

## A Retrospective Surveillance of the Occurrence of Flaviviruses in *Aedes* Mosquitoes in Phetchabun Province

### การสำรวจหาเชื้อไวรัสในตระกูลเฟลิวีไวรัสย้อนหลังในยุงลายในจังหวัดเพชรบูรณ์

Chanya Jetsukontorn (ชัยญา เจตศุคนธร)\* Akanitt Jittmittraphat (อภนิษฐ จิตตรีมิตรภาพ)\*\*  
Dr.Pornsawan Leangwutiwong (ดร.พรสวรรค์ เหลืองวุฒิวงษ์)\*\*\* Dr.Nathamon Kosoltanapiwat  
(ดร.ณฐมน โกศลธนาภักดิ์)\*\*\* Dr.Patchara Sriwichai (ดร.พัชรา ศรีวิชัย)\*\*\*

#### ABSTRACT

In Thailand, flaviviruses outbreak such as DENV was reported in Phetchabun during 2004-2005 which had many cases of patients who came to hospital with the most common symptoms of DENV and ZIKV infection such as fever, arthralgia, myalgia, headache and rash. According to that outbreak, 939 *Aedes aegypti* and *Aedes albopictus* collected from Amphoe Muang, Phetchabun during 2004-2005 were pooled in groups of up to 10 individuals according to genus/species, and sampling location. Then, they were screened with PCR assay for Pan-flavivirus, ZIKV and DENV, respectively. Two pools gave positive for DENV serotype 4, whereas no positive pool for ZIKV. The minimum infection rate of Dengue virus in this study was 2.13. These finding showed that no ZIKV circulation in mosquitoes in this area at that time. However, these finding confirmed dengue outbreak in Amphoe Muang, Phetchabun during 2004-2005.

#### บทคัดย่อ

ในประเทศไทยเมื่อปี พ.ศ. 2547-2548 พบผู้ป่วยจำนวนมากในจังหวัดเพชรบูรณ์ที่เข้ารับการรักษาที่โรงพยาบาลด้วยอาการของการติดเชื้อไวรัสเดงกีและไวรัสซิกา คือ มีไข้ อ่อนเพลีย ปวดศีรษะ ปวดข้อ ปวดกล้ามเนื้อ และมีผื่นแดง ทำการเก็บตัวอย่างยุงลายในพื้นที่เขตอำเภอเมือง จังหวัดเพชรบูรณ์ไว้ในช่วงปี พ.ศ. 2547-2548 ซึ่งมียุงลายทั้งหมด 939 ตัว นำมาตรวจหาไวรัสในตระกูลเฟลิวีไวรัส คือ ไวรัสซิกาและไวรัสเดงกีโดยการรวม 10 ตัวอย่างตามชนิดและตำแหน่งที่เก็บของยุงลายและนำมาตรวจด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส การศึกษานี้ไม่พบการติดเชื้อไวรัสซิกาในตัวอย่างยุงลาย แต่ยุงลายจำนวน 2 pools พบการติดเชื้อไวรัสเดงกีซีโรทัยป์ 4 และมีค่าอัตราการติดเชื้อไวรัสเดงกีในตัวอย่างยุงลายเท่ากับ 2.13 จึงสรุปว่า เมื่อปีพ.ศ. 2547-2548 ณ อำเภอเมือง จังหวัดเพชรบูรณ์ ประเทศไทยไม่มีการติดเชื้อไวรัสซิกาในยุงลาย และการระบาดในครั้งดังกล่าวเกิดจากยุงลายที่ติดเชื้อไวรัสเดงกี

**Keywords:** Flavivirus, *Aedes* mosquitoes

**คำสำคัญ:** เฟลิวีไวรัส ยุงลาย

\* Student, Master of Science Program in Tropical Medicine, Faculty of Tropical Medicine, Mahidol University

\*\* Lecturer, Department of Microbiology and Virology, Faculty of Tropical Medicine, Mahidol University

\*\*\* Assistant Professor, Department of Microbiology and Virology, Faculty of Tropical Medicine, Mahidol University

## Introduction

Viruses in the genus *Flavivirus*, family *Flaviviridae* are related to the medically important mosquito-borne diseases, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Dengue (DENV), Zika virus (ZIKV), and Yellow fever virus (YFV) (Paixao et al., 2016) They are transmitted to humans primarily by the bite of the infected mosquitoes (Marchette et al., 1969). Symptoms of dengue virus and zika virus are generally mild and self-limiting; rash, fever, pain and headache. Normally, these symptoms can resolve in about a week without medical treatment (Naeem, 2016). However, there is now scientific confirmed that ZIKV is a cause of Guillan-Barré syndrome (GBS) and microcephaly (Cao et al., 2017; Mlakar et al., 2016; Rubin et al., 2016). and DENV can cause Dengue Haemorrhagic Fever which can be found in dengue epidemics in Thailand and has become a leading cause of hospitalization and death among children and adults (World Health Organization, 2018)

*Aedes* mosquitoes are major vectors of ZIKV and DENV which widely distributed globally, and native habitats of most species are warm tropical and sub-tropical regions. The primary mosquito vector is the *Aedes aegypti* species and the *Aedes albopictus* species is a secondary mosquito vector (Kraemer et al., 2015). These mosquitoes typically lay eggs near standing water in things like buckets, bowls, animal dishes, flower pots, and vases. They prefer to bite people, and live indoors and outdoors near people. *Aedes* mosquitoes are hard to control because they live in or around houses. They have adapted themselves to humans' habits. In addition, their eggs can survive drying for more than a year (Hunter, 2017). Mosquito acquisition of the virus likely occurs during a blood meal; after uptake, the virus replicates and is transmitted to a reservoir animal at the next blood meal (Colpitts et al., 2012). The most important control strategies of ZIKV and DENV is controlling of vectors (Falcao et al., 2016). Serological and entomological virus infection was investigated in different areas to see the association between virus and vector in the outbreak areas.

In Thailand, flaviviruses outbreak such as dengue virus was reported in Phetchabun in 2004-2005 which had many cases of patients who came to hospital with the most common symptoms of DENV and ZIKV infection such as fever, arthralgia, myalgia, headache and rash. Therefore, the study of flaviviruses infection in mosquitoes should be investigated for knowing whether mosquitoes have already infected which ZIKV in the past or not. Moreover, detection of infections in field-caught mosquitoes is imperative for determining the natural vectors. According to the outbreak in Phetchabun, we did a retrospective study of flaviviruses infection from mosquitoes which caught from Phetchabun Province in 2004-2005. 98 pools of 939 *Aedes aegypti* and *Aedes albopictus* that were collected in field were screened with PCR assay for Pan-flavivirus, ZIKV and DENV, respectively. Then we calculated the minimum virus infection rate (MIR) in this study area.

## Objective of the study

The aim of this study was to detect flaviviruses in *Aedes* mosquitoes which were collected in field in Phetchabun Province

## Materials and methods

### Mosquitoes Collection and Identification

Phetchabun province in Thailand was chosen as mosquito's collection site because it was high incidence of

flaviviruses outbreak such as DENV which was reported in Phetchabun during 2004-2005. There were many cases of patients who came to hospital with the most common symptoms of DENV and ZIKV infection such as fever, arthralgia, myalgia, headache and rash. *Aedes* mosquitoes were collected in Amphoe Muang, Phetchabun Province from 20 villages which the houses were checked as stand in the community and had not been sprayed with insecticide for at least 3 weeks. The mosquitoes were captured during 09.00-12.00 o'clock both indoor and outdoor of the houses. Mosquitoes in all states were captured by landing and the resting state mosquitoes were captured by nets. Mosquitoes were collected in rainy season from May to September, 2004 and dry season from March to April, 2005. Mosquitoes were transported live to the laboratory. The mosquitoes were only killed by deep-freezing in the refrigerator before separated by species on the chilling table according to mosquito identifying key by Huang (2004). *Aedes* mosquitoes were kept at -80 °C until used.

#### **Ethical approval**

Ethical approval for the study was obtained from the Faculty of Tropical Medicine - Animal Care and Use Committee (FTM-ACUC) Mahidol University, Bangkok; Protocol No. 023-2018E.

#### **Mosquitoes pooled**

According to the three basic body segments of mosquitoes, all mosquitoes were separated into 3 parts; head, thorax and abdomen. Head of mosquitoes were pooled according to genus/species, and sampling location for pan-flavivirus detection. Each pool consisted of a maximum of 10 *Aedes* mosquitoes. Then pools which gave positive results for pan-flaviviruses were detect ZIKV and DENV using thorax and abdomen.

#### **RNA extraction**

Before extraction, mosquito pools were homogenized in the PowerBead Tubes, Garnet 0.70 mm. with 100 µL cell culture medium (MEM) for 2 min at 25 Hz using the TissueLyser LT, Qiagen. Homogenates were centrifuged for 1 min at 13000 rpm. 350 µL of Buffer RLT was added to the same tube with PowerBead for 2 min at 25 Hz and followed the extraction step using the RNeasy kit, Qiagen according to the manufacturer's instructions. Finally, 30 µL RNase-free water was used to elute the sample. RNA was stored at -80 °C until used.

#### **Detection of viruses**

Reverse transcription PCR was used to detect Pan-Flavivirus with Primer FLAVI\_F and FLAVI\_R using SuperScript One-step RT-PCR with Platinum Taq, Invitrogen. Positive pools with Pan-flavivirus were used to test for ZIKV and DENV, respectively. ZIKV in mosquitoes were screened through real-time quantitative polymerase chain reaction (RT-qPCR) using the Luna® Universal One-Step RT-qPCR Kit, Biolab with primer ZIKV\_F and ZIKA\_R. While, DENV in mosquitoes were tested by PCR followed the original method of Lanciotti et al. in 1992 which is divided into 2 steps. First, the RT-PCR product was performed in cDNA by using D1 and D2 primers that amplify C protein and prM protein region. Second, RT-PCR product was reamplified in this step. Five primers were used for the nested-PCR. The PCR products were evaluated by 1.5% agarose gel electrophoresis.

**Table 1** Primers and Nucleotide sequences which were used in this study.

| Primer name | Nucleotide sequence (5' to 3') |
|-------------|--------------------------------|
| FLAVI_F     | TACAACATGATGGGVAARAGWGARAA     |
| FLAVI_R     | AGCATGTCTTCCGTGGTCATCCA        |
| ZIKA_F      | AGGATCATAGGTGATGAAGAAAAGT      |
| ZIKA_R      | CCTGACAACACTAAGATTGGTGC        |
| D1          | TCAATATGCTGAAACGCGCGAGAAACCG   |
| D2          | CAAAGCYCCTTCMGMYGACAT          |
| TS1         | MGCYTCAGTGATTCKAGG             |
| TS2         | CGCCACAAGGGCCATGAACAG          |
| TS3         | TAACATCATCATGAGACAGAGC         |
| TS4         | CTCTGTTGTCTTAAACAAGAGA         |

**Table 2** Primers of each virus detection and their product size (base pairs).

| Detection of | Step          | serotype      | primer                 | Product size (bp) |
|--------------|---------------|---------------|------------------------|-------------------|
| PAN-FLAVI    | RT-PCR        |               | FLAVI_F and<br>FLAVI_R | 1084              |
| ZIKV         | Real-time PCR |               | ZIKA_F and<br>ZIKA_R   | -                 |
| DENV         | RT-PCR        | All serotypes | D1 and D2              | 511               |
|              | Multiplex-PCR | Serotype 1    | D1 and TS1             | 482               |
|              |               | Serotype 2    | D1 and TS2             | 119               |
|              |               | Serotype 3    | D1 and TS3             | 290               |
|              |               | Serotype 4    | D1 and TS4             | 392               |

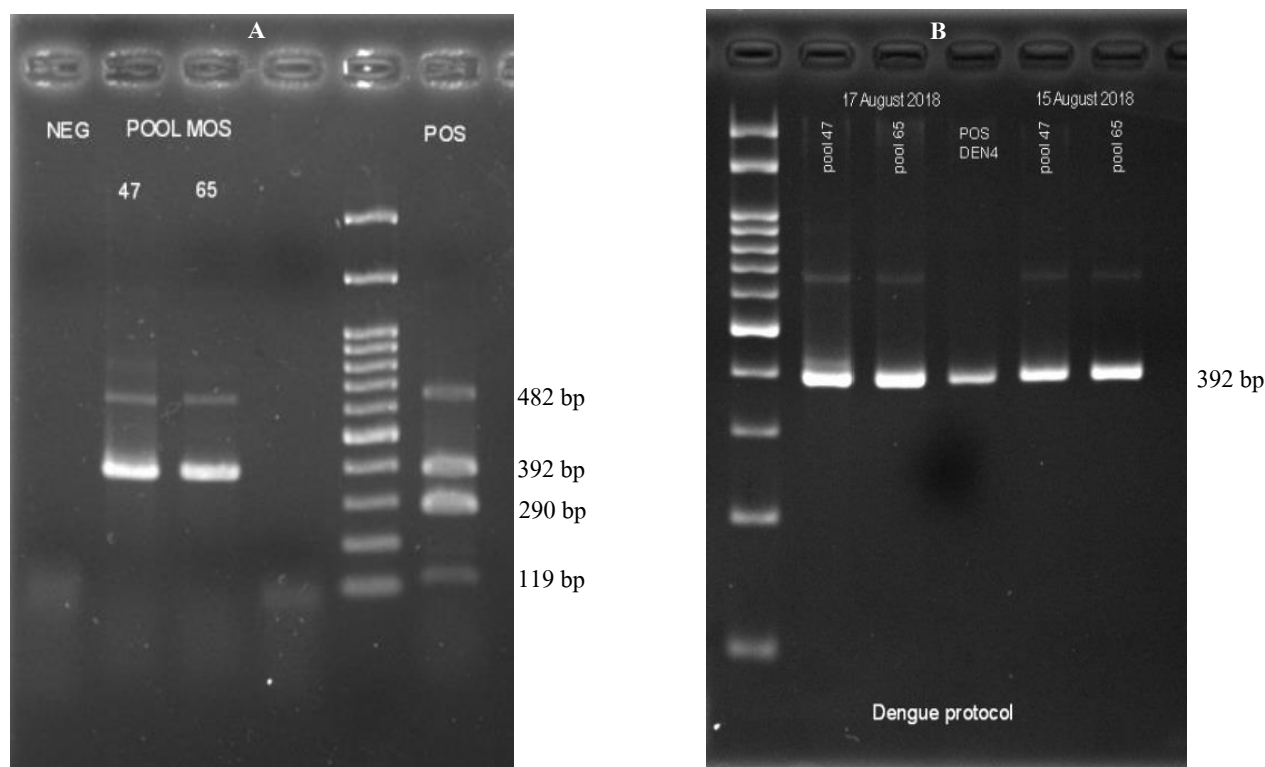
### Data analysis

*Aedes* mosquitoes from field-caught area were detected for minimum infection rate (Condotta et al., 2004) as follows: Minimum infection rate (MIR) = (no.positive pools/no.mosquitoes tested) × 1000

### Results

675 *Aedes aegypti* and 264 *Aedes albopictus* were used in this study. In total, 98 pools of mosquitoes, which were pooled according to genus/species, and sampling, were screened for pan-flavivirus by RT-PCR and gave 19 positive pools. After that, pan-flavivirus positive pools were detected for ZIKV by real-time PCR. RT-qPCR Ct-values of all tested pools were mostly around 40, unlike the positive control was around 28 or below. Lastly, detection of DENV, two pools tested

positive for DENV and showed a band intensity of amplified DNA fragments at 511 bp in the RT-PCR step, whereas nested-PCR showed a clearly band at 392 bp as shown in figure 1.



**Figure 1** Agarose gel analysis of the DNA product from nested-PCR amplification with primer TS1, TS2, TS3, and TS4.

(A) DNA product from nested-PCR amplification with primer TS4 to confirm DENV serotype 4. (B)

## Discussion

In this study, Amphoe Muang, Phetchabun was chosen as studied site because it had high incidence of patients who came to hospital with the most common symptoms of DENV and ZIKV infection such as fever, arthralgia, myalgia, headache and rash in 2004-2005. In field-caught mosquitoes, both *Aedes aegypti* and *Aedes albopictus* were captured, most of them were *Aedes aegypti* (71.88%), and the less were *Aedes albopictus*. This showed that *Ae. aegypti* is closely associated with human. Testing pools of mosquito instead of individual mosquito should be used for saving time and cost in the study. Previous study suggested that pool sizes as large as 200 can be used without a meaningful loss of information when Real Time RT-PCR, cell culture, RAMP, and VecTest assays are utilized to detect arbovirus in the samples (Sutherland and Nasci, 2007). Thus, in this study we decided to use 10 individuals' mosquitoes per pool according to species and collection site. The results gave 19 pools of pan-flavivirus positive whereas only 2 out of 19 pools gave positive for DENV and no positive pool of ZIKV. This result indicated that other pan-flavivirus might be found in these samples and should be investigated further. For DENV detection, two pools gave positive for DENV serotype 4. Thus, the dengue minimum infection rate in this study was 2.3.

## Conclusion

These findings showed that no ZIKV circulation in mosquitoes in this area at that time. However, these finding confirmed DENV was found circulating in Amphoe Muang, Phetchabun in 2004-2005.

On the whole, this study showed *Aedes aegypti* played an important role in DENV infection. People should be protecting themselves by avoiding mosquitoes-biting especially in daytime because they are most active during daylight. Therefore, the best effective method for controlling dengue virus infection is to control vector. The results that obtained from this study were useful to see the association between virus infection cases and mosquitoes in Thailand for controlling vectors which transmission of flaviviruses in the future.

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