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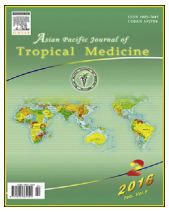

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Immunological evidence of Zika virus transmission in Thailand

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ABSTRACT

Objective: To identify immunological evidence of Zika virus transmission in Thailand.
Methods: To undertake a preliminary serosurvey of possible exposure to Zika virus, 21 serum samples from cohort of acute undifferentiated fever patients were examined for immunoreactivity to Zika, Dengue, Japanese encephalitis and Chikungunya envelope antigens by Western blot analysis.
Results: Twenty of the 21 serum samples showed immunoreactivity to at least one of the antigens, with seven samples showing immunoreactivity to all antigens. Of particular note, two serum samples showed immunoreactivity only to Zika envelope antigen, with no immunoreactivity to other envelope antigens.
Conclusions: This study presents the first evidence of Zika virus transmission in Thailand, although as yet the relationship between transmission and possible cases of Zika fever in Thailand requires further investigation.

1. Introduction

The family *Flavivirus* within the genus *Flaviviridae* contains some of the most important mosquito transmitted viruses that affect humans and animals. Particularly important mosquito transmitted viruses in this family include Dengue virus (DENV), Yellow fever virus, Japanese encephalitis virus (JEV) and West Nile virus, which combined cause millions of infections every year around the world. However, many of the other viruses in this family also cause disease in humans or animals, but are generally believed to have a more limited geographical range and to cause few infections annually [1].

The mosquito transmitted Zika virus (family *Flavivirus*, genus *Flaviviridae*) was first isolated from a sentinel monkey in the Zika forest near Entebbe, Uganda [2], and the first reported cases of human infection occurred in Nigeria in 1954 [3]. Since then, significant outbreaks of Zika disease have occurred in Yap Island in the Federated States of Micronesia, and more recently in French Polynesia and New Caledonia with this last outbreak causing more than 8 000 suspected cases [4]. Isolated cases have been reported from the Philippines [5], Cambodia [6] and Indonesia [7,8] and autochthonous transmission of Zika has been reported in Brazil [9]. The virus has additionally been detected in mosquitoes in Malaysia [10], and in travelers returning from Malaysia [11] and Thailand [12,13]. However, there have been no direct reports of Zika fever in the Thai population.

The main vector of Zika virus in Africa is the *Aedes africanus* mosquito [14], but *Aedes aegypti* mosquitoes, the main vector of dengue in much of Southeast Asia and elsewhere, are also capable of transmitting Zika virus [15] and it is probable that other *Aedes* species such as *Aedes albopictus*, *Aedes polynesiensis* and *Aedes hensilli* are also capable of transmitting Zika virus to humans [16].

Zika fever has significant similarities with dengue fever, although there is no abrupt clinical onset. The main symptoms are fever, rash, headache, muscle and joint pain, odema of the hands and feet and non-purulent conjunctivitis [17–19]. While the course of the disease is believed to be relatively self-limiting, lasting some 4 d–7 d, the recent outbreak in French Polynesia was marked by a number of cases of Guillain-Barre syndrome as well as other complications after the initial Zika virus infection [20].

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Given the general similarity of Zika fever to Dengue fever (sudden onset of fever, headache, rash), it is possible that cases of Zika disease remain unreported in Thailand as a consequence of a mis-identification of the disease as dengue fever, compounded by the lack of specific investigation for Zika fever. The well known cross reaction of antibodies between flaviviruses could cause Zika infections to be serologically diagnosed as dengue fever [21].

We have therefore undertaken a preliminary retrospective serosurvey of 21 serum samples from a previously described cohort of acute undifferentiated fever patients collected in Thailand [22] to determine if there is any evidence of exposure to Zika virus.

2. Materials and methods

2.1. Serum samples

The serum samples used in this study were collected as part of a previously described prospective hospital based study on adult patients with acute undifferentiated fever undertaken at a hospital in Nakhon Ratchasima province in Northeastern Thailand [22]. The study was approved by appropriate Ethical Review subcommittees and written inform consent was obtained from all study participants as previously documented [22].

2.2. Synthetic gene construction

The entire *capsid* (*C*), *premembrane/membrane* (*prM*), and *envelope* (*E*) gene sequence for Zika virus was commercially synthesized (GeneScript, USA Inc.) based on the sequence of a Cambodian isolate of Zika virus (isolate FSS13025), Genbank number AFD30972.1. The Zika synthetic gene was codon-optimized for efficient expression in mammalian cells. The Zika synthetic clone contained 2 385 bp of *C*, *prM*, *E* of Zika virus in the pUC57 plasmid vector.

A DNA fragment containing 19 amino acids of the capsid region immediately upstream of *prM*, as well as the *prM* and *E* sequences was amplified from the codon optimized construct using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA) with *Nhe*I-19CprME-Zika-Fw forward primer (5′-GAGCTAGCCACCATGGGAAGAGGGAC CGATACAAGC-3′) and 19CprME-Zika-*EcoR*I-Rw reverse primer (5′-CGGAATTCTTATGCGGACACTGCGGTGGA-CAGAAAA-3′). After double digestion with *Nhe*I and *EcoR*I restriction enzymes the product was ligated into *Nhe*I and *EcoR*I double digested pCDNA™3.1(+) vector. The ligated products were transformed into competent DH5-α *Escherichia coli*. After confirmation of the sequence by DNA sequence analysis, 3 μg of the pCDNA3.1+_19CprME-Zika plasmid was transfected into 1.3×10^6 cells of HEK293T/17 cells in 6-well plate by the $CaCl_2$ method. Cells were cultured in Opti-MEM and the culture supernatant was collected at 2 d post-transfection.

2.3. Viral infection

HEK293T/17 cells were infected with either Chikungunya virus (CHIKV) E1: 226V (ECSA: Thai isolate), DENV-2 (16681) or JEV (BJ-1) at MOI of 1 according to our standard protocols [23–25] and cultured in Opti-MEM for two days after which the culture supernatant was collected.

2.4. Western blot analysis

Opti-MEM, culture supernatant from the pCDNA3.1+_19CprME-Zika transfection and culture supernatants from CHIKV, DENV-2 and JEV infections were resuspended in a non-reducing loading dye and electrophoresed through 10% SDS-PAGE gels with a protein marker (161-0373, precision plus protein™ all blue prestained standards). Proteins were transferred onto nitrocellulose membranes (Hybond™ECL™ code: RPN303D, GE Healthcare, Little Chalfont, UK). The membranes were blocked with 5% skim milk for 1 h and incubated with following antibodies; a 1:1 000 dilution of HB112 (a mouse monoclonal anti-flavivirus group antigen antibody [26]), a 1:1 000 dilution of a mouse monoclonal anti-Alphavirus antibody (sc-58088, Santa Cruz Biotechnology, Santa Cruz, USA) or a 1:1 000 dilution of human serum for 1 h. After washing, membranes were incubated for 1 h with an appropriate secondary antibody, namely either a HRP-conjugated rabbit anti-mouse IgG (A9044, Sigma St. Louis, USA) or a HRP-conjugated goat anti-human IgG (62-8420, Thermo Fisher Scientific). The membranes were washed and incubated with chemiluminescent substrate (RPN2232, GE Healthcare) prior detecting the signal by the chemiluminescent Western blot imaging system Azure c400.

3. Results

A synthetic gene construct containing the codon optimized sequence of a recently reported Cambodian Zika virus isolate (Genbank number AFD30972.1) encompassing the last 19 codons of the *C* sequence and the *prM* and *E* genes was transfected into Hek293T/17 cells and the supernatant collected on day two post transfection. In parallel HeK293T/17 cells were infected with CHIKV, JEV or DENV-2 and supernatants were collected on day two post infection. Aliquots of these supernatants were electrophoresed though standard SDS-PAGE gels, and the proteins were transferred to nitrocellulose membranes. A total of 26 filters were prepared. Five of the filters were used in control Western blots with a mouse monoclonal pan flavivirus antibody (HB112), with an anti-alphavirus antibody, a combination of both antibodies and with two secondary antibodies (a rabbit anti-mouse IgG and a goat anti-human IgG). Results (Figure 1) show the pan-flavivirus antibody was able to detect Zika, DENV and JEV envelope antigens with no cross reactivity to CHIKV envelope antigen, while conversely the anti-alphavirus antibody was able to detect CHIKV envelope antigen with no cross reactivity to the flaviviruses (Zika, DENV and JEV). The two secondary antibodies showed no immunoreactivity to any of the envelope antigens. Equivalent filters were then used in western blot analyses with 21 serum samples individually. Results showed a complex pattern of immunoreactivity (Table 1 and Figure 1). Only 1 sample showed no immunoreactivity with any of the antigens. Immunoreactivity to the CHIKV envelope antigen was seen in 13/21 (61%) of samples, while the corresponding figures for JEV, Zika and DENV envelope antigens were 14/21 (66%), 16/21 (76%) and 17/21 (80%) respectively. A total of 7 samples showed immunoreactivity to all antigens.