Journal of Clinical Virology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Short communication

# Seroprevalence of anti-H5 antibody in rural Cambodia, 2007

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ARTICLE INFO

*Article history:*
Received 23 September 2009
Received in revised form 21 February 2010
Accepted 26 February 2010

*Keywords:*
H5N1 virus
Cambodia
Risk factors
Neutralizing antibodies
Seroprevalence
Environmental exposure

ABSTRACT

*Background:* Since 2005, eight patients with H5N1 infection were laboratory confirmed in Cambodia. Despite the widespread of highly pathogenic avian influenza H5N1 virus and the intense exposure to poultry, there is growing evidence that H5N1 viruses may not be easily transmitted to human.
*Objectives:* To evaluate the frequency of H5N1 transmission in rural Cambodia, to identify potential risk factors for H5N1 in humans and to explore the extent of asymptomatic and clinically mild illness among humans.
*Study design:* A seroepidemiologic survey was conducted, 9 weeks after the recognition that H5N1 infection caused the death of a 13 years old female in April 2007. Blood specimens were collected from 700 participants for H5N1 serological testing. All participants were interviewed with standardized questionnaire to collect information about poultry exposure.
*Results:* Eighteen (2.6%) of the 700 villagers were tested positive cases for H5N1 antibodies. These 18 individuals were more likely than seronegative participants to report bathing or swimming in the community pond ($p = 0.04$).
*Conclusions:* The seroprevalence of H5N1 antibodies was higher than previously reported in the other investigations conducted in Cambodia and Thailand. This finding reinforces the overwhelming evidence that the virus continues to circulate widely in settings where human have high exposure to poultry. Our results, provides additional evidence suggesting that bathing or swimming in the community ponds, remains important potential risk factor for H5N1 infection. Both wild birds and domestic poultry have free access to these ponds which are also used for aquaculture through the dumping of poultry feces for fish feeding.

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## 1. Background

Since 2005, eight patients with H5N1 disease were laboratory confirmed in Cambodia.[1] Despite the widespread of highly pathogenic avian influenza H5N1 virus and the frequent exposure to poultry, there is growing evidence that H5N1 may not be easily transmitted to human.[2] Close contact with infected birds is assumed to be the main route of transmission; however, several studies also identified environmental factors as potential sources of transmission.[3]

## 2. Objectives

To evaluate the frequency of H5N1 transmission, to identify potential risk factors in humans and to explore the extent of asymptomatic and clinically mild illness among humans, the study describes a seroepidemiology survey after the identification of a fatal case on H5N1 infection, in April 2007.

## 3. Study design

During the investigation, we interviewed all residents of the affected village using a standardized questionnaire to collect demographic data and information about poultry exposure. In addition, 5 mL of blood was collected for serological testing. Repeated home visits were made in order to include absent household members. The study was approved by the Cambodian National Ethical Committee. Informed written consent was obtained from all subjects

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doi:10.1016/j.jcv.2010.02.021

or from a guardian (in children under 18 years old) prior to interview.

All sera were screened using H5 hemagglutinin pseudotyped lentiviral particles (H5pp) expressing the hemagglutinin of a 2005 Cambodian H5N1 virus according a protocol previously described.[4] In principle, H5pp followed the same steps as conventional microneutralization (MN) except that the neutralization of infection was detected by measuring the reduction of endpoint chemiluminescent signal compared to controls performed in the absence of sera (equivalent to 0% neutralization) and in absence of H5pp (equivalent to 100% neutralization), respectively. The neutralization titer was defined as the reciprocal of the dilution that matches the positivity criteria (0% neutralization) after fitting with the Hill model.[5] When the titer of this screening test was ≥1:20, samples were tested by haemagglutination inhibition (HI) test with the use of horse's red blood cell according the method described by Rowe et al.[6] and by standard MN assay, both using the strain A/Cambodia/R0405050/2007(H5N1) isolated from the 13-year-old female case. For standard MN assay, 100 tissue culture infectious dose 50 (100 TCID50) of the R045050 strain were incubated with serially two-fold diluted sera (starting from 1:10) for 1 h at room temperature prior to addition to Madin Darby Canine Kidney (MDCK) cells. Cell monolayers were incubated for a further 3 days and examined for cytopathic effect. Determination of the endpoint neutralizing antibody titers was performed in four wells per dilution. The neutralizing titer was defined as the reciprocal of the highest dilution of serum at which the infectivity of 100 TCID50 of H5N1 virus for MDCK cells was completely neutralized in 50% of the wells. The titer was calculated by the Reed–Muench method.[7] A recent study demonstrated that at the positivity threshold ≥1:80, the performance of the H5pp test compared favorably with MN and that at a lower cut-off titer (≥1:20), the sensitivity of H5pp improved while specificity dropped.[8] According WHO recommendations, a serum was considered as positive when the neutralization antibody titer was ≥1:80 and when the HI test antibody titer was ≥1:160.[9]

SPSS for Windows version 11.0 (*SPSS Inc. Chicago, IL, USA*) was used for all statistical analyses. We compared proportions using Fisher's exact test, means using the ANOVA test and medians using Kruskal–Wallis test. The strength of the association between the antibodies response and the risk factors was estimated from the odds ratios (OR). A logistic regression was performed to test the association in a multivariate model using the Wald test.

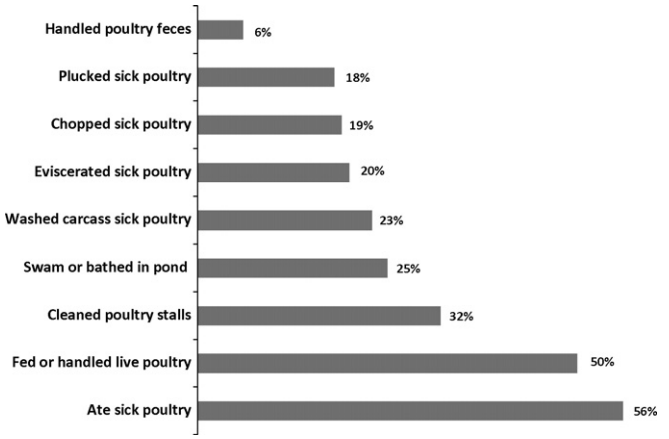


**Fig. 1.** Characteristics of 700 participants in a H5N1 seroprevalence survey, Kampong Cham province, Cambodia, June 2007.

## 4. Results

Among the 703 residents that were at home during both the exposure and the survey periods, 700 agreed to participate in the study. The median age was 18 years and 45% were males. During the study period, most participants noted they had swam or bathed in community ponds and reported close poultry contact (Fig. 1).

Eighteen (2.6%) of the 700 residents [95% CI: 1.6–4.1] tested positive for H5N1 antibodies. Of the 18 individuals with positive antibodies, 8 (44.4%) were males and the median age was 14.5 years, which was not significantly different from the median age of the overall study participants ($p > 0.20$). No seropositive individual reported a febrile illness episodes during the study period but 11 (61.1%) reported close contact with sick poultry. Two family clusters were identified. In cluster A, two of the three seropositive individuals reported close contact with the index case (but none of them were blood relatives with her). In cluster B, the two seropositive individuals were blood relatives and close contacts of the index case (Table 1).

**Table 1**
Characteristics of the 18 participants with H5N1 antibodies, Kampong Cham province, Cambodia, June 2007.

| | H5N1 seropositive cases | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| Gender | M[a] | F[a] | F | F | F | M | F | F | M | M | F | F | F | M | M | M | M | F |
| Age | 3 | 4 | 7 | 7 | 7 | 9 | 10 | 10 | 12 | 17 | 19 | 20 | 22 | 40 | 47 | 75 | 76 | 77 |
| Swam/bathed in pond | +[a] | + | –[a] | – | + | + | – | + | + | – | – | – | + | + | – | – | – | – |
| Cleaned poultry stalls | – | – | – | – | – | – | + | – | – | – | + | + | + | – | – | – | – | + |
| Collected poultry feces (manure) | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + |
| Touched/fed live poultry | + | – | – | – | – | + | – | – | – | – | – | + | + | + | + | – | – | + |
| Attended cockfight | – | + | – | – | – | – | – | – | + | – | – | – | – | – | + | – | – | – |
| Touched sick poultry | + | + | – | – | – | + | – | – | – | – | – | – | + | + | + | – | – | + |
| Plucked sick poultry | + | – | – | – | – | – | – | – | – | – | – | – | + | + | + | – | – | + |
| Eviscerated sick poultry | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | – | – | + |
| Washed sick poultry carcasses | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | – | – | + |
| Chopped/butchered sick poultry | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | – | – | + |
| Cooked sick poultry | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | – | – | – |
| Ate sick poultry | + | + | + | – | + | + | – | + | – | – | – | + | + | + | + | – | – | + |
| Any contact with sick poultry[b] | + | + | + | – | + | + | – | + | – | – | – | + | + | + | + | – | – | + |
| Any symptoms[a] | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| Close contact with H5N1 case | – | – | – | – | – | – | – | – | + | + | – | – | – | – | – | – | – | – |
| Serological Family cluster | – | – | – | – | – | – | – | – | B | B | – | – | A | A | – | – | – | A |
| Relative of the H5N1 case | – | – | – | – | – | – | – | – | + | + | – | + | + | + | – | – | – | – |

[a] M = male, F = female, + = positive/yes, – = negative/no, any symptom = fever or cough or dyspnea during the study period.
[b] Contact with sick poultry = those who touched or plucked or eviscerated or washed carcass or chopped or cooked or ate sick poultry.

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**Table 2**
Univariate analysis of factors associated with H5N1 antibodies, Kampong Cham province, Cambodia, June 2007.

| Variables (%) | Seronegatives, $n = 682$ | Seropositives, $n = 18$ | Odds ratios | 95% CI | $p$ Value |
|---|---|---|---|---|---|
| Sex, female | 373 (54.7) | 10 (55.6) | 1.03 | 0.40–2.66 | 0.94 |
| Age, median | 18.0 | 14.5 | – | – | 0.58 |
| Swam/bathed in pond | 164 (24.0) | 8 (44.4) | 2.52 | 0.98–6.51 | 0.05 |
| Cleaned poultry stalls | 218 (32.0) | 5 (27.8) | 0.82 | 0.29–2.33 | 0.71 |
| Collected poultry feces for manure | 44 (6.5) | 1 (5.6) | 0.85 | 0.11–6.56 | 1.00 |
| Touched/fed live poultry | 343 (50.3) | 7 (38.9) | 0.63 | 0.24–1.64 | 0.34 |
| Attended cockfight | 81 (11.9) | 3 (16.7) | 1.47 | 0.33–5.54 | 0.47 |
| Touched sick poultry | 209 (30.6) | 7 (38.9) | 1.44 | 0.55–3.77 | 0.46 |
| Plucked sick poultry | 127 (18.6) | 5 (27.8) | 1.68 | 0.59–4.80 | 0.33 |
| Eviscerated sick poultry | 134 (19.6) | 4 (22.2) | 1.17 | 0.38–3.61 | 0.77 |
| Washed sick poultry carcasses | 153 (22.4) | 5 (27.8) | 1.33 | 0.47–3.79 | 0.59 |
| Chopped/butchered sick poultry | 132 (19.4) | 5 (27.8) | 1.60 | 0.56–4.57 | 0.37 |
| Cooked sick poultry | 132 (19.4) | 4 (22.2) | 1.17 | 0.38–3.61 | 0.78 |
| Ate sick poultry | 379 (55.6) | 11 (61.1) | 1.26 | 0.48–3.29 | 0.64 |
| Any contact with sick poultry[a] | 398 (58.4) | 11 (61.1) | 1.12 | 0.43–2.93 | 0.82 |
| Close contact with H5N1 case | 53 (7.8) | 2 (11.1) | 1.48 | 0.00–7.00 | 0.65 |
| Relative of the H5N1 case | 203 (29.8) | 5 (27.8) | 0.91 | 0.32–2.58 | 0.86 |

[a] Contact with sick poultry = those who touched or plucked or eviscerated or washed carcass or chopped or cooked or ate sick poultry.

In univariate analysis, seropositive participants were more likely than seronegative participants to report bathing or swimming in the pond (44.0% versus 24%, OR 2.53 [95% CI: 0.98–6.51], $p = 0.05$). This association persisted when adjusting by age, gender and poultry handling practices in logistic regression model (adjusted OR 2.96 [95% CI: 1.05–8.38], $p = 0.04$, *data not shown*). None of the sick-poultry handling practices was significantly associated with anti-H5N1 immune response. No differences were observed with regard to contact with the confirmed H5N1 case (Table 2).

## 5. Discussion

The seroprevalence of H5N1 antibodies was substantially higher than previously reported in recent investigations conducted in Cambodia and Thailand.[2,10–12] This high proportion of seropositive villagers reinforces the overwhelming evidence that the virus continues to circulate widely in settings where human-poultry interactions are intense. However, the presence of H5N1 antibodies may not imply recent exposure given that the virus has been widely circulating since 2004 and that no paired samples were collected. Nevertheless, our recent data suggest that a substantial proportion of asymptomatic persons having a positive microneutralization test become negative after 1 year[2] (*Institut Pasteur in Cambodia unpublished data*).

Surprisingly, none of the sick-poultry handling practices was found to be associated with anti-H5N1 immune response. In contrast, this study provided additional evidence to support recent studies[13,14] suggesting that bathing or swimming in the community ponds, remained important potential risk factor for H5N1 infection. Both wild birds and domestic poultry have free access to these ponds which are also used for fish farming through the dumping of poultry feces for fish feeding.[15] In human, it has been suggested the possibility of H5N1 viral transmission, either through the direct intranasal or conjunctival inoculations or through the ingestion/inhalation of contaminated water, while, for example, swimming, in these ponds.[16]

Interestingly two family clusters of serological cases have been identified. On the other hand, many seronegative participants were also related to the index H5N1 patients suggesting that family clustering in this setting may not necessarily indicate genetic susceptibility to the virus, as it may also result from common household exposures to the same risk factors.[17]

Our results need, however, to be interpreted in light of an important limitation: many of the study participants were aged >50 years which may have led to some false positive serology results as suggested by Rowe et al.[6]. We however chose to account for these older participants as recent data showed only 6.8% and 0.8% of cross-neutralizing antibodies with a titer $\geq$1:20 in elderly $\geq$60 years old using H5pp and MN methods, respectively.[18] Nevertheless, our results remained unchanged when excluding individuals aged >50 years from the analysis (*data not shown*).

These results confirm the risk of H5N1 infection through exposure to a contaminated environment. Further environmental investigations are required to fully evaluate the virus survival and to better understand the transmission routes in the environment. As a result, in outbreak settings, limitation of interactions between humans and poultry as well as restricted access to potentially contaminated environments should remain a public health priority.

## Conflict of interest

No conflict of interest.

## Ethical approval

Ethical approval provided by the Cambodian National Ethical Review Board.

## Acknowledgements

We thank the Ministry of Health staffs who were responsible for the human case investigation and are grateful to Chan Malen for her support in conducting the survey. The study was generously supported by the World Health Organization, Phnom Penh Cambodia, the Institut Pasteur in Cambodia, and by a grant from the AFD (Agence Française pour le Développement) through SISEA (Surveillance and Investigation of epidemic Situations in South-East Asia), a regional project coordinated by the International Network of Pasteur Institutes.

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