3.2 (1.7–6.1)[a,b,c]	3.5 (1.6–7.5)[c,d]	3.1 (1.4–6.7)[a,b,c]
Yes	543	Reference	Reference	Reference	Reference
Ever received vaccination for human influenza[f]					
Yes	13	5.3 (0.9–21.5)[a,b]	—	2.4 (0.1–17.6)[a,b]	—
No	781	Reference	—	Reference	—
Ever used tobacco products					
Yes	268	2.2 (1.2–4.0)[c,d]	—	2.4 (1.1–5.0)[a,c]	—
No	532	Reference	—	Reference	—
Chronic breathing problems					
Yes	37	2.9 (1.1–7.8)[c,d]	—	5.0 (1.8–14.1)[a,c]	4.0 (1.3–11.7)[a,b,c]
No	763	Reference	—	Reference	Reference

Abbreviations: OR, odds ratio; CI, confidence interval.

[a] Binary logistic regression (Negative = titer < 1:10, Positive = titer ≥ 1:10).

[b] Exact conditional method used.

[c] Statistically significant data ($P < .05$).

[d] Proportional odds model used.

[e] Negative = titer < 1:40, Positive = titer ≥ 1:40.

[f] These covariates have missing data.

that then contaminated outdoor water supplies. An environmental survey conducted in 2006 in Cambodia detected H5N1 HPAI viral RNA in 27 of 77 environmental samples collected from mud, pond water and plants, and soil (35%) [30].

For the 3.5% of the cohort with elevated antibody titers against A/Thailand/384/2006(H5N1) HPAI virus, participants exposed to poultry had a higher odds (OR = 3.3; 95% CI, 1.1–13.1) of seropositivity, although this was not significant after including other covariates in the final multivariate model. This virus was isolated from a human in Thailand, but information regarding this isolate has not been published. Having an indoor water source was also protective against seropositivity, suggesting that this virus may also be contaminating environmental water sources. Older participants (>60 years) and those with history of

chronic breathing problems were also more likely to have an elevated antibody titer against the 2006 H5N1 virus, which, as with the H9N2 virus, suggests that older adults with distressed respiratory systems may be more susceptible to influenza virus infection.

Whereas older cohort members had higher adjusted odds ratios for infections with both 2005 and 2006 H5N1 viruses (adjusted OR = 31.2; 95% CI, 5.0–infinity and adjusted OR = 8.2; 95% CI, 1.9–75.2, respectively), exposure data collected for this study did not provide an explanation. Perhaps more cumulative time of at-risk exposure led to a higher likelihood of exposure to the virus, or as an older generation, these participants are more often preparing foods with unsafe methods.

Although it is of note that poultry exposure was not associated with LPAI and HPAI virus infection, this is not an unusual finding. A seroprevalence study conducted by Cavailler et al in August 2007 in Cambodia found 18 (2.6%) of 700 participants to be seropositive for antibodies against a Cambodian H5N1 HPAI, yet the authors also did not find poultry exposure to be associated with previous H5N1 infection; only reportedly bathing or swimming in the community pond was significant (adjusted OR = 2.96; 95% CI 1.1–8.4) [31]. Also, a 2005 seroprevalence study of 4 Thai villages with at least 1 human H5N1 HPAI case found that whereas most participants were exposed to backyard poultry and a quarter of them were exposed to sick or dead chickens, no participant had serological evidence of H5N1 AIV infection [32].

This study had a number of limitations. For multiple reasons, only adults $\geq$ 20 years of age were enrolled. Previous studies have shown that younger children are also at risk of avian influenza virus infections [33, 34], so our sampling approach may have excluded a large subset of the at-risk population. A further limitation may be the specificity of our serological assays. We could have missed important evidence of previous LPAI infections if the viruses we used in this study were different from the virus strains circulating in Thailand prior to study enrollment. Similarly, although we tried to control for cross-reactivity from human influenza virus infections through 4 different human virus assays, another human influenza virus such as the pandemic H2N2 might have contributed some cross-reactivity to explain our seroreactivity against the AI viruses. Transportation of specimens from the villages to the KAVRU laboratory to the reference laboratories was thoughtfully planned and carefully executed; however, factors outside the control of study staff may have led to the degradation of sera samples during transport.

Despite its limitations, this study effectively established a cohort predominantly exposed to poultry for prospective studies of acute influenza-like illnesses (ILI). Following this enrollment phase, these 800 participants (with replacement enrollment following drop-outs) are now being monitored on a weekly basis for ILI. In the event a cohort member develops an ILI, a family investigation is conducted to examine possible person-to-person transmission. Sera samples are also being collected annually to monitor for changes in influenza antibody titers. With such evidence of previous infections with avian influenza viruses in this cohort, we expect some interesting prospective analyses.

Notes

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