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Absence of neutralizing antibodies against influenza A/H5N1 virus among children in Kamphaeng Phet, Thailand

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Abstract

Background—Influenza A/H5N1 actively circulated in Kamphaeng Phet (KPP), Thailand from 2004–2006. A prospective longitudinal cohort study of influenza virus infection in 800 adults conducted during 2008 to 2010 in KPP suggested that subclinical or mild H5N1 infections had occurred among this adult cohort. However, this study was conducted after the peak of H5N1 activity in KPP. Coincidentally, banked serum samples were available from a prospective longitudinal cohort study of primary school children who had undergone active surveillance for febrile illnesses from 2004 to 2007 and lived in the same district of KPP as the adult cohort.

Objectives—We sought to investigate whether subclinical or mild H5N1 infections had occurred among KPP residents during the peak of H5N1 activity from 2004 to 2006.

Study design—H5N1 microneutralization (MN) assay was performed on banked serum samples from a prospective longitudinal cohort study of primary school children who had undergone active surveillance for febrile illnesses in KPP. Annual blood samples collected from 2004 to 2006 from 251 children were selected based on the criteria that they lived in villages with documented H5N1 infection.

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Competing interests: None declared.

Ethical approval: Testing of banked samples was approved by the Institutional Review Boards of the Thailand Ministry of Public Health, the Walter Reed Army Institute of Research, and the University of Florida.

Result—No H5N1 neutralizing antibodies were detected in 753 annual blood samples from 251 children.

Conclusion—During 2004 to 2006, very few subclinical or mild H5N1 infections occurred in KPP. Elevated H5N1 MN titers found in the adult cohort in 2008 were likely due to cross-reactivity from other influenza virus subtypes highlighting the complexities in interpreting influenza serological data.

Keywords

Avian influenza; H5N1; microneutralization; serology; subclinical infection

Background

Since 2003, over 600 human cases of highly pathogenic avian influenza (HPAI) H5N1 have been documented in 16 countries. In Thailand, the first H5N1 human case was diagnosed in 2004[1]; subsequently, 25 other cases were documented from 2004 to 2006. The case fatality rate was 68% consistent with the estimated global case fatality rate of >50%[2]. The high fatality rate of H5N1 is based on a relatively small denominator presuming that few subclinical and mild H5N1 infections occur. However, the actual rate of subclinical and mild human infections has not been well defined largely because estimates are based on serological assay cut points designed to be more specific than sensitive[2]. H5N1 seroprevalence rates in potentially exposed individuals have ranged from 0 to 11.7% depending on the population studied, sampling method used, laboratory assay performed, and criteria for seropositivity[2, 3]. Subclinical infections have rarely been well documented by detecting viable H5N1 virus[4].

We previously conducted a prospective longitudinal cohort study of influenza virus infection from 2008 to 2010 in 800 adults (age range 20–84 years) in Kamphaeng Phet province (KPP), Thailand[5, 6]. Blood samples were collected at enrollment in 2008, then 12 and 24 months thereafter. Active surveillance for influenza-like illness was performed during the two-year study period. We reported that 5.6% and 3.5% of participants at enrollment had neutralizing antibody titers of ≥1:10 to A/Thailand/676/2005(H5N1) and A/Thailand/384/2006(H5N1), respectively, as measured by microneutralization (MN) assay[5]. However, only one participant was seropositive to H5N1 (with a titer of 1:20) at 24 months, and none of the H5N1 seropositive participants at enrollment remained seropositive at 12 or 24 months[6]. Because KPP was a center for H5N1 transmission activity in Thailand from 2004 to 2006 to include the first case of probable human-to-human transmission[5], a possible explanation for these findings was that cohort participants had been exposed during the peak of H5N1 activity in KPP from 2004 to 2006 with a decline in H5N1 neutralizing antibodies thereafter, given that H5N1 antibodies may last for 3–5 years after symptomatic infection[7] and a shorter period of time after subclinical infection[8].

Coincidentally, we had conducted a prospective longitudinal cohort study of over 2000 primary school children (age range 4–15 years) from 2004 to 2007 in the same district of KPP as the adult cohort. Blood samples were collected in May and December each year

from 2004 to 2007, with active surveillance for febrile illnesses performed during the rainy season from June to December[9].

Objectives

To investigate whether a substantial number of subclinical or mild H5N1 infections had occurred among KPP residents.

Study design

H5N1 MN assays were performed on banked serum samples from the child cohort study collected during the period 2004 to 2006. Blood samples collected from three consecutive years from each of 251 children living in villages with documented H5N1 infection in poultry or people during 2004 to 2006 were selected. All samples underwent testing for neutralizing antibodies to A/Thailand/676/2005(H5N1) and A/Thailand/384/2006(H5N1) using the same MN assay as was used for the adult cohort[5, 6]. Briefly, 100 median tissue culture infective doses ($TCID_{50}$) of virus was added to a 1:10 dilution of serum and incubated at 37°C for 1 hour. Madin-Darby Canine Kidney (MDCK) cells in log-phase growth were adjusted to 2×10^5 cells/ml; 100 μl of cells were added to each well, and the plate was incubated at 37°C for an additional 24 hours. The monolayers were fixed with acetone, and influenza virus was measured by ELISA using mouse monoclonal antibody against influenza A nucleoprotein (MAB8251, EMD Millipore, Billerica, MA, USA) as the primary antibody and goat anti-mouse IgG (474–1802, Kirkegaard, Gaithersburg, MD, USA) conjugated to horseradish peroxidase as the secondary antibody. Following the final wash, 0.1 ml of 3,3',5,5'-tetramethylbenzidine (50-76-03, Kirkegaard, Gaithersburg, MD, USA) was added and incubated at room temperature for 10 minutes. Optical density (OD) of the plates was read at 450nm. The ELISA endpoint titer was expressed as the reciprocal of the highest serum dilution with OD less than X, where X = [(average OD of virus control wells) + (average OD of cell control wells)]/2.

Results

Of 753 samples tested, all were found to be seronegative for H5N1 by MN assay (titer <1:10), for a seroprevalence of 0/251 (upper bound of 95% confidence interval 1.5%). This result was in contrast to the H5N1 MN findings from the adult cohort.

Discussion

Given the obvious age difference between the child and adult cohorts, it is possible that the adult cohort had environmental exposures distinct from the children. In the adult cohort, lack of an indoor water source was found to be a risk factor for elevated H5N1 neutralizing antibodies supporting the possibility that certain exposures (potentially differing with age) could predispose to H5N1 infection[5]. Unfortunately, detailed environmental exposure histories were not available for the child cohort. A study in Cambodia also found an increased likelihood of having influenza H5N1 antibodies in individuals who reported bathing or swimming in household ponds[10]. The H5N1 seropositivity rate in that study was quite low at 1%. Interestingly, all seven seropositive individuals in the Cambodia study were

≤18 years old as opposed to our child cohort in which no seropositive individuals were identified. This difference may have been due to the sample size (upper bound of seropositivity rate in our study was 1.5%), or perhaps because blood was collected in the Cambodian study only seven weeks after H5N1 infection was documented in the vicinity whereas our child study collected blood annually. There may also have been differences in environmental exposures in KPP compared to Cambodia, particularly as 6 of the 7 seropositive subjects in Cambodia lived in the same village.

The most likely explanation, however, for the discrepancy in the H5N1 seropositivity rates between the child and adult cohorts lies in the differences in the immune history of adults as compared with children. Adults are more likely to have a complex history of influenza virus exposures that have contributed to their antibody repertoire, making them more likely than younger children to develop subtype cross-reactive antibodies[11].

Even within the adult cohort itself, participants ≥60 years of age were more likely to have elevated H5N1 antibody titers than participants 20–39 years old[5] (adjusted odd ratio=31.2, 95%CI:5.0-infinity, and adjusted odd ratio=8.2, 95%CI:1.9–75.2, for 2005 and 2006 H5N1 viruses, respectively). Furthermore, elevated antibody titers to A/New Caledonia/20/99(H1N1) as measured by hemagglutination inhibition (HI) assay were associated with elevated H5N1 MN titers[5], suggesting the possibility of cross-reactivity. A recent study using banked sera from U.S. military personnel, in whom H5N1 infection has never been documented, demonstrated 14% seroprevalence to H5 pseudotyped lentiviral particles as measured by MN assay, suggesting that much of this seroprevalence was due to cross-reactivity[12]. The potential for cross-reactivity in the adult cohort in KPP may have been further accentuated by the relatively low 1:10 cut off titer used to determine H5N1 MN seropositivity. The optimal criteria to determine seropositivity for H5N1 serological assays has been the subject of much recent discussion[13].

Taken together, the most likely scenario consistent with the H5N1 MN results from the adult and child cohorts is that very few subclinical and mild H5N1 infections occurred in KPP. The elevated H5N1 MN titers found in the adult cohort in 2008 were more likely due to cross-reactivity from other influenza virus subtypes. Our findings highlight the complexities in interpreting influenza serological data and further emphasize the pressing need for more specific serological assays to evaluate avian influenza viruses.

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Highlights

- Influenza A/H5N1 actively circulated in Kamphaeng Phet, Thailand from 2004–2006.
- A prospective child cohort had undergone active fever surveillance from 2004–2007.
- No positive H5N1 microneutralization result occurred in 753 banked blood samples.
- Very few subclinical/mild H5N1 infections occurred in Kamphaeng Phet.