Prevalence of Dengue Virus in *Aedes* Mosquitoes During Dry Season by Semi-Nested Reverse Transcriptase-Polymerase Chain Reaction (Semi-Nested RT-PCR)

Rupporn Tuksinvaracharn*, Pongsakorn Tanayapong*,
Saravech Pongrattanaman*, Pokrath Hansasuta MD, DPhil (Oxon)**,
Parvapan Bhattarakosol PhD**, Padet Siriyasatien MD, DTM&H, PhD***

** Undergraduate student, M.D. programme, Faculty of Medicine, Chulalongkorn University*
***Department of Microbiology, Faculty of Medicine, Chulalongkorn University*
****Department of Parasitology, Faculty of Medicine, Chulalongkorn University*

Dengue hemorrhagic fever remains a major health concern in Thailand. Much effort has focused on the prevention and control of the disease. Detection of dengue virus infection rate in mosquitoes would evaluate dengue control programs and predict the epidemics of dengue hemorrhagic fever. To determine dengue virus infection rate in mosquitoes by Semi-nested RT-PCR. A total of 400 mosquitoes were collected from Rom Kao Community representing a crowded community and another 9 non-crowded communities in Bangkok. Mosquitoes were then divided into 40 pools, each contained 10 mosquitoes. A total of 391 Aedes aegypti and 9 Aedes albopictus were screened for dengue virus. The mosquito infection rate in the Rom Klao community was 5% of the mosquito pool equal to that found in non-crowded communities. Both groups were found to have dengue virus serotype 3. The present study suggests a circulation of dengue virus serotype 3 in both crowded and non-crowed communities, the infection rates of which are indifferent during the dry season.

Keywords : *Dengue virus, Aedes aegypti, Aedes albopictus, Mosquito infection rate*

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Dengue hemorrhagic fever (DHF) continues to present a major health problem in many areas of the world especially in Southeast Asia. The infection is caused by the dengue virus which has four serotypes (Den1, Den2, Den 3 and Den 4). Dengue virus is transmitted to humans via mosquito bites. In Thailand, there are two major dengue vectors described, *Aedes aegypti* and *Ae. albopictus* [1,2].

The first outbreak in Thailand was documented in 1958, and a total of 2158 cases were reported. The prevalence of the disease has increased dramatically in recent decades. Morbidity and mortality rate of DHF is highest in children especially at the age of 5-9 and 10-14. More recently, DHF in adults has been documented in Thai patients [3]. At present, the transmission of the disease has spread throughout the country [4]. Due to the lack of specific treatment of the dengue virus and unavailability of effective vaccine against the virus, the interruption of pathogen transmission by mosquito control provides the only effective approach to the control of dengue infection[5,6]. The most effective strategy to control a dengue outbreak is to eliminate mosquitoes and larval breeding habitats. The strategy is commonly operated before the rainy season when the population densities of mosquitoes are lower, and hence easier to control [7-9]. The surveillance of dengue infection in mosquitoes at such times provides useful data for prevention and control of the disease before the beginning of an epidemic in the rainy season. In Thailand, the dengue control relies solely on surveillance for *Aedes* larval habitat and mosquito population density. Although the surveillance has been regularly operated for many years, it has never prevented an outbreak of dengue infection in Thailand. This phenomenon coincided with the reemergence of dengue outbreaks in Singapore despite the reduction of *Aedes* mosquito population to a relatively low level[10]. As described previously, entomologic surveillance *per se* may not serve as an effective tool to forecast a dengue outbreak. Monitoring of the dengue virus in field mosquitoes is more relevant and can complement the current dengue surveillance. It directly detects the prevalence of dengue virus serotypes [11] and pockets of

Correspondence to : Siriyasatien P. Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

infected mosquitoes, thus serving as a sensitive indicator for mosquito control. The present study demonstrates data of a dengue vector survey and prevalence of dengue infection from field-caught mosquitoes from crowded and non-crowded communities in Bangkok during the dry season.

Material and Method

Mosquito collection

This project was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Mosquitoes were collected from crowded and non-crowded communities in Bangkok using human bait collection according to WHO methods [6]. Rom Klao community in Lad Krabang district was chosen and defined as a crowded community (crowded community is a highly urbanized area with a house density of at least 15 houses per 1600 square meters: defined by Bangkok Metropolitan Authority). Ten female mosquitoes were collected daily from each house from 10.00 am to 5.00 pm from the 25th of March to the 10th of April 2003. The total number of female mosquitoes was 400, which were equally collected from crowded and non-crowded communities. All mosquitoes were aliquoted, ten female mosquitoes per microcentrifuge tube and stored at -70°C until used.

Detection of dengue virus

Viral RNA extraction

Viral RNA was extracted from 10 pooled mosquitoes. The mosquito's wings and legs were removed then the mosquitoes were quickly frozen by dry ice and ground in 150 l of RNase-free water and centrifuged at 12,000 rpm for 5 minutes. 100 l of supernatant was preceded to RNA extraction using RNeasy mini kit (QIAGEN, Germany). The procedure followed the manufacturer's recommendation. Total RNA was eluted in 30 l of RNase-free water.

Oligonucleotide primers

The primers' sequence designed by Lanciotti *et al*[12] was used in the present study. Six oligonucleotide primers within the core and pre-membrane protein gene (C-prM) of dengue viruses were used. Two consensus primers (D1 and D2) were designed to be homologous to the genomic RNA of all four dengue serotypes, whereas the type-specific nucleotide primers (TS1, TS2, TS3 and TS4) were designed to anneal specifically to each of their respective genomes. These primers were positioned such that a differently sized product was generated from each type.

Semi-nested RT-PCR

The procedure performed in the present study was modified from the study by Lanciotti *et al.* The first step RT-PCR was performed using Superscript one step RT-PCR with Platinum *Tag* (Invitrogen, USA). The protocol followed the manufacturer's instruction. In 50 l of reaction was performed by mixing 25 l of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM $MgSO_4$), 1.8 l of 50 mM $MgSO_4$, 0.5 l of 25 pmol primers (D1 and D2) of each primer, 1 l of RT/Platinum *Taq* Mix (Invitrogen), 2 l of RNA template and RNase-free water to the total of 50 l. The thermal cycler was programmed to begin with one cycle of 55°C for 30 min followed by a 2-min incubation at 94°C and 40 cycles of 94°C for 15 sec, 55°C for 20 sec and 68°C for 3 min.

The second step amplification reaction was attempted to identify type-specific DNA products. A typical 25 l of PCR reaction was performed containing 2.5 l of 10X buffer, 1.5 l of 50 mM $MgCl_2$, 1 l of 25 pmol of each primer (D_1, TS_1, TS_2, TS_3 and TS_4), 0.5 unit of *Taq* polymerase (GibcoBRL), 4 l of 20 mM DNTP mixture, 10 l of product from the first step and RNase-free water to 25 l. The thermal cycler was programmed to begin with one cycle of 94°C for 5 min incubation followed by 30 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, the last 1 cycle of 72°C for 5 min and final holding at 4°C. 10 l of the product from the second step was electrophoresed through a 1.5% Agarose gel at 100 volts, stained with ethidium bromide and visualized on a UV transilluminator.

Partially extracted dengue virus antigens from mice microglia were used as positive controls. The antigens were purchased from the Ministry of Public Health, Thailand. They were standard Den-1 Hawaii, Den-2 strain TR 1751, Den-3 strain H87 and Den-4 strain H241. Uninfected laboratory-reared *Aedes aegypti* mosquitoes were used as negative controls.

Results

Mosquito collection

During the study period, there was no dengue case reported from the area of study. A total of 400 female mosquitoes were collected. The mosquitoes were identified by morphometric analysis, 391 were *Ae. aegypti* and 9 were *Ae. albopictus*. All *Ae. albopictus* mosquitoes were collected from the non-crowded community. The landing activities of *Aedes* mosquitoes collected from Rom Klao community seemed to have two highest peaks, one in the early morning and the other in the late afternoon (Fig. 1). *Ae. aegypti*

was found only in the crowded community while both species (*Ae. aegypti* and *Ae. albopictus*) were captured in the non-crowded community. The number of *Ae. aegypti* was greater than that of *Ae. albopictus*.

Sensitivity of semi-nested RT-PCR

In order to determine the sensitivity of the test, dengue virus serotype 3 strain H87 was used. The amount of dengue virus was determined by hemagglutination test and converted into viral particles (10^7 particles/ml is equivalent to 1 HA unit). Serial 10-fold dilutions of extracted dengue virus serotype 3 were reverse transcribed and amplified. The limit of detection was approximately 25 viral particles/ l (Fig. 2).

Detection of Dengue viruses in mosquitoes

A total of 400 female mosquitoes were grouped into 40 pools (20 pools from each community). Dengue virus was detected in 2 pools, one from the crowded community and another from the non-crowded community. Only dengue virus serotype 3 was found. Both infected mosquito pools contained only *Ae. aegypti* mosquitoes (Fig. 3).

Discussion

The prevalence of *Ae. aegypti* and *Ae. albopictus* mosquitoes in the present study were 97.75 and 2.25% respectively. The magnitude of differences between the two species in the present result is different from that of the previous study by Thavara *et al* on Samui Island, Thailand during the rainy season. *Ae. aegypti* in their study contributed 75.4% of indoor biting activity, whereas *Ae. albopictus* contributed 24.6% of indoor biting activity. Relatively low frequencies of *Ae. albopictus* in the present study

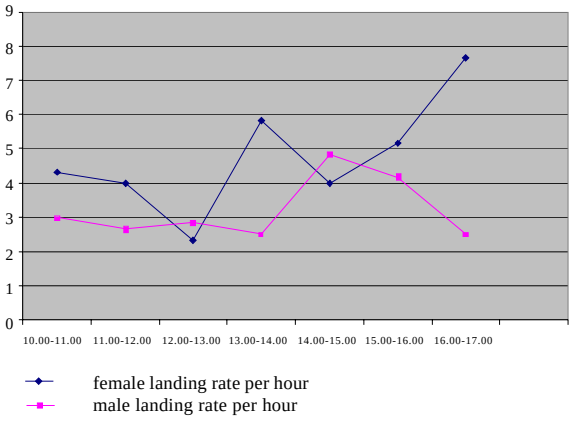

Fig. 1 Graph illustrating *Aedes* mosquito landing rate between 10.00 am to 5.00 pm

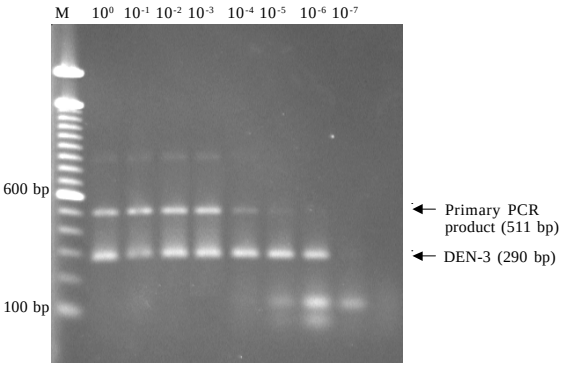

Fig. 2 Agarose gel electrophoresis of the second-round semi-nested PCR quantitated Dengue serotype 3 RNA. Serial 10-fold dilutions were reverse transcribed and amplified as indicated above each lane. Molecular weight (M) markers are shown of the left

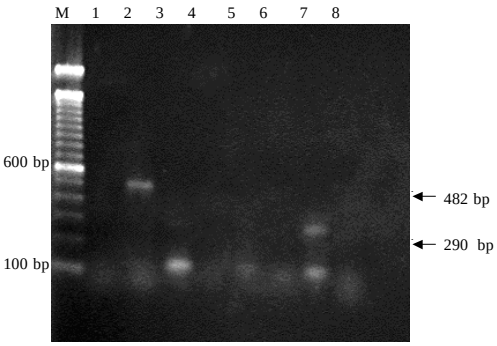

Fig 3. Agarose gel electrophoresis of dengue virus detection in field caught mosquito. Molecular weight (M) markers are shown of the left., lane 1: virus-free mosquito sample were as negative control, lane 2: dengue virus serotype 1 was used as a positive control, lane 3-8:Field caught mosquito samples, dengue virus serotype 3 was detected in lane 7

may be due to the lack of their natural habitats during the dry season. In addition, *Ae. albopictus* breeding potential is expected to decline in highly urbanized areas due to a reduction of outdoor natural site. Moreover, the biphasic landing activities demonstrated in the present study was similar to that reported by Thavara *et al*. However, the landing activities shown in the present study were peaked at early and late afternoon (Fig. 1), while Thavara *et al* reported the peaks of landing activities during the morning and afternoon hours.

There have been a number of studies on detection of dengue virus in field-caught mosquitoes. The mosquito infection rates in Thailand ranged from 3.6% to 61.5% in previous studies [10,13-16]. The rates of infection were different due to various factors

including virus stain, susceptibility of mosquito species, sensitivity of virus detection techniques, study sites, and period of investigation. Indeed, the differences of these infection rates were primarily due to the sensitivity of detection employed in each study. The technique used in the present study, semi-nested RT-PCR, is believed to be one of the most sensitive methods to detect RNA virus in specimens. The present study showed the detection of 25 viral particles.

Although the authors proposed that dengue virus infected mosquitoes were more prevalent in crowded communities, the present result did not agree with the hypothesis. The number of infected mosquitoes in crowded and non-crowded communities was not different from the infection rate of 5% from both communities. The incidence of dengue infection in crowded communities is usually higher than that of non-crowded communities. The reason for this, despite equally prevalent infected mosquitoes, might be that more people would be accessible to the mosquitoes in a crowded area *vis- -vis* in a non-crowded community.

Ae. aegypti mosquitoes were confirmed to be the most important vectors for dengue virus transmission in the present study. *Ae. albopictus* mosquitoes, however, should be underestimated in this context, since the present study was done during the dry season when their natural habitats were depressed. Moreover, preference was given to *Ae. aegypti* mosquitoes because collection of mosquitoes in the present study was done indoors.

Dengue virus serotype 3 was identified in mosquito pools both from crowded and non-crowded communities. Interestingly, dengue virus serotype 3 was recently not isolated from patients from Bangkok Metropolitan areas where the present study was carried out. Whether this study result would predict dengue serotype prevalence in the following dengue season remains to be seen and warrants further study.

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การสำรวจความชุกของเชื้อ Dengue virusในยุงลายช่วงฤดูแล้งโดย วิธี Semi-nested Reverse Transcriptase - Polymerase Chain Reaction (Semi- nested RT- PCR)

รับพร ทักษิณวราจาร, พงศกร ตนายพงศ์, สราเวช พงศ์รัตนามาน, ปกรัฐ หังสสูต, ภาวพันธ์ ภัทรโกศล, เผด็จ สิริยะเสถียร

ไข้เลือดออกยังคงเป็นปัญหาสาธารณสุขที่สำคัญของประเทศไทยและหลายประเทศในเอเชียตะวันออกเฉียงใต้ สาเหตุของโรคเกิดจากเชื้อ Dengue virus ซึ่งมีอยู่ 4 serotypes คือ DEN-1, DEN-2, DEN-3 และ DEN-4 โดยมียุงลายบ้าน (Aedes aegypti) และยุงลายสวน (Aedes albopictus) เป็นพาหะนำโรคที่สำคัญ เนื่องจากยังไม่มียาและวัคซีนสำหรับการรักษาและป้องกันไข้เลือดออก การควบคุมยุงลายซึ่งเป็นพาหะนำโรคจึงเป็นวิธีที่มีประสิทธิภาพที่สุดในการป้องกันและควบคุมการระบาดของโรค ปัจจุบันการเฝ้าระวังโรคโดยอาศัยปริมาณลูกน้ำยุงลาย และจำนวนยุงลายเป็นหลักแต่ยังไม่สามารถควบคุมการระบาดของโรคได้อย่างมีประสิทธิภาพ ดังนั้นการสำรวจปริมาณยุงลายที่มีเชื้อไวรัสไข้เลือดออกจึงควรนำมาพิจารณาร่วมในการเฝ้าระวังโรค สำหรับประเทศไทยจำนวนผู้ป่วยโรค ไข้เลือดออกจะเปลี่ยนแปลงขึ้นลงตามฤดูกาล โดยมักจะมีการระบาดในฤดูฝน ดังนั้นการตรวจหาเชื้อไวรัสไข้เลือดออกในยุงลายช่วงฤดูแล้งอาจสามารถนำมาใช้ในการทำนายการระบาดของโรคที่จะเกิดขึ้นในฤดูฝน เนื่องจากปริมาณยุงลายในช่วงฤดูแล้งมีน้อยและจำนวนยุงลายที่มีเชื้อไข้เลือดออกอาจมีไม่มาก ดังนั้นการตรวจหาเชื้อไวรัสในยุงลายจึงต้องเป็นวิธีที่มีความไวและความจำเพาะสูง วิธี semi-nested RT-PCR เป็นเทคนิคที่มีความไว และความจำเพาะสูงพอที่จะตรวจหาเชื้อไวรัสแม้จะมีปริมาณน้อยในยุง ผู้วิจัยได้ทำการสำรวจโดยการจับยุงลายในกรุงเทพมหานครจำนวน 400 ตัว โดยจับจากชุมชนร่มเกล้าซึ่งเป็นชุมชนแออัด และจากบ้านที่ไม่อยู่ในชุมชนแออัดจาก 9 เขต พบว่ายุง 391 ตัวเป็นยุงลายบ้านและ 9 ตัวเป็นยุงลายสวน ยุงถูกแบ่งเป็น 40 กลุ่มเพื่อหาไวรัสไข้เลือดออกโดยวิธี RT-PCR พบว่าทั้ง 2 แหล่งมียุงติดเชื้อไข้เลือดออก 5% และเป็น serotype 3 จากการสำรวจหาเชื้อไข้เลือดออกในยุงลายจากชุมชนทั้ง 2 แห่งในช่วงฤดูแล้ง ไม่พบว่ามีความแตกต่างกัน