J Vector Borne Dis 44, December 2007, pp. 272–276

ELISA as an alternative tool for epidemiological surveillance for dengue in mosquitoes: a report from Thailand

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Abstract

Background & objectives: Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are the re-emerging infectious diseases caused by the four serotypes of dengue (DEN) virus, type 1 to 4, belonging to the family *Flaviviridae* and genus *Flavivirus*. In the absence of a safe and effective mass immunisation, the prevention and control of dengue outbreaks depend upon the surveillance of cases and mosquito vector. The aim of this work is to test enzyme-linked immunosorbent assay (ELISA) tool for the virological surveillance of dengue.

Methods: Virus-infected *Aedes* mosquitoes were collected from the field in order to serve as an early warning monitoring tool for dengue outbreaks. In a prospective field study conducted from April to September 2000, female adult *Aedes* mosquitoes were caught from selected dengue-sensitive area in Chombung district, Ratchaburi province and assayed by ELISA.

Result: Approximately 18.3% were found positive for dengue virus.

Conclusion: This can imply that ELISA can be an alternative tool for epidemiological surveillance for dengue in mosquitoes.

Key words *Aedes* mosquito – Dengue – ELISA – surveillance

Introduction

Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are the re-emerging infectious diseases caused by the four serotypes of dengue (DEN) virus, type 1 to 4, belonging to the family *Flaviviridae* and genus *Flavivirus*. They are arthropod-borne viruses with positive stranded RNA genome of approximately 11 kb. These viruses are major public health concern for many tropical and subtropical regions of the world, causing endemicity of periodical or annual outbreak of disease[1–2]. Dengue infection has expanded to other geographical areas such as the Americas due to changes in human ecology and behaviour[3–4]. Hundreds of thousands of cases of dengue and dengue hemorrhagic fever are reported each year in tropical regions of the Americas, Africa, Asia and Oceania[5].

In the absence of a safe and effective mass immuni-

sation, the prevention and control of dengue outbreaks depend upon the surveillance of cases and mosquito vector[6–8]. Vector surveillance allows timely implementation of emergency mosquito control measures such as insecticidal fogging to kill adults and destruction of breeding places to limit an impending outbreak. In Thailand, a comprehensive mosquito control programme against mosquito breeding incorporates source reduction, public health education and community participation. Virologic surveillance, which involves monitoring of dengue virus infection in human, has been used as an early warning system to predict outbreak.

Such surveillance based on the isolation and identification of dengue viruses infecting the human population provides an important means of early detection of any changes in the prevalence of dengue virus serotype(s)[9–10]. Monitoring of the dengue virus type(s) infecting *Aedes* mosquitoes during epidemic period will complement the current virologic surveillance for dengue outbreaks[11–12]. Therefore, identification and typing of dengue viruses isolation from field-caught mosquitoes and clinical specimens are important for epidemiological and clinical investigations.

The aim of this work is to test ELISA tool for the virological surveillance of dengue virus-infected *Aedes* mosquitoes from the field in order to serve as an early warning monitoring tool for dengue outbreaks.

Material & Methods

Field collection of mosquitoes

Adult mosquitoes collection: Aedes aegypti mosquitoes for field study were collected at weekly intervals during April to September 2000 from both indoor (in rooms in houses) and outdoor (in front and back yards) in dengue-endemic areas. Specific sampling locations were selected by GIS software programme Mapinfo® and Trimble GPS on the basis of a history of high disease incidence, *Aedes* density, and human population density. Additional indoor *Aedes* mosquitoes were caught during outbreaks in Chombung district, Ratchaburi province areas where cases occurred.

For each indoor station, *Aedes* mosquitoes were captured by plastic vials from households between 0900 and 1200 hrs by the researcher and a vector control officer using themselves as baits or resting inside houses. For outdoor station, *Aedes* mosquitoes were caught by the researcher and a vector control officer using battery-operated, manual aspirators. It normally took 10–15 min to collect *Aedes* mosquitoes between 0900 and 1200 hrs in each area. Overall 240 mosquitoes were caught for further laboratory studies.

Identification of mosquitoes: The *Aedes* mosquitoes were identified to species on chilled table by the researcher and confirmed by an Armed Forces Research Institute of Medical Sciences (AFRIMS) entomologist. All males and females were stored at –70°C in pools of 30 mosquitoes until testing.

Artificially infection of *Aedes* mosquitoes

Virus seed strains: The prototype dengue virus strains in this study were used as positive controls in all tests which consisted of DEN-1 (Hawaiian) titer 1.0×10^6 PFU/ml, DEN-2 (New Guinea C) titer 7.5×10^6 PFU/ml, DEN-3 (H87) titer 2.5×10^5 PFU/ml and DEN-4 (H241) titer 2.5×10^5 PFU/ml. These viruses were obtained from Department of Virology, the US Army Medical Components (AFRIMS), Thailand.

Preparation of mosquito for inoculation: There were four strains of dengue viral seed used for inoculation in this experiment; DEN-1, DEN-2, DEN-3 and DEN-4. Each strain of dengue virus requires 100 of adult *Ae. aegypti* females. These mosquitoes were seven days old and were Bangkok strain (BKK2).

Aedes mosquitoes inoculation: The adult *Ae. aegypti* females were infected by intrathoracically inocula-

tion of DEN virus seed strains. The inoculation technique was modified from the method described by Rosen & Gubler[11]. In groups of 100, each mosquito received an inoculum of 0.017 µl containing concentration of titer 1.0×10^6 PFU/ml, 7.5×10^6 PFU/ml, 2.5×10^5 PFU/ml and 2.5×10^5 PFU/ml of DEN-1, -2, -3 and -4, respectively. After injection, mosquitoes were held at 30°C, 60–70% relative humidity, 14 days for viral replication and were maintained on 10% sucrose solution. They were then individually separated by pools, each containing approximately 30 mosquitoes, and were stored separately in cryogenic vial in –70°C freezer for further viral detection studies by ELISA method. All assay data of individual mosquitoes were analysed. Strains and concentration of DEN viruses for inoculation are presented in Table 1.

Indirect dengue Ag-capture ELISA

Antisera: Goat-anti-human IgG, goat-anti mouse IgG-HRP conjugated and mouse flavivirus-specific monoclonal antibodies (4G2) were obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA and Department of Virology, the US Army Medical Components (AFRIMS), Thailand respectively.

Preparation of mosquitoes for ELISA: The adult *Ae. aegypti* females were dissected individually to separate head, thorax and abdomen. The thorax was triturated with 1 ml plastic mosquito tissue grinders in a solution of 20% acetone-extracted normal human serum in PBS, pH 7.4, 0.05% Tween-20 and 0.02% NaN_3 in PBS, pH 7.4. The suspensions produced were stored at –70°C until testing.

Procedure of ELISA: Optimal dilutions of the sensitising antibody (goat anti-human IgG) and capture antibody (anti-flavivirus human IgG) were determined by serial cross-titration of reagents with DEN control antigens. Although satisfactory results were obtained when dilutions of antibodies were made in PBS, pH 7.4, the lowest background readings were obtained when 20% normal human serum in PBS was used as dilutant. Several blocking buffers were compared for their ability to reduce non-specific binding of proteins. The optimal time and temperature for incubations of the test antigen were also determined. Monoclonal antibodies (4G2) to flavivirus viruses, prepared as mouse ascitic fluids, were evaluated to determine the antibody which could best serve as a detector antibody for DEN viruses used in the study. Several enzyme-labeled conjugates and substrates were also compared to optimise the reaction.

The procedure described by Sithiprasasna[13] was adopted to optimise detection of antigen in this study. ELISA plates (96-well U-bottom polystyrene plates,Titertek.Flow) were sensitised coated with an anti-human goat globulin, diluted 1: 800 in 0.1 M carbonate buffer, pH 9.0 by adsorbing 100 µl/well at 24°C for 4 h . Plates were stored at 4°C overnight. Just prior to use in the assay and between each of the incubations that followed, the sensitised plates were washed six times, each time with 200 µl/well of 0.01M PBS, pH 7.4, containing 0.05% Tween-20 (PBS-TW). To block non-specific protein binding, wells were flooded with 1% casein in PBS-TW at

Table 1. Strains and concentration of DEN viruses for artificial inoculation of *Aedes aegypti*

Serotype of dengue virus	Strains	Viral titer concentration (PFU/ml)	Volume (µl)
DEN-1	Hawaiian	1.0×10^6	0.017
DEN-2	New Guinea C	7.5×10^6	0.017
DEN-3	H87	2.5×10^5	0.017
DEN-4	H241	2.5×10^5	0.017

24°C for 2 h. The polyclonal anti-flavivirus human IgG capture antibody was then diluted 1:100 in PBS, added to the wells (50 µl/well) and incubated at 24°C for 2 h. The test samples were then added to duplicate wells (50 µl/well) and incubated at 4°C overnight. Mouse anti-flavivirus monoclonal-detector antibody (4G2), diluted 1:1000 in 20% acetone-extracted normal human serum in PBS pH 7.4, was then added to all wells (50 µl/well) and incubated at 24°C for 2 h. Goat anti-mouse IgG conjugated to horse-radish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) was diluted 1:1000 in 20% acetone-extracted normal human serum in PBS and incubated in the well at 24°C for 2 h. A small quantity (67 µl) of the substrate solution 5 mg O-phenyline-diamine (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) diluted in 10 ml citrate phosphate buffer, pH 5.0 and 33 µl of 3% hydrogen peroxide were finally added to each well and incubated for 10 min in darkness. The reaction was stopped by addition of 50 µl of 4M H_2SO_4/well and the absorbance was read at 492 nm. All optical density (OD) values were read directly from a Dynatech MR 600 reader to a microcomputer, and the data were stored using Lotus 1-2-3 spread sheet software. Specimens were considered positive if their OD values were greater than the mean value plus three standard deviation (S.D.) of the negative controls. Negative controls consisted of suspensions of triturated mosquitoes of the same stage and species as that place in the test wells, and of a number equal to the maximum number of specimens in test. Positive controls consisted of various serial dilutions of the virus seed.

Results & Discussion

In this study, the adult *Ae. aegypti* females were dissected individually into head, thorax, abdomen and further subjected for ELISA assay. There were 240 mosquito thorax specimens to be subjected for dengue virus detection by ELISA. Specimens were considered positive if their OD values were greater than the mean value plus three S.D. of the negative control. An average of 18.3% (44 of 240) of *Ae. aegypti* tested positive for dengue virus infection by ELISA (Table 2).

Table 2. The numbers of field-caught and artificially inoculated *Ae. aegypti* positive for DEN viruses by ELISA

Type of mosquitoes	No. tested	No. positive	Percent
Artificially-infected	16	16	100
Field-caught	240	44	18.3

All were positive for DEN 1-4 virus serotypes.

Dengue virus infections are becoming an increasingly important international health problem with explosive outbreaks occurring in many parts of the world. Since transoverial transmission of dengue virus is well established. Research work has already done in Malaysia utilising dengue virus detection in field population of *Ae. aegypti* and *Ae. albopictus* as predictive model for forecasting impending outbreak[14]. A rapid, sensitive and specific tool for both diagnostic and epidemiological purposes is therefore needed. According to this circumstance, laboratory technique should be developed as epidemiological tools for the virologic surveillance of the type-specific detection of dengue viruses in artificially infected and in field-caught adult *Aedes* mosquitoes.

In Thailand, dengue hemorrhagic fever was first reported in 1958 and in recent years many outbreaks have occurred and an endemic disease caused a major public health problem[15]. There were 60,330; 37,929; 99,410; 126,348; 24,826 and 17,582 cases of DF/DHF reported to the Epidemiological Division, Ministry of Public Health during 1995–2000, respectively[16]. In 2000, the number of reported cases from the central region was the highest, followed by the northeastern, the northern and the southern region of Thailand[17].

In a prospective field study conducted from April to September 2000, female adult *Aedes* mosquitoes

were caught from selected dengue-sensitive area in Chombung district, Ratchaburi province and assayed by ELISA. Approximately 18.3% were positive for dengue virus.

Indeed, ELISA for detection of other virus antigens in mosquitoes has ever been reported. An ELISA for detection of virus antigen in field collecetd mosquitoes and preserved dry at room temperature ranging from 1–20 months has been certified for detection of JE virus as a part of epidemiological surveillance in JE endemic areas in India[18]. This can imply that ELISA can be an alternative tool for epidemiological surveillance for dengue in mosquitoes.

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Received: 18 May 2007 *Accepted in revised form:* 5 July 2007