รายงานผลการวิจัย

เรื่อง

ผลของการติดเชื้อไวรัสไข้เลือดออกสองชนิดในยุงลายบ้าน (*Aedes aegypti*)

ทุนวิจัยรัชดาภิเษกสมโภช คณะแพทยศาสตร์

ประจำปี 2553

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

โดย

เผด็จ สิริยะเสถียร

พฤษภาคม 2555

ACKNOWLEDGEMENTS

The successful completion of this research entitle “Effect of double dengue serotypes infection in dengue mosquito (*Aedes aegypti*)” was made possible through the invaluable contribution of a number of people.

I would like to thank staffs at Entomology unit, Department of Parasitology, Faculty of Medicine Chulalongkorn University and National Institute of Health, Department of Medical Sciences, Ministry of Public Health.

This study was supported by a Ratchdapisak Sompoj Foundation, Chulalongkorn University.

I hope that the results of this research would be benefits to dengue control of Thailand.

Padet Siriyasatien

ชื่อโครงการวิจัย ผลของการติดเชื้อไวรัสไข้เลือดออกสองชนิดในยุงลายบ้าน (*Aedes aegypti*)

ชื่อผู้วิจัย เผด็จ สิริยะเสถียร

เดือน และ ปี ที่ทำวิจัยเสร็จ พฤษภาคม 2555

บทคัดย่อ

ผู้วิจัยรายงานผลการทดสอบในห้องปฏิบัติการเพื่อชักนำให้เกิดการติดเชื้อไวรัสไข้เลือดออกในยุงลายบ้านด้วยเชื้อไข้เลือดออกมากกว่า 1 ชนิด โดยใช้วิธีการให้ยุงดูดกินเลือดผ่านเยื่อสังเคราะห์ ซึ่งใช้ไวรัสไข้เลือดออกที่มีความเข้มข้น 10^3 pfu/ml เมื่อทดสอบโดยใช้ไวรัสไข้เลือดออกทั้ง 4 ซีโรไทป์ผสมในเลือดและให้ยุงลายเพศเมียจำนวน 40 ตัว ดูดกินเลือด หลังจากนั้น 1 สัปดาห์ได้ทำการตรวจหาเชื้อไข้เลือดออกโดยวิธี RT-PCR พบว่ายุง 7 ตัวติดเชื้อไข้เลือดออกซีโรไทป์ที่ 3 ยุง 2 ตัวติดเชื้อไข้เลือดออกซีโรไทป์ที่ 4 และมีการติดเชื้อซีโรไทป์ที่ 3 และ 4 ร่วมกันในยุง 4 ตัว การทดสอบโดยให้ยุงติดเชื้อไข้เลือดออกซีโรไทป์ที่ 1 และ 2 เพื่อยืนยันว่าไวรัสทั้งสองซีโรไทป์ที่ใช้ในการทดสอบนี้สามารถติดต่อสู่ยุงลายได้ จากยุงที่ใช้ทดสอบทั้งหมด 46 ตัว พบยุงติดเชื้อไข้เลือดออกซีโรไทป์ที่ 1 จำนวน 8 ตัวและซีโรไทป์ที่ 2 จำนวน 2 ตัว และตรวจไม่พบการติดเชื้อร่วมกันระหว่าง 2 ซีโรไทป์นี้ ผลการทดลองแสดงให้เห็นว่าเชื้อไข้เลือดออกแต่ละซีโรไทป์มีความสามรถในการติดต่อสู่ยุงลายบ้านแตกต่างกัน นอกจากนี้การทดสอบความสามารถในการเจริญของเชื้อไข้เลือดออกเทียบกับเชื้อชิคุนกันยาในเซลล์เพาะเลี้ยง C6/36 พบว่าเซลล์เพาะเลี้ยงยุงลายสวน C6/36 ถูกใช้สำหรับประเมินการติดเชื้อร่วมกันระหว่างเชื้อไวรัสไข้เลือดออกซีโรไทป์ที่ 3 และชิคุนกันยา การติดเชื้อไวรัสประเมินโดยวิธีการ one-step duplex reverse transcriptase

PCR (D-RT-PCR) ซึ่งเมื่อทำการติดเชื้อไวรัสทั้งสองชนิดในปริมาณเท่าๆกันและเมื่อทำการติดเชื้อไวรัสในปริมาณที่แตกต่างกันโดยใช้ชิคุนกันยามีปริมาณมากกว่าเชื้อไข้เลือดออกพบว่าสามารถตรวจพบเชื้อไวรัสทั้งสองชนิดได้ อย่างไรก็ตามเมื่อติดเชื้อชิคุนกันยาในปริมาณน้อยกว่าเชื้อไข้เลือดออกกลับตรวจพบเฉพาะเชื้อไข้เลือดออกเท่านั้น ซึ่งแสดงให้เห็นว่าเชื้อไข้เลือดออกสามารถกดการเจริญของเชื้อชิคุนกันยาได้ นอกจากนี้การศึกษาในภาคสนามโดยการจับยุงลายมาตรวจหาเชื้อไข้เลือดออกพบว่าอัตราการติดเชื้อไข้เลือดออกในยุงที่จับมาในแต่ละฤดูกาลมีความแตกต่างกัน การศึกษาครั้งนี้ให้ข้อมูลเกี่ยวข้องกับปฏิกิริยาของเชื้อก่อโรคในโฮสท์ ในยุงพาหะทั้งในระดับห้องปฏิบัติการและในภาคสนาม ซึ่งจะสามารถนำมาใช้ในการทำนายการระบาดของโรคและควบคุมโรคต่อไป

Project Title	Effect of double dengue serotypes infection in dengue mosquito (*Aedes aegypti*)
Name of the Investigator	Padet Siriyasatien
Year	May, 2012

Abstracts

We demonstrated the results of laboratory induced dengue virus infection more than 1 serotype in female *Aedes aegypti* through membrane feeding apparatus. Forty female mosquitoes were allowed to feed human blood contained 4 dengue serotypes at the concentration of 10^3 pfu/ml. One week after infection, dengue virus were detected by RT-PCR technique. Seven and two mosquitoes were positive for dengue serotype 3 and 4, respectively. Mix infection of dengue virus serotype 3 and 4 was found in 4 female mosquitoes. To determine the infectivity of dengue serotype 1 and 2, both serotypes were used to infect 46 female mosquitoes. Eight and two mosquitoes were infect with serotype 1 and 2, respectively while no co-infection of these two serotypes were observed. The results showed that difference in capability of infection between dengue serotype in mosquito. Furthermore, co-infection between dengue virus serotypes 3 (DEN 3) and chikungunya (CHIKV) virus was also determined in *Ae. albopictus* (C6/36) by one-step duplex reverse transcription polymerase chain reaction (D-RT-PCR). The D-RT-PCR showed positive for both viruses either infection with equal titer of multiplicity of infection of both viruses and infection with higher titer of CHIKV than DENV 3. In contrast, co-infection with DENV 3 higher titer than CHIKV was shown only positive D-RT-PCR dengue virus. We demonstrate that inhibition of

CHIKV replication by DENV 3 depends on virus titer. Field studies of dengue infection rates in *Ae. aegypti* female obtained from various seasons also showed that dengue infection rate in the mosquito vector depended on time and season. Thus, this study provides the interaction between pathogen against the host cells, in the mosquito vector both in laboratory and in the field which could be applied for predicting the outbreak and furthermore for effectively controls these mosquitos borne disease in the future.

CONTENTS

	Page
ACKNOWLEDGEMENTS	i
THAI ABSTRACT	ii
ENGLISH ABSTRACT	iv
CONTENTS	vi
LIST OF TABLE	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xi
CHAPTER I INTRODUCTION	1
CHAPTER II MATERIALS AND METHODS	5
2.1 Mosquito rearing	5
2.2 Viral propagations	5
2.3 Calculating viral titer by standard plaque assay	6
2.4 Viral detection in *Ae. aegypti*	6
2.5 One - step duplex reverse transcription polymerase chain reaction (D-RT-PCR)	7
2.6 Field study areas	7

2.7 *Ae. aegypti* collection 8

2.8 Collection of blood specimens 8

2.9 Viral determination in blood samples 9

2.10 Biting activity test 9

2.11 Incidence of DHF in the study areas 10

CHAPTER III RESULTS 11

3.1 Laboratory determination of DENV infection in *Ae. aegypti* mosquitoes 11

3.2 Determination of virus production from single infection and co-infection 13

3.3 Determination of viral production from super infection on C6/36 cell cultures 13

3.4 Dengue infection rate from field captured mosquitoes 15

3.5 Seasonal dengue infection in *Ae. aegypti* females 17

3.6 Transovarial dengue transmission in *Ae. aegypti* larvae and males 18

3.7 Serotyping of dengue viruses in *Ae. aegypti* and DHF patients 19

3.8 Seasonal biting activity of *Ae. aegypti* females 21

CHAPTER IV DISCUSSION 24

4.1 Determination of viral production from super infection on C6/36 cell cultures 24

4.2 Laboratory induced DENV infection in Ae. aegypti mosquitoes and Field studies 27

REFERENCES 32

PUBLICATIONS 43

LIST OF TABLE

Page

Table 1 The results of dengue and chikungunya virus infected C6/36 cell cultures. The secretion fluids were evaluated by D-RT-PCR after 7 days' post-infection 14

LIST OF FIGURES

Page

Figure 1 Artificial blood-feeding of mosquitoes 11

Figure 2 RT-PCR amplification of DENV serotypes 1-4 infection in female mosquitoes were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide 12

Figure 3 The 2% agarose gel and stained with ethidium bromide demonstrated the DENV serotypes 1 and 2 infection in female mosquitoes. 12

Figure 4 The positive results of mixed dengue and chikungunya mixed-infection was evaluated by D-RT-PCR 15

Figure. 5 Agarose gel analysis of the PCR products generated by multiplex RT-PCR assay 16

Figure 6 The dengue infection in *Ae. aegypti* females collected in each season of the 4 study provinces 17

Figure 7 The transovarial dengue transmission in *Ae. aegypti* larvae and adult males collected from the 4 study provinces 19

Figure 8 Serotyping of dengue viruses in *Ae. aegypti* in the 4 study provinces 20

Figure 9 Serotyping of dengue viruses in patients with suspected dengue in the 4 study provinces 21

Figure 10 Seasonal biting activity of *Ae. aegypti* females 22

Figure 11 Incidence of DHF in the study areas of the 4 provinces 23

LIST OF ABBREVIATIONS

%	percent
µg	microgram
µl	microlitre, 10^{-6} litre
µM	micromolar
bp	base pair
cm	centimeter
cDNA	complementary deoxyribonucleic acid
ddH2O	deionized distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EDTA	ethylene diamine tetraacetic acid
et al.	et alii (latin), and others
g	gram
L	litre
M	molar(s)
mg	milligram
ml	milliliter, 10^{-3} litre
mM	millimolar
NCBI	National Center for Biotechnology Information
mRNA	messenger RNA

mt DNA	mitochondrial DNA
ng	nanogram, 10^{-9} gram
^{o} C	degree Celsius
OD	optical density
PCR	polymerase chain reaction
RT-PCR	Reverse-transcriptase PCR
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	Ribosomal RNA
TAE	Tris sodium acetate ethylene diamine tetraacetic acid (EDTA)
Taq	*Thermophilus aquaticus* (polymerase)
UV	ultraviolet
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Dengue virus (DENV) and chikungunya virus (CHIKV) are medically important viruses causing morbidity among millions of people worldwide. Both viruses cause public health problems in Southeast Asia. There have been outbreaks of these viruses reported from India, Singapore, Malaysia and Thailand (Simon *et al*, 2008; Yamamoto *et al*, 2010; Ho *et al*, 2011). The first reported case of chikungunya fever in Thailand occurred in 1954 cause by the Asian genotype of chikungunya virus. The main vector for CHIKV is *Ae. Aegypti* (Pongsiri *et al*, 2010). The outbreaks of chikungunya fever in Southeast Asia in 2008-2009 were related to the East, Central and South African genotype (ECSA) which is transmitted by *Ae. Albopictus* (Auksornkitti *et al*, 2010). The ECSA genotype is closely related to the virus isolated from earlier outbreaks in Malaysia and Singapore (Rianthavorn *et al*, 2010). The genotype has a different amino acid sequence at A226V, which allows more case transmission by *Ae. albopictus* than *Ae. aegypti*.

DENV infection is a public health problem in Thailand which reported in Annual Epidemiological Surveillance Report in 2009 (AESR, 2009). The number of cases of DENV infection increased to 56,651 with 50 deaths in 2009, a 2.23 fold increase from the previous year (AESR, 2009). Dengue fever (DF) and dengue hemorrhagic fever (DHF) are diseases transmitted by the bite of *Aedes* mosquitoes, principally *Ae. aegypti*, infected with any one of the four dengue viruses, DENV 1-4 (WHO, 2011). DF/DHF currently occurs in over 100 countries worldwide and an estimated 2.5 billion people are at risk of infection. Approximately 975 million people live in urban areas in tropical and sub-tropical countries in Southeast Asia, the Pacific and the Americas (WHO, 2007). In other countries, Africa and Eastern Mediterranean

including rural communities are increasingly being affected with dengue transmission as well. There are up to 50 million cases reported annually with 500,000 cases of DHF and 22,000 deaths mainly among children (WHO, 2011). DHF in Thailand was first reported in Bangkok in 1949 (Wangroongsarb, 1997), whereas the first epidemic occurred in 1958 (Ungchusak and Kunasol, 1988). The highest mortality rate of DHF (1.88 per 100,000 populations) was documented in 1987 (Nisalak *et al*. 2003). After first DHF outbreak appeared, the disease has spread across the country and become a major public health problem. At present, there is no specific medication for treatment of dengue infection available. The prevention and control measures of dengue disease have emphasized vector mosquito management. Attempts have been made to focus on surveillance for planning to reduce disease burden, changing behaviors to improve vector control and responding to disease epidemic (WHO, 2000).

Since 2009, CHIKV infections have occurred in Phuket, Songkhla, Pattani, Narathiwat, and Yala provinces of Thailand with 24,029 cases have been reported. Symptoms of chikungunya fever are similar to dengue fever such as prolonged joint pain, swelling of joints, stiffness, muscle pains, headaches, fatigue, nausea, vomiting and rashes (Pardigon 2009; Rianthavorn *et al,* 2010). Detection and evaluation are very important especially in dengue-endemic areas.

Dengue is an endemic disease in Thailand and co-circulation of all four serotypes has been reported by the dengue epidemiological surveillance network. After the initial large outbreaks of chikungunya fever in Thailand, co-circulation of all four dengue serotype are found every year (Nimmannitya *et al*, 1969; Thavara *et al*, 2006; Fried *et al*, 2010; Tang *et al*, 2010; Zhao *et al*, 2010). ELISA tests on sera obtained from Thai patients were positive for antibody against dengue virus. Dengue virus serotype 1 (DENV 1) was shown the primary infection and

DENV 3 was a secondary infection. DENV 3 was shown to be the predominant infection among the Thai people, compared with the other serotypes, during the dengue outbreak in Thailand, in 1962 (Nimmannitya *et al*, 1969). Additionally, DENV 3 was found in blood specimen and mosquitoes sample which collected from recently chikungunya outbreak in South of Thailand (Raekiansyah *et al*, 2005; Sriprom *et al*, 2010; Zhao *et al*, 2010). Thus, co-infection or co-circulation of all four dengue serotypes, or with other arboviruses, in Thailand is very interesting, although still not well understood.

Double infection between DENV and CHIKV in human sera has never been reported in Thailand, but it has been reported in other countries, such as India, Malaysia, and Germany (Nayar *et al*, 2007; Ezzedine *et al*, 2008; Kukreti *et al*, 2008; Chahar *et al*, 2009; Leroy *et al*, 2009; Schilling *et al*, 2009). Furthermore, CHIKV and DENV co-infection in mosquitoes was revealed in field population, and heterogeneous infection between four serotypes of dengue viruses has been demonstrated. Rarely recent information has been recorded regarding to CHIKV and DENV co-infection. Thus needs further study to investigate how their endemic outbreaks are still occur annually in Thailand (Mavale *et al*, 2010; Thavara *et al*, 2009). The aims of this study are focus on characterization of DENV 3 and CHIKV co-infection *in vitro*. In this study, we used *Aedes albopictus* (C6/36) cell line as a host to determine the effect of DENV and CHIKV co-infection in *Aedes albopictus* (C6/36) cell line. Effect of co-infection between DENV and CHIKV in the mosquitoes would be valuable information to forecast the diseases outbreak and furthermore for disease control. For field study, *Ae. aegypti* larvae and adults were collected in three seasons from the 4 central provinces of Thailand for detection and serotyping of dengue viruses. Data on DHF patients reported in those provinces and the suspected patients confirmed as DHF were taken into consideration with data on dengue infection in mosquitoes for

planning in response to imminent dengue occurrence. Seasonal biting activity of *Ae. aegypti* females was studied and used as supporting factor on explanation of dengue incidence. The data obtained herein might be employed in constituting an early warning monitoring system for dengue outbreak in all those endemic areas.

CHAPTER II

MATERIALS AND METHODS

2.1 Mosquito rearing

Ae. albopictus mosquitoes were maintained in an insectary of the Department of Medical Sciences, National Institute of Health, Thailand. Conditions were set at 28^{o}C±1^{o}C at 80%±5% relative humidity under 12/12 hours light/dark photo-period. Adults were supplied with a damp cotton wool pad containing 10% sucrose solution as a carbohydrate source. For blood feeding, female mosquitoes were allowed to artificial blood-feeding at 30 minutes. Group of mosquitoes were reared simultaneously from the same cohort of eggs. Adult female mosquitoes aged 3-5 days were used in this study, the mosquitoes were allowed to feed on 10% sucrose solution. For DENV infection, DENV were mixed with EDTA blood and allowed mosquitoes to feed for 30 minutes (Figure 1). Fully engorged mosquitoes were collected and used for DENV further detection experiments.

2.2 Viral propagations

Ae. albopictus mosquito (C6/36) cell cultures were maintained at 28^{o}C in an incubator for virus propagation. CHIKV and DENV 3 were derived from infected mosquitoes collected from southern Thailand. DENV 3 and CHIKV were propagated on a monolayer C6/36 cell line. The supernatant was obtained and used to calculate the viral titer via a plaque assay. The experiments were divided into three groups: single infection, mixed infection (co-infection), and superinfection. Equal volumes and MOIs of the two viruses were used in the single-infection and superinfection treatment groups. Mixed infections used both equivalent titers and different titers. The superinfection group received a double infection treatment, which involved non-infected cells being exposed to the first viral treatment for 1 hour, removing the primary virus with an

acid glycine buffer, and exposing the cells to a secondary virus (Hung *et al*, 1999). The experiments were done separately in triplicate replications.

2.3 Calculating viral titer by standard plaque assay

The LLC-MK_2 (Rhesus monkey kidney cell) monolayer was grown in 6 well plates at a concentration 4 x 10^5 cell/well. DENV and CHIKV were diluted and infected on LLC-MK_2 cell separately. Each well was covered with 1% methylcellulose and stained with 2% crystal violet. Plaque number were counted, and plaque performing unit (PFU/ml) calculations conducted before MOI=1 and 0.1 to other experiments. The viral titer was measured before and after infection, and the experiment was done separately in triplicate replications.

2.4 Viral detection in *Ae. aegypti*

The method for detection of dengue viruses in *Ae. aegypti* larvae and adults was modified from that described by Tuksinvaracharn *et al*. (2004). Mosquito wings and legs were removed and the remaining bodies were processed for RNA extraction using Invisorb® Spin Virus RNA Mini Kit (Invitex Gmbh, Germany). Five oligonucleotide primers used in this study were designed by Lanciotti *et al*. (1992), D1 and 4 type-specific primers (TS1, TS2, TS3 and TS4). Multiplex RT-PCR was performed using BluePrint™ One Step RT-PCR Kit. Target RNA was amplified in 25-ul volume containing 2X One Step BluePrint™ Buffer, 25 pmol of each primer, One Step BluePrint™ RT Enzyme Mix, and RNase-free water. The thermal cycler was programmed to incubate at 50°C for 30 minutes, 94°C for 2 minutes and then to proceed with 40 cycles of 94°C for 30 sec, 50 °C for 30 sec and 72°C for 30 sec, the last 1 cycle of 72°C for 7 minutes and final holding at 4°C. A 6-ul product was electrophoresed through a 2% Agarose gel at 100 volts, stained with ethidium bromide, visualized on a UV transilluminator and confirmed the positive bands by nucleotide sequencing analysis. Mosquitoes intrathoracically inoculated

with dengue viruses were used as positive controls. These viruses were DENV 1 strain Hawaii, DENV 2 strain TR 1751, DENV 3 strain H87, and DENV 4 strain H241. Uninfected laboratory-rearing *Ae. aegypti* mosquitoes were used as negative controls. The larval or adult pools positive for dengue viruses were determined dengue infection rate which was calculated from the number of positive pools divided by the total number of tested pools x 100.

2.5 One - step duplex reverse transcription polymerase chain reaction (D-RT-PCR)

The RNA viral genome was extracted from infectious C6/36 cell culture fluid by using the Nucleospin® RNA II kit (Macherey-Nagel). One-step RT-PCR was performed by SuperScript® III One-Step RT-PCR test Kit (Invitrogen) with Platinum® *Taq* DNA Polymerase in a total volume 25 µl; contained 100 ng of RNA template, 12.5 µl (2X) Reaction mix buffer, 0.5 µl sense (2.5 µM), 0.5 µl anti-sense (2.5 µM) and 0.5 U of SuperScript III RT/ Platinum *Taq*. The specific primers were designed by previously described (Dash, *et al*, 2008). The amplification program included: a reverse transcription at 55 ^{o}C for 30 minutes, a polymerase activation step at 94 ^{o}C, 2 minutes, followed by 40 cycles of 30 seconds at 94 ^{o}C and 1 minute at 55 ^{o}C, extension at 72 ^{o}C 10 minutes. The D-RT-PCR products were analyzed on 2.0% agarose using gel electrophoresis.

2.6 Field study areas

Four provinces in Central region of Thailand: Nakhon Pathom, Nonthaburi, Ratchaburi and Samut Sakhon were chosen as the study areas. In each province, three districts (2 sub-districts per district, 2 villages per sub-district, and 40 dwellings per village) were conducted for collection of *Ae. aegypti* larvae and adults. The selection of those areas was based on three reasons: 1) the provinces at least once ranked on top fifteen DHF incidence reported in Thailand

between 2002 and 2006, 2) the abundance of *Ae. aegypti* mosquitoes, and 3) the travel convenience for mosquito collection.

2.7 *Ae. aegypti* collection

The *Ae. aegypti* larvae collected from clean water-containing containers indoor and adults using human bait (WHO, 1997) were carried out twice a season between March 2007 and February 2008; there are three seasons in Thailand: the winter, from November to February, the hot season, from March to May, and the rainy season, from June to October. The dwellings for larval and mosquito collections were selected from the villages which had experienced recent DHF cases. The mosquito collection time was started from 09.00 am to 05.00 pm. Six volunteers usually settled down in dark areas of the room where most biting activity occurs. All bared their legs between knee and ankle, collected all landing and biting mosquitoes individually with plastic vial and capped it. The collectors caught mosquitoes indoors for 20 minutes per dwelling. The collected larvae and mosquitoes were visually identified for *Ae. aegypti* species. The live mosquitoes were inactivated in a refrigerator, separated by localities and sexes. All *Ae. aegypti* larvae and mosquitoes were pooled and then kept in cryogenic vials (5 larvae or mosquitoes/pool/vial) to store in liquid nitrogen for subsequent dengue viral detection.

2.8 Collection of blood specimens

Blood specimens were taken from DHF patients admitted to hospitals in the study areas. The blood samples were drawn into tubes with EDTA anticoagulant, and centrifuged to obtain plasma. The plasma specimens were kept in liquid nitrogen tanks and then transported to the Arbovirus Laboratory, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand for determination of dengue serotypes.

2.9 Viral determination in blood samples

RT-PCR was performed to determine dengue serotypes (Yenchitsomanus *et al*. 1996; Chanama *et al*. 2004). A 100 µl of patient plasma was extracted for viral RNA using QIAamp viral RNA mini kit (Qiagen Gmbh, Hilden, Germany). One-step RT-PCR kit (Qiagen) was used together with type-specific primers in RT-PCR reactions. Positive and negative controls were added in each run as well. PCR products were electrophoresed through agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

2.10 Biting activity test

The study of seasonal biting activity pattern of *Ae aegypti* mosquitoes was simulated and conducted between March 2007 and February 2008 at the research station in Bang Bua Thong district, Nonthaburi. This station where a married couple live, contains basic facilities like the general dwellings. Twenty earthen jars (200 liters in capacity) were fully filled with city hydrant water. Five hundred large *Ae. aegypti* pupae which mostly become female mosquitoes, were picked out for the experiment. Twenty five pupae were added to each jar for adult emergence. After 5-day pupal addition, the biting activity test of *Ae. aegypti* was performed through 24-hour period from 06.00 am to 06.00 am of the following day once a month. Three volunteers sit down on the small plastic chairs provided in a row. Five meter distance was set up for each volunteer. All bared their legs between knee and ankle, collected all the landing mosquitoes individually with plastic vial and capped it. The collectors caught mosquitoes indoors by the first 20-minutes period of each hour. The collected mosquitoes were visually identified for *Ae. aegypti* females and pooled together with those captured in the same hour of other months by the same season. The seasonal biting rate was calculated and expressed as an average number of all mosquitoes collected in each season /person/hour.

2.11 Incidence of DHF in the study areas

DHF data reported from the study areas in 2007 was obtained from the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. The data was expressed as the morbidity rate of DHF per 100,000 populations.

CHAPTER III

RESULTS

3.1 Laboratory determination of DENV infection in *Ae. aegypti* mosquitoes

Laboratory induced dengue virus infection more than 1 serotypes in female *Ae. aegypti* through membrane feeding apparatus. Forty female mosquitoes were allowed to feed human blood contained 4 dengue serotypes at the concentration of 10^3 pfu/ml (Figure 1). One week after infection, dengue viruses were detected by RT-PCR technique. Seven and two mosquitoes were positive for dengue serotype 3 and 4, respectively. Mix infection of dengue virus serotype 3 and 4 was found in 4 female mosquitoes (Figure 2). To determine the infectivity of dengue serotype 1 and 2, both serotypes were used to infect of 46 female mosquitoes. Eight and two mosquitoes were infected with serotypes 1 and 2, respectively while no co-infection of these two serotypes was observed (Figure 3).

Figure 1 Artificial blood-feeding of mosquitoes

Figure 2 RT-PCR amplification of DENV serotypes 1-4 infection in female mosquitoes were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Lane 100 bp: molecular mass marker (100 basepairs [bp], lane D1-D4: positive control of DENV serotypes 1-4 respectively, lane 4, 11, 16, 19-21, 23-27, and 33: mosquitoes sample.

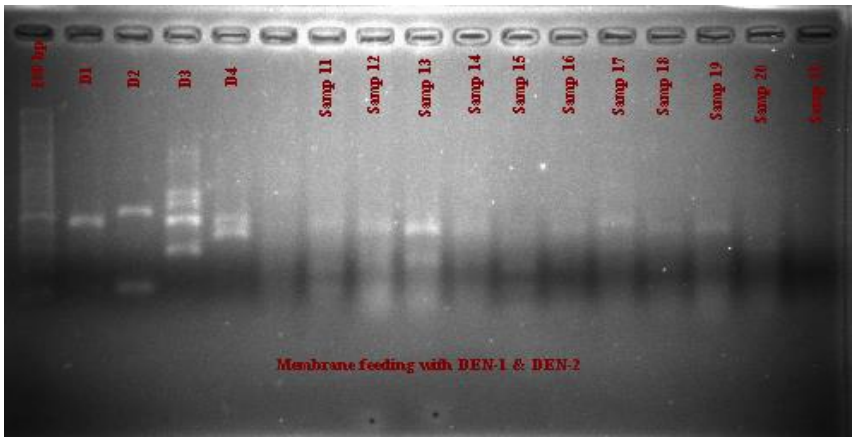

Figure 3 The 2% agarose gel and stained with ethidium bromide demonstrated the DENV serotypes 1 and 2 infection in female mosquitoes. Lane 100 bp: molecular mass marker (100 basepairs [bp], lane D1-D4: positive control of DENV serotypes 1-4 respectively, lane samp 11-21: mosquitoes sample.

3.2 Determination of virus production from single infection and co-infection

The D-RT-PCR was used to determine viral secretion in cell culture-fluid after 7 days' post-infection. The single infection samples were collected and the supernatant were utilized for viral RNA extraction and nucleotide sequencing. The partial *E1* gene sequence was compared with the NCBI database to evaluate the CHIKV genotype. These genotypes are closely related to the East, Central and South African genotypes (ECSA), which were also determined to be the cause of the recent chikungunya disease in Singapore and Malaysia. The CHIKV (ECSA) strain and DENV 3 were combined into a mixed infection in C6/36 cell cultures. The result of mixed infection analysis, with equal titers of each virus, showed both dengue and chikungunya virus infection in the same culture (Figure 4). The result consisted of two viruses that were able to replicate in mosquito cell line (Figure 4, Lane 6). We also evaluated mixed infection using unequal titer of the two viruses at MOIs of 0.1 and 10. The results were similar to infection with both viruses (data not showed). When a lower DENV 3 titer (MOI=0.1) than CHIKV (MOI=1.0) titer was used, the D-RT-PCR results also showed infection of two viruses, which was similar to co-infection with equal titers (Figure 4, Lane 8). Unexpectedly, when we used a higher titer of DENV 3 (MOI=1.0) than CHIKV (MOI=0.1) mixed-infection, only one positive result of D-RT-PCR product at position 490 bp of DENV 3 *C-prM* gene occurred, while the CHIKV product disappeared completely (Figure 4, Lane 7).

3.3 Determination of viral production from super infection on C6/36 cell cultures

We also determined viral infection by changing the order of virus infection in a series of infections, or super infection. In one treatment group, the cells were first exposed to dengue virus, followed by CHIKV (in equal titer). D-RT-PCR results showed a positive signal for both viruses in the same culture (Figure 4, Lane 9). Reversal in the order of virus exposure yielded the

same results (Figure 4, Lane 10). Although, increasing the viral titer 10-fold, to a ratio 1:10 (MOI=1.0 and 10.0) to the infections, the results were similar to equal titer (data not show). Our finding concluded that dengue and chikungunya virus co-infection results in infection by both viruses, no matter the order, with only one exception: co-infection with a lower CHIKV titer than DENV 3 results in a CHIKV decrease or disappearance. These data demonstrate that DENV 3 and CHIKV infections depend on the amount of virus titer and not infection order. Thus, the two viruses can potentially infect host cells concurrently, as long as the viral concentration is high enough (Table 1).

Table 1 The results of dengue and chikungunya virus infected C6/36 cell cultures. The secretion fluids were evaluated by D-RT-PCR after 7 days' post-infection.

Viral infection in *Aedes albopictus* (C6/36) cell	MOI*	D-RT-PCR	
		DENV	CHIKV
DENV 3	1.0	+	-
CHIKV	1.0	-	+
DENV 3 + CHIKV	1.0 + 1.0	+	+
DENV 3 higher than CHIKV	1.0 + 0.1	+	-
DENV 3 lower than CHIKV	0.1 + 1.0	+	+
DENV 3 following with CHIKV	1.0, 1.0	+	+
CHIKV following with DENV 3	1.0, 1.0	+	+

* The multiplicity of infection (MOI) is the ratio of infectious agents (e.g. virus or phage) to infection targets (e.g. cell)

+ Positive results, - Negative results

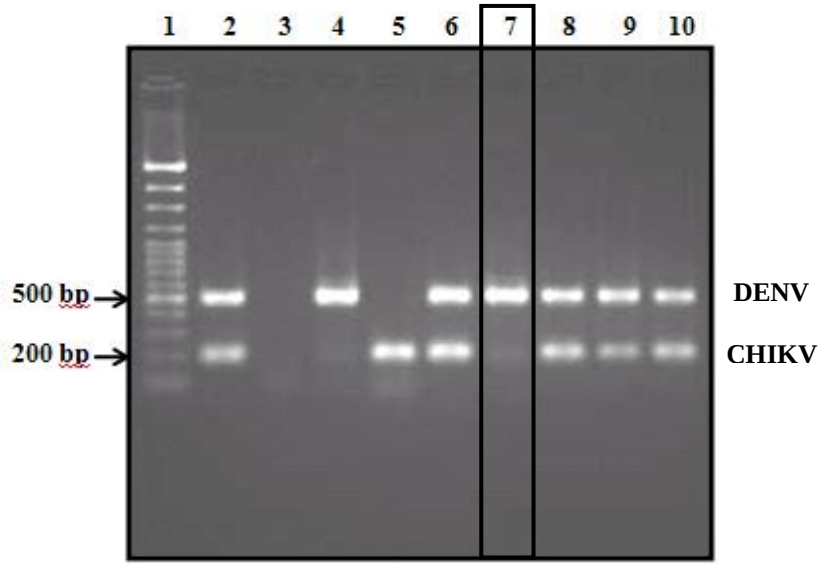

Figure 4 The positive results of mixed dengue and chikungunya mixed-infection was evaluated by D-RT-PCR. Lane 1: 100 bp DNA ladder (Fermentus), Lane 2: Positive control dengue and chikungunya virus, Lane 3: negative control, Lane 4: single infection with DENV 3 virus, Lane 5: single infection with CHIKV, Lane 6: Mixed infection, with equal titer of DENV 3 and CHIKV, Lane 7: Mixed infection, with higher dengue-virus titer than CHIKV, Lane 8: Mixed infection with lower dengue-virus titer than CHIKV, Lane 9: super infection with DENV 3 following CHIKV, Lane 10: super infection with CHIKV following DENV 3. All samples were separated by 1% agarose gel electrophoresis.

3.4 Dengue infection rate from field captured mosquitoes

RNA extracted from *Ae. aegypti* larval and adult pools was processed for dengue detection by multiplex RT-PCR using dengue serotype-specific primers. The PCR products generated were analyzed by agarose gel electrophoresis to visualize the characteristic band sizes

of 482 bp (DENV 1), 119 bp (DENV 2), 290 bp (DENV 3), 392 bp (DENV 4) and co-existence of more serotypes against positive controls as shown in Figure 5A and Figure 5B, respectively.

A **B**

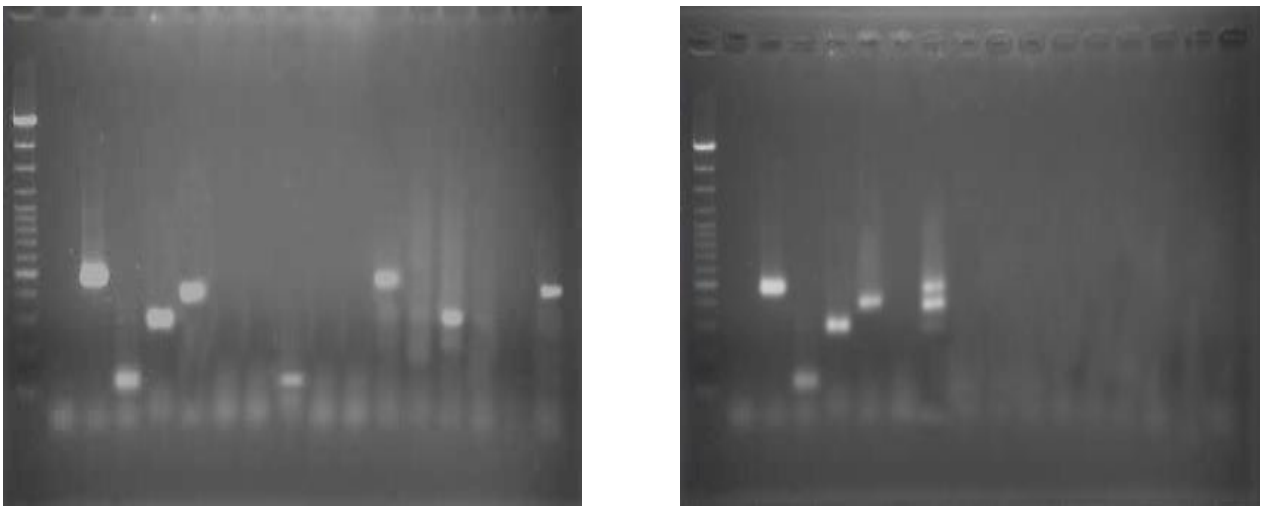

Figure 5 Agarose gel analysis of the PCR products generated by multiplex RT-PCR assay. (A.) Amplification of DNA amplicons reverse-transcribed from dengue RNA extracted from *Ae. aegypti* pools, M = DNA marker, Lane 1: negative control (uninfected *Ae. aegypti* RNA), Lane 2: positive control (DENV 1 = 482 pb), Lane 3: positive control (DENV 2 = 119 bp), Lane 4: positive control (DENV 3 = 290 bp), Lane 5: positive control (DENV 4 = 392 bp), Lane 8: sample positive for DENV 2, Lane 11: sample positive for DENV 1, Lane 13: sample positive for DENV 3, Lane 16: sample positive for DENV 4, Lanes 6,7,9,10,12,14 and 15: samples negative for dengue viruses. (B.) Co-existence of two dengue serotypes found in individual pools, M = DNA marker, Lane 1: negative control, Lane 2: positive control (DENV 1), Lane 3: positive control (DENV 2), Lane 4: positive control (DENV 3), Lane 5: positive control (DENV 4), Lane 7: sample positive for DENV 1 and DENV 4, Lanes 6 and 8-16: samples negative for dengue viruses.

3.5 Seasonal dengue infection in *Ae. aegypti* females

Ae. aegypti female pools grouped by season and province were detected for dengue infection. In Figure 6, data showed that the seasonal dengue infection rates in the female mosquitoes collected from Nakhon Pathom, Nonthaburi, Ratchaburi and Samut Sakhon were highest in hot season with 77.5% (31/40 pools), 75.5% (77/102 pools), 70% (7/10 pools), and 64.4% (29/45 pools), respectively. Followed by in winter in Nakhon Pathom, Samut Sakhon and Ratchaburi, the infection rates were 31.6% (30/95 pools), 29.4% (15/51 pools), 14.3% (6/42 pools) ,while those in rainy season were 29.4% (82/279 pools), 25% (31/124 pools) and 8% (9/113 pools), respectively. Only in Nonthaburi, the infection rate in rainy season with 49.5% (53/107 pools) was higher than that in winter with 11.8% (10/85 pools).

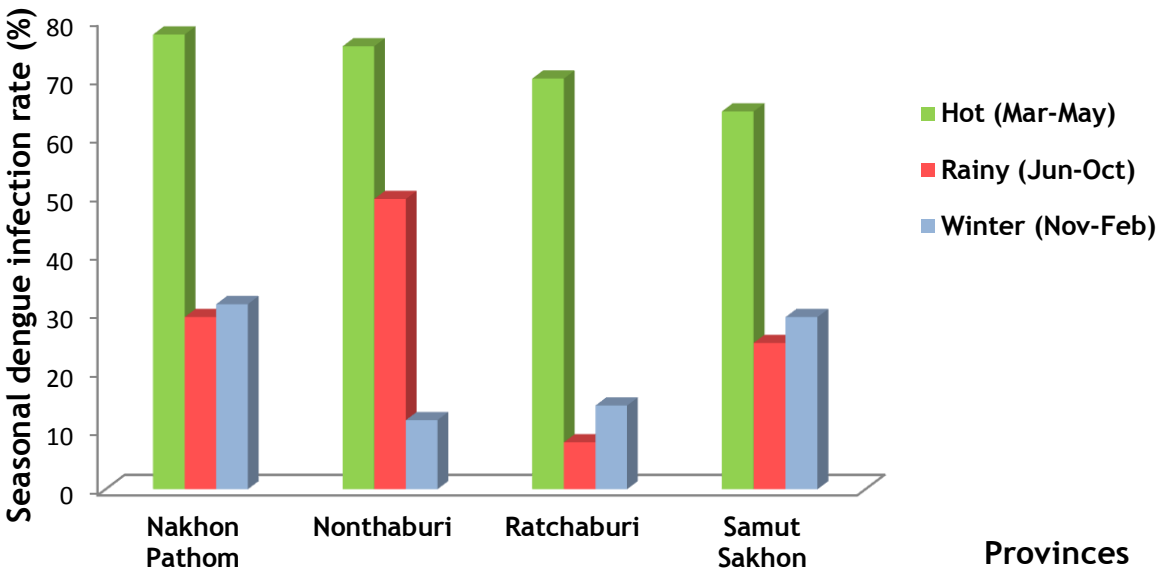

Figure 6 The dengue infection in *Ae. aegypti* females collected in each season of the 4 study provinces. All female mosquito pools separated by season and province were determined for dengue infection rate.

3.6 Transovarial dengue transmission in *Ae. aegypti* larvae and males

All *Ae. aegypti* larvae or males collected from each province were demonstrated for the event of transovarial dengue infection; the infected female mosquitoes transmitted dengue viruses to their offspring via the eggs. It revealed that the occurrence of transovarial dengue transmission was found indeed in both local *Ae. aegypti* larvae and males of all the 4 provinces investigated (Figure 7). The transovarial dengue infection rates in the mosquito larvae obtained from Nakhon Pathom, Nonthaburi, Samut Sakhon, and Ratchaburi were 46.9% (99/211 pools), 45.0% (143/318 pools), 31.8% (49/154 pools), and 18.3% (11/60 pools), respectively. In adult males, the dengue infection rates gained from Nonthaburi, Samut Sakhon, Nakhon Pathom and Ratchaburi were 46.3% (57/123 pools), 39.4% (71/180 pools), 37.7% (85/225 pools), and 12.0% (14/117 pools), respectively.

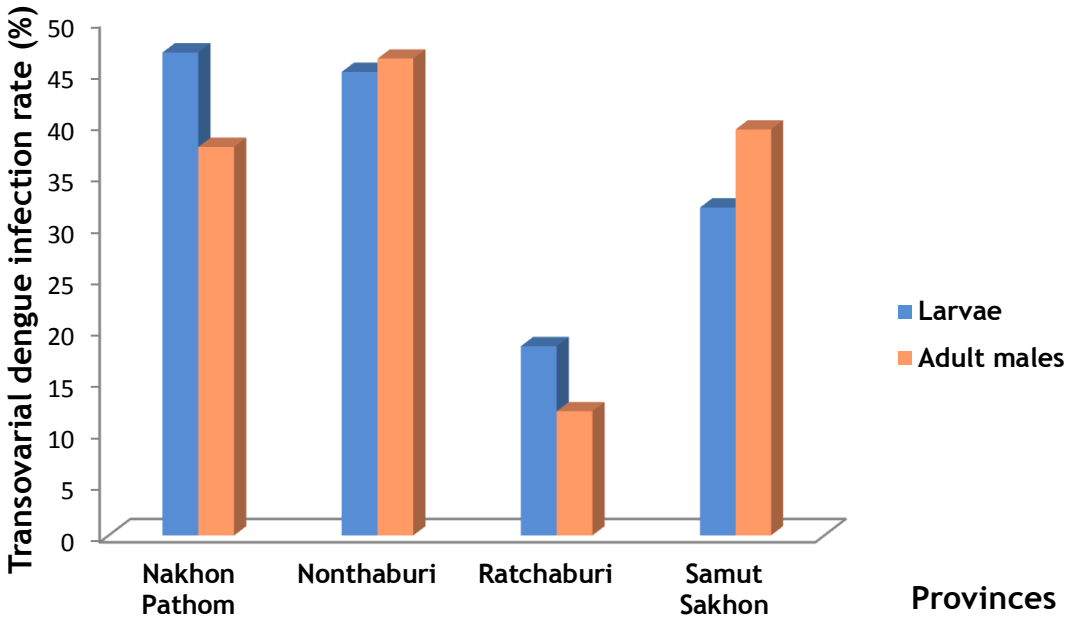

Figure 7 The transovarial dengue transmission in *Ae. aegypti* larvae and adult males collected from the 4 study provinces. All of the larval and adult male pools positive for dengue viruses from each province was determined for the transovarial dengue infection rate.

3.7 Serotyping of dengue viruses in *Ae. aegypti* and DHF patients

All larval and mosquito pools collected from all the 4 study provinces and found positive for dengue viruses were grouped into 5 categories: 4 dengue serotypes and coexistence of more serotypes. The result showed that all four serotypes of dengue viruses were detected in both *Ae. aegypti* larvae and adults in all provinces examined. Of 2,798 pools tested, 1,047 pools were positive for dengue viruses. Among four serotypes presented, DENV 3 and DENV 1 were the two most prevalent serotypes with the frequencies of 17.0% (178 pools) and 16.9% (177 pools),

followed by DENV 2 and DENV 4 with the frequencies of 14.7% (154 pools) and 8.2% (86 pools) as shown in Figure 8. In separated positive pools, there was 43.2% of co-existence of more serotypes found in individual pools. It revealed that the multiple dengue serotypes are circulating in a dwelling. Of 908 blood specimens taken from suspected patients admitted in all those provinces confirmed for dengue serotypes, only 415 suspected patients were positive for dengue serotypes, The proportion of each serotype showed that DENV1 and DENV 3 were the two most predominant serotypes with the frequencies of 51.3% (253 pools) and 28.8% (142 pools), followed by DENV 2 and DENV 4 with the frequencies of 14.4% (71 pools) and 5.5% (27 pools). There was no double or multiple dengue infection reported among all those serotyped DHF patients (Figure 9).

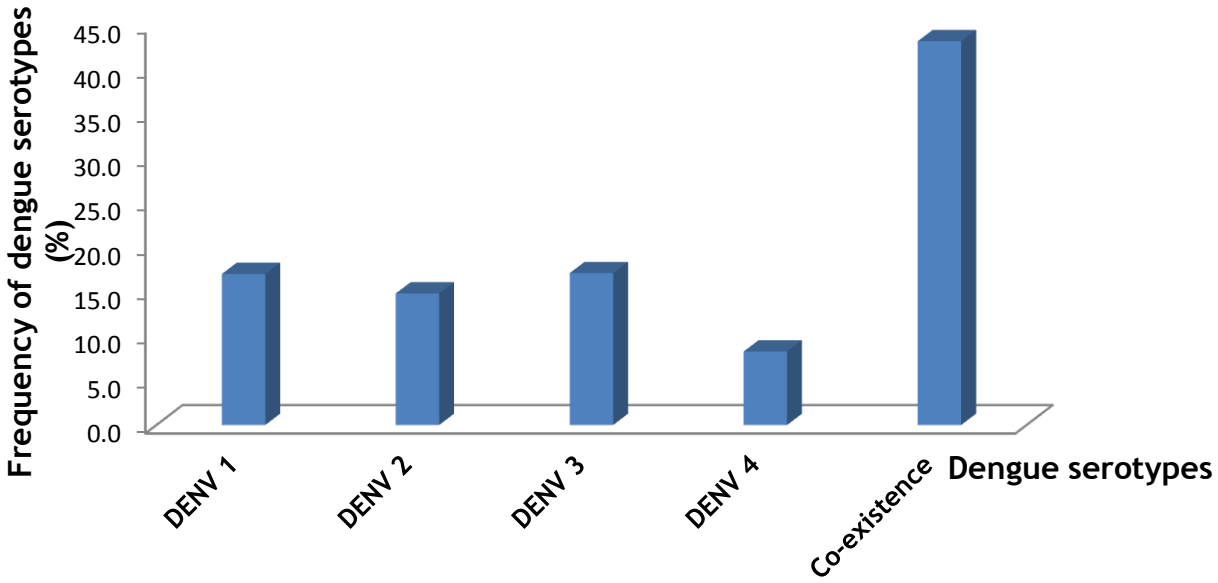

Figure 8 Serotyping of dengue viruses in *Ae. aegypti* in the 4 study provinces.

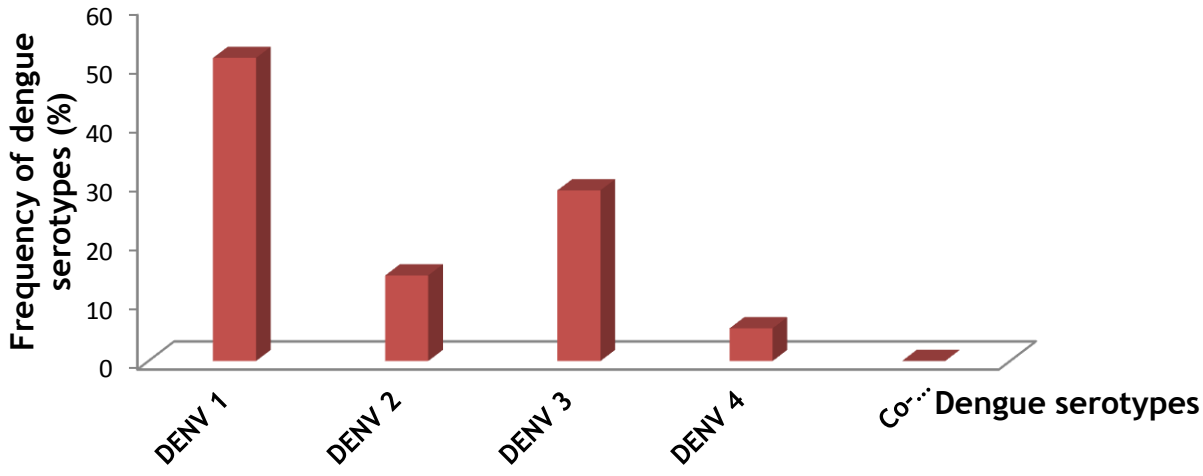

Figure 9 Serotyping of dengue viruses in patients with suspected dengue in the 4 study provinces.

3.8 Seasonal biting activity of *Ae. aegypti* females

The study of seasonal biting activity was carried out once a month for 12 months. Adult mosquitoes emerged from pupal addition to earthen jars were captured over 24-hour period. The *Ae. aegypti* females collected in each hour were pooled with those captured in the same hour of other months by the same season for the determination of seasonal biting rate. The data obtained from 3 seasons revealed that the highest biting activity occurred in hot season with the biting rate of 30 mosquito/person/hour, followed by in rainy season and in winter with the biting rates of 19.8 and 15.8 mosquito/person/hour (Figure 10). The peak of biting activity in hot season was present in the afternoon at 14.00-15.00 h, while that in rainy season and winter happened in the morning at 08.00-09.00 h and 09.00-10.00 h, respectively. In all three seasons, the biting activity decreases after sunset at 18.00-19.00 in winter and delayed an hour at 19.00-20.00 h in hot and

rainy season. An increase in biting activity started up again after sunrise at 06.00-07.00 h in hot and rainy seasons and deferred an hour at 07.00-08.00 h in winter.

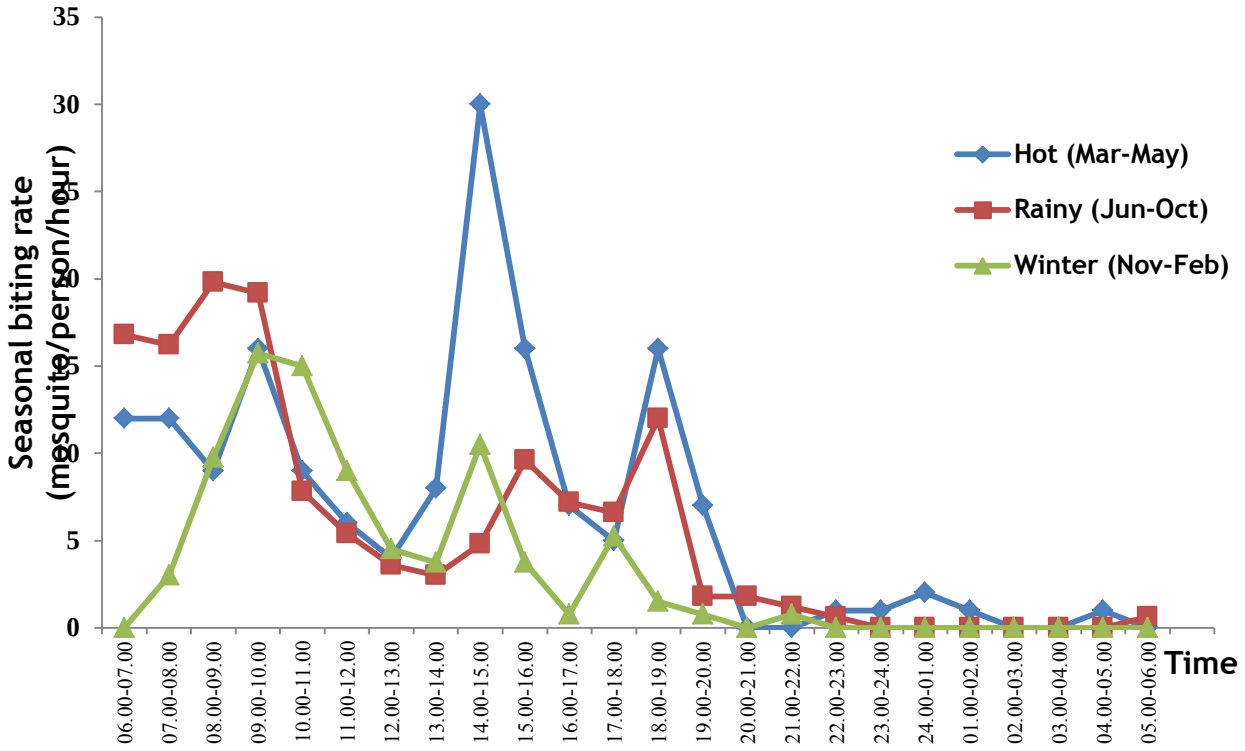

Figure 10 Seasonal biting activity of *Ae. aegypti* females. The experiment was conducted once a month throughout 12 months during March 2007 and February 2008.

DHF cases reported in various districts of the study areas in 2007 were expressed as morbidity rate per 100,000 populations. As shown in Figure 11, the morbidity rates of DHF in all the 4 study provinces were highest in rainy season with 129.3 (Nonthaburi), 123.8 (Nakhon Pathom), 115 (Ratchaburi) and 107.3 (Samut Sakhon). Following that, the morbidity rate of DHF

among those provinces appeared to be low in winter and hot season with less than 50 per 100,000 populations.

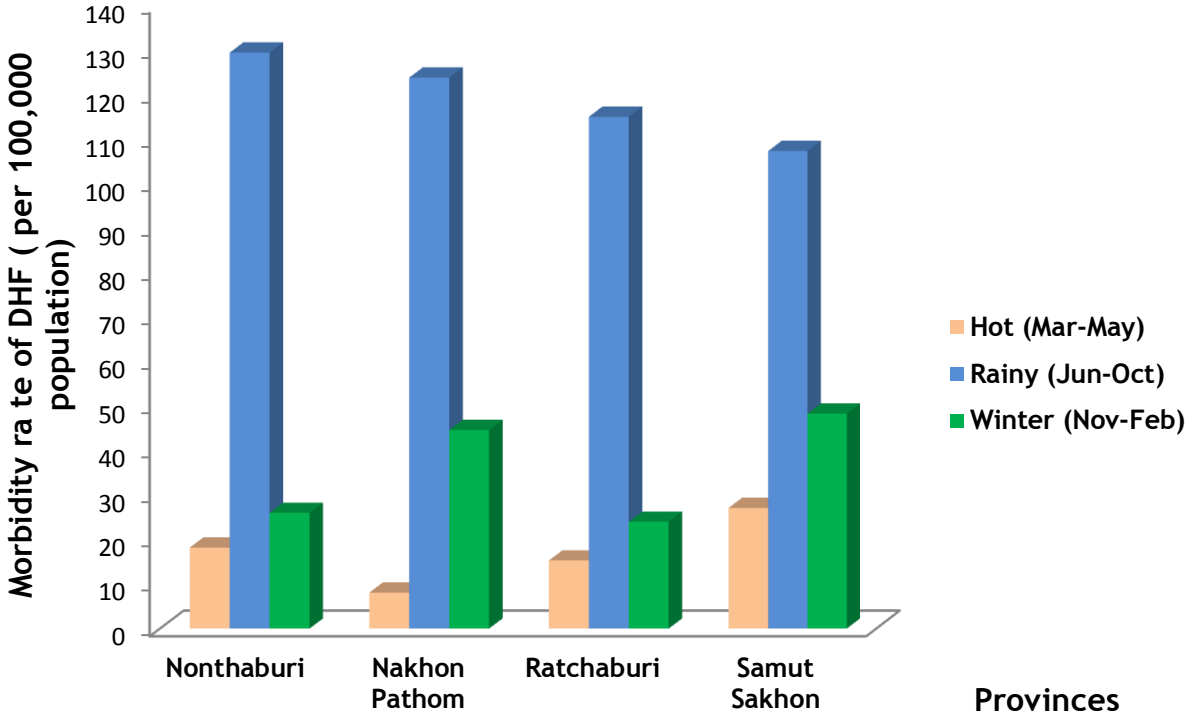

Figure 11 Incidence of DHF in the study areas of the 4 provinces**.** DHF cases reported in the study areas of various provinces were expressed as morbidity rate per 100,000 populations.

CHAPTER IV

DISCUSSION

4.1 Determination of viral production from super infection on C6/36 cell cultures

The results are the first reported that illustrated of co-infection between DENV 3 and CHIKV which derived from field caught mosquito vectors in Thailand. We demonstrated single infection of DENV 3 or CHIKV in C6/36 cell line which showed the 80% cytopathic effect (CPE) (data not shown), the viral production are increased until 7 day post-infection that similar to previously reported by Sakoonwatanyoo *et al*. (2006). Mixed-infection sample between DENV 3 and CHIKV with equal titer or variable titer of the virus by using higher concentration of CHIKV (MOI=1.0) than DENV 3 (MOI=0.1), also demonstrated both viruses in the supernatant. The D-RT-PCR technique was used to determine the virus production by detecting the *C-prM* gene from DENV 3 and *E1* gene of CHIKV respectively (Figure 4). The sensitivity of this assay was found to be better than conventional virus isolation and could detect as low as 100 copies of genomic RNA, which is equivalent to respective virus-specific RT-PCR (Dash *et al*, 2008). The benefit of this assay could also be able to detect dual infection of CHIK and DENV in one reaction. We applied this technique to differential diagnosis of these 2 infections in one culture. The results were also similar to previously reported. In addition, plaque assay was performed in order to determine whether the infectious viral particles were presented in the samples of both positive and negative for D-RT-PCR. The results of positively D-RT-PCR were demonstrated in plaque detection and the negatively D-RT-PCR sample has unseen on plaque assay.

The competitive suppression were presented in the sample of mixed infection which using higher titer DENV 3 (MOI=1.0) than CHIKV (MOI=0.1). Only 490 bp of DENV 3 was

detected while CHIKV RNA was not able to detect by D-RT-PCR (Figure 4), and plaque assay before viral infection was available and the number of infectious particles was compared to single infection was decreased. In contrast to other experiments (equal titer of both viruses, DENV 3 lower titer than CHIKV and super infection) both viruses were detected by D-RT-PCR. These findings may be implied that DENV 3 has highly competitive infection or it can suppress CHIKV replication. Sakoonwatanyoo *et al*. (2006) mentioned that specific receptors of DENV 3 were presented in C6/36 cell and enrolled for the virus to enter into the insect cells. Competition between DENV 3 and CHIKV in our experiments would relate to the receptors of the insect cell. There are two important glycoproteins with molecular mass 80 and 67 kDa that are specific to the DENV 2 (María de Lourdes Muñoz *et al*, 1998). These glycoproteins are presented in midgut of *Ae. aegypti* mosquito and *Ae. albopictus* cell line. Previously report showed difference proteins binding between DENV and CHIKV (Mourya *et al*, 1998; Tio *et al*, 2005; Mercado-Curiel *et al*, 2006). Nevertheless, the transcriptional and mechanism of them are not clearly understood (Mercado-Curiel *et al*, 2006). In the natural situations, surveys of the dengue vectors by Thavara *et al* (2006), more than one dengue serotypes were detected in one mosquito vector, even though it did not uptake the virus at the same time (Thavara *et al*, 2006).

Asymmetric competitive suppression between two types of virus (DENV 2 and DENV 4) has been demonstrated in co-infection and super infection experiments in mosquito cell line. The results reported that infectious DENV 2 were higher suppression than DENV 4 in mixed infectious trial (Hanley *et al*, 2008). These results were supposed to intra-host competition among different strains of DENV. The competitive suppression from our experiment showed that DENV has higher concentration than CHIKV and these results might be used to describe how reemergence outbreak between DENV and CHIKV were occurred in co-circulation area. DENV

and CHIKV are transmitted by the same mosquito species and viral transmissions are related to vector competence. Degree of transmission is related to vector competence and extrinsic or intrinsic factors in mosquito (Beerntsen *et al*, 2000). One of the extrinsic factor is the viral concentration, when mosquitoes infected by two viruses at the same time, such as DENV has a higher concentration could be able to suppress CHIKV replication in the mosquitoes. The intrinsic factors of mosquito vectors competence would relate to specific receptors for the virus to enter the mosquito cells as described previously.

CHIKV showed a lower yielded when compared with co-infection or single infection treatments which demonstrates in Figure 4, Lane 9 and 10 (Richards *et al*, 2010; van den Hurk AF *et al*, 2010). These results are similar to previously reported which super infection between DENV 2 and DENV 4 was found lower yield compare with single infection control (Pepin *et al*, 2008). The results are supported about asymmetric competitive suppression. Because of CHIKV cannot be replicated in the high concentration of DENV 3 sample which is due to intrinsic or extrinsic factor of these virus and host cell.

In conclusion, our findings might be applied to predict the evolutionary epidemiology of medically important viruses and supported the information of silencing outbreaks. The competitive suppression is related to decrease virus transmissions while mixed infection and super infection were supported two virus co-circulations in endemic area. These results are supported to field mosquitoes vector that mosquito vector have a chance to transmission two types of virus by one mosquito. Thus, to prevent viral transmission we should be avoid mosquito bites and elimination of mosquito breeding sites because unavailable vaccination of both virus (Hanley *et al*, 2008). Further study is required to determine how to control and treat chikungunya and dengue virus within the Thai population.

4.2 Laboratory induced DENV infection in *Ae. aegypti* mosquitoes and Field studies

In experiment 2, the study of DENV 1 and DENV 2 inhibition in mosquito vectors were determined by using RT-PCR. The percentage of DENV 1 and DENV 4 transmission was calculated as 47.5 (19/40) and 35(14/40) respectively. These are likely to both dengue strain co-infected in mosquitoes dependently or both of them may not directly effect on each other of which are may not involve the competition inhibit between DENV 1 and DENV 2 to enter the mosquito cell via the cell specific report (Potiwat *et al*, 2011)

Virological observation of dengue viruses in field caught *Ae. aegypti* populations has been suggested for prediction of the dengue outbreak (Urdaneta *et al*, 2005; Chen *et al*, 2010). However, in appearance of the disease epidemic, the environmental factors such as temperature, rainfall and humidity affecting dengue transmission should be considered as well (Ooi and Gubler, 2009). As shown in our study, the highest dengue infection rates in *Ae. aegypti* females collected from all the 4 provinces were obtained in hot season. It can be explained that a moderately high temperature in environment may influence on vector efficiency and dengue virus propagation. Scott *et al* (2000) reported that temperature involves in transition rate from larva to pupa and pupa to young adult because it accelerates the development rate of *Ae. aegypti*. A warmer temperature may allow mosquito vectors to adapt themselves, survive in unsuitable conditions and reach maturity more rapidly. Consequently, mosquito biology is presumably changed by size reduction of mosquito larvae that develop smaller adults with high metabolism rates, more frequent blood feeding, and more often egg-laying (Jetten and Focks, 1997; Barbazan *et al*, 2002; McMichael *et al*, 2007). Besides, the environmental temperature made an obvious effect on the extrinsic incubation period (EIP) of arboviruses in their vectors (Lindsay and Mackenzie, 1997; McMichael *et al*. 2007). The increase in environmental temperature can

reduce the length of viral extrinsic incubation periods in mosquito vectors (Harrington *et al*, 2001; Keating, 2001). As reported by Watts *et al* (1987), only mosquitoes infected with high dose of DENV 2 and incubated at 30°C with 12-day EIP, and at 32-35°C with 7-day EIP can transmit the virus to monkeys but no viral transmission by mosquitoes maintained at 26°C. In addition, a 5-day decrease in EIP resulted in a three-fold higher transmission rate of dengue (Koopman *et al*, 1991).

In theory, it mentioned that high temperatures should accelerate biting and egg laying activities of mosquitoes (Reiter, 2001). Our result indicated that it is possible due to highest biting rate of *Ae. aegypti* females obtained in hot season. In fact, the frequency in mosquito biting until complete feed is unknown and could be 2 or more from interrupted feeding attempts with resumption on the same or different host (Focks *et al*, 1995). The warmer temperature with 2 or 3 degree induced doubling of the expected number of replete feeds which is equivalent to a doubling of *Ae. aegypti* density (Focks and Barrera, 2006). Therefore, the mosquito vectors feeding on infected human multiply or the infected mosquitoes biting human more frequently can enhance the natural cycle of dengue disease involving human-mosquito-human transmission. However, a morbidity rate of DHF reported in our study areas was highest in rainy season which populations of the mosquito females peaked each year during May-June in Thailand (Scott *et al*, 2000). The peak for DHF cases occurred by about 2 months after the peak for *Ae. aegypti* populations (Halstead, 1966). Additionally, our results supported the former observations on the seasonal feeding pattern of *Ae. aegypti* suggesting that annual DHF epidemics was more likely the result of increased frequency of feeding on humans during the hot-dry and rainy seasons (Yasuno and Pant, 1970; Pant and Yasuno, 1973). This study revealed that warmer temperature in hot season was positively associated with high biting rate of *Ae. aegypti* mosquitoes.

As well-known, *Ae. aegypti* females feeding on viremic humans in acute phase of infection become infected with dengue viruses. Subsequently, the mosquitoes maintain the viruses for life and transmit them to susceptible individuals (WHO, 1998). More efficiently, the infected female mosquitoes are able to pass on the viruses to their offspring through the eggs as demonstrated in *Ae. aegypti* larvae and adult males both infected experimentally and collected from the field (Joshi *et al*, 2002; Usavadee *et al*, 2006; Thongrungkiat *et al*, 2011). Therefore, we demonstrated this phenomenon and found the occurrence of transovarial transmission of dengue virus in local *Ae. aegypti* collected from all the 4 study provinces. It elucidated that there have been the transovarial maintenance of dengue viruses in those endemic areas. This vertical transmission may be one of the factors that provide a mechanism to viruses to survive in dry season and winter with low mosquito populations (Rosen *et al*, 1983). Strikingly, the transovarially infected mosquitoes are also capable of transmitting the virus through the bite (Mourya *et al*, 2001). Hence, the transovarial transmission of dengue virus is an important phenomenon for retention of the viruses during inter-epidemic periods

Determination of dengue serotypes in *Ae. aegypti* mosquitoes could be used as an important surveillance indicator to investigate the epidemiological situation of DHF (Kow *et al*, 2001; Mendez *et al*, 2006; Chen *et al*, 2010). Our study showed that all four dengue serotypes were found in the *Ae. aegypti* larvae and adults in which DENV 3 and DENV 1 were the two most prevalent serotypes. Serotyping of dengue viruses in blood specimens of DHF patients showed similar result as obtained in mosquitoes that DENV 1 and DENV 3 were the two most predominant among those four serotypes. Additionally, it was found that there has been co-existence of more serotypes of dengue viruses circulating in the same household of the study areas. Previous work reported that the occurrence of multiple infections with different DENV

serotypes was possible in regions of hyperendemicity (Mackenzie *et al*, 2004). As reported by Gubler *et al* (1985), there was a case of natural concurrent infection with DENV 1 and DENV 4 with mild symptoms during a 1982 outbreak in Puerto Rico. However, a more severe symptom of dengue disease, DHF, is caused by secondary infection with a different dengue serotype (Halstead *et al*, 1970; Vaughn *et al*, 2000; Nisalak *et al*, 2003) due to antibody-dependent enhancement of disease (Porterfield, 1986). The understanding of the presence of multiple serotypes of dengue viruses co-circulating in those endemic areas can ignite public awareness for people who have never experienced dengue infection or were previously infected with different serotypes from those circulating in their investigated dwellings. Moreover, it is also essential to perceive the dengue infection history of the patients confirmed by serotyping techniques. Taken together with the use of data on dengue infection in the mosquitoes, these can be considerable data in forecasting future dengue outbreaks

Since no specific treatment or effective vaccine against all four serotypes of dengue virus is currently available. Preventive measures have emphasized vector control and personal protection measures. However, public health practitioners still lack adequate knowledge on mosquito biology and ecology which is necessary to operate the control of mosquito vector efficiently. For personal and household protection, the commercial products have been less used in response to any public health concerns, but employed to mitigate the nuisance of biting mosquitoes. Furthermore, an insufficiency of data on dengue infection and biting behavior of mosquito vector is an important reason that causes failure of dengue disease control. In this study, it provided the valuable data on vector control that revealed highest dengue infection rate and biting rate of *Ae. aegypti* females found in hot season including the occurrence of transovarial transmission found in all the 4 study provinces. These are beneficial to public health

practitioners in planning properly for vector control, environmental management and source reduction at the appropriate time for prevention or diminution of dengue outbreak usually occurred during the rainy season. The data obtained from our study could be used as an early warning monitoring system for dengue outbreaks and for the detection of newly introduced virus serotype in endemic areas. However, the use of only one or two control measures will not achieve in dengue disease control effectively, the integrated control measures should be taken into account and practiced regularly for the sustainable dengue disease control.

REFERENCES

Auksornkitti V, Pongsiri P, Theamboonlers A, *et al*. Whole-genome characterisation of Chikungunya virus from *Aedes albopictus* collected in Thailand. Ann Trop Med Parasitol 2010; 104: 265-9.

Barbazan P, Yoksan S, Gonzalez JP. Dengue hemorrhagic fever epidemiology in Thailand: description and forecasting of epidemics. Microbes Infect 2002; 4: 699–705.

Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. Microbiol Mol Biol Rev 2000; 64: 115-37.

Chahar HS, Bharaj P, Dar L, Guleria R, Kabra SK, Broor S. Co-infections with chikungunya virus and dengue virus in Delhi, India. Emerg Infect Dis 2009; 15: 1077-80.

Chanama C, Anantapreecha S, A-nuegoonpipat A, *et al*. Analysis of specific IgM responses in secondary dengue virus infections: levels and positive rates in comparison with primary infections. J Clin Virol 2004; 31: 185-9.

Chen CF, Shu PY, Teng HJ, *et al*. Screening of dengue virus in field-caught *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) by one-step SYBR green-based reverse transcriptase-polymerase chain reaction assay during 2004-2007 in Southern Taiwan. Vector Borne Zoonotic Dis 2010; 10: 1017-25.

Dash PK, Parida M, Santhosh SR, *et al*. Development and evaluation of a 1-step duplex reverse transcription polymerase chain reaction for differential diagnosis of chikungunya and dengue infection. Diagn Microbiol Infect Dis 2008; 62: 52-7.

Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux AB. Chikungunya virus and Aedes mosquitoes: saliva is infectious as soon as two days after oral infection. PLoS ONE 2009; 4: e5895.

Ezzedine K, Cazanave C, Pistone T, *et al*. Dual infection by chikungunya virus and other imported infectious agent in a traveller returning from India. Travel Med Infect Dis 2008; 6: 152-4.

Focks DA, Daniels E, Haile DG, *et al*. A simulation model of the epidemiology of urban dengue fever: literature analysis, model development, preliminary validation, and samples of simulation results. Am J Trop Med Hyg 1995; 53: 489-506.

Focks DA and Barrera R. Dengue Transmission Dynamics: Assessment and Implications For Control. Working paper for the Scientific Working Group on Dengue Research, convened by the Special Programme for Research and Training in Tropical Diseases, Geneva, http://www.who.int/tdr/publications/publications/swg_dengue_2.htm

Fried JR, Gibbons RV, Kalayanarooj S, *et al*. Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. PLoS Negl Trop Dis 2010; 4: e617.

Gubler DJ, Kuno G, Sather GE, *et al*. A case of natural concurrent human infection with two dengue viruses. Am J Trop Med Hyg 1985; 34: 170-3.

Halstead SB. Epidemiological studies of Thai haemorrhagic fever, 1962-64. Bull World Health Org 1966; 35: 80-1.

Halstead SB, Nimmannitya S, Cohen SN. Observations related to pathogenesis of dengue haemorrhagic fever IV. Relation of diseases severity to antibody response and virus recovered. Yale J Biol Med 1970; 42: 311-28.

Hanley K, Nelson J, Schirtzinger E, Whitehead S, Hanson C. Superior infectivity for mosquito vectors contributes to competitive displacement among strains of dengue virus. BMC Ecology 2008; 8: 1.

Harrington LC, Harrington JP, Buonaccorsi JD, *et al*. Analysis of survival of young and old *Aedes aegypti* (Diptera: Culicidae) from Puerto Rico and Thailand. J Med Entomol 2001; 38: 537–47.

Ho K, Ang LW, Tan BH, *et al*. Epidemiology and control of chikungunya fever in Singapore. J Infect 2011; 62(4):263-70.

Hung SL, Lee PL, Chen HW, Chen LK, Kao CL, King CC. Analysis of the steps involved in Dengue virus entry into host cells. Virology 1999; 257: 156-67.

Jetten TH and Focks DA. Potential changes in the distribution of dengue transmission under climate warming. Am J Trop Med Hyg 1997; 57: 285-97.

Joshi V, Mourya DT, Sharma RC. Persistence of dengue-3 virus through transovarial transmission passage in successive generations of Aedes aegypti mosquitoes. Am J Trop Med Hyg 2002; 67: 158-61.

Keating J. An investigation into the cyclical incidence of dengue fever. Soc Sci Med 2001; 53: 1587–97.

Koopman JS, Koopman DR, Prevots MA, *et al*. Determinants and predictors of dengue infection in Mexico. Am J Epidemiol 1991; 133: 1168–78.

Kow CY, Koon LL, Yin PF. Detection of dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Singapore by type-specific PCR. J Med Entomol 2001; 38: 475-9.

Kukreti H, Chaudhary A, Rautela RS, *et al*. Emergence of an independent lineage of dengue virus type 1 (DENV-1) and its co-circulation with predominant DENV-3 during the 2006 dengue fever outbreak in Delhi. Int J Infect Dis 2008; 12: 542-9.

Lanciott R, Calisher C, Gubbler DJ, *et al*. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992; 30: 545-51.

Leroy EM, Nkoghe D, Ollomo B, *et al*. Concurrent Chikungunya and Dengue Virus Infections during Simultaneous Outbreaks, Gabon, 2007. Emerg Infect Dis 2009; 15: 591-3.

Lindsay M and Mackenzie J. Vector-borne viral diseases and climate change in the Australian region: major concerns and the public health response in Climate changes and human health in the Asia-Pacific region. Australian Medical Association (1997); 47–62.

Mackenzie JS, Gubler DJ, Petersen LR. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nature Med 2004; 10: 98–109.

Mavale M, Parashar D, Sudeep A, *et al*. Venereal transmission of chikungunya virus by *Aedes aegypti* mosquitoes (Diptera: Culicidae). Am J Trop Med Hyg 2010; 83: 1242-4.

McMichael AJ, McMichael A. Haines R, *et al*. Climate changes and human health, World Health Organization, Geneva, 1996. World Academy of Science, Engineering and Technology 2007; 33.

Méndez F, Barreto M, Arias JF, *et al*. Human and mosquito infections by dengue viruses during and after epidemics in a dengue-endemic region of Colombia. Am J Trop Med Hyg 2006; 74: 678-83.

Mercado-Curiel RF, Esquinca-Aviles HA, Tovar R, Diaz-Badillo A, Camacho-Nuez M, Munoz Mde L. The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells. BMC Microbiol 2006; 6: 85.

Mourya DT, Ranadive SN, Gokhale MD, Barde PV, Padbidri VS, Banerjee K. Putative chikungunya virus-specific receptor proteins on the midgut brush border membrane of *Aedes aegypti* mosquito. Indian J Med Res 1998; 107: 10-4.

Mourya DT, Gokhale, Basu A, Barde PV, Sapkal GN, Padbidri VS, Gore MM. Horizontal and vertical transmission of dengue-2 virus in highly susceptible strains of *Aedes aegypti* mosquitoes. Acta Virol 2001; 45(2): 67-71.

Muñoz ML, Cisneros A, Cruz J, Das P, Tovar R, Ortega A. Putative dengue virus receptors from mosquito cells. FEMS Microbiol Lett 1998; 168: 251-8.

Nayar SK, Noridah O, Paranthaman V, et al. Co-infection of dengue virus and chikungunya virus in two patients with acute febrile illness. Med J Malaysia 2007; 62: 335-6.

Nimmannitya S, Halstead SB, Cohen SN, Margiotta MR. Dengue and Chikungunya Virus Infection in Man in Thailand, 1962-1964: I. Observations on Hospitalized Patients with Hemorrhagic Fever. Am J Trop Med Hyg 1969; 18: 954-71.

Nisalak A, Endy TP, Nimmannitya S, *et al*. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg 2003; 68: 191-202.

Ooi EE and Gubler DJ. Global spread of epidemic dengue: the influence of environmental change. Future Virol 2009; 4(6), 571-80.

Pant CP and Yasuno M. Field studies on the gonotrophic cycles of *Aedes aegypti* in Bangkok, Thailand. J Med Entomol 1973; 10: 219-23.

Pardigon N. The biology of chikungunya: A brief review of what we still do not know. Pathologie Biologie 2009; 57: 127-32.

Pepin KM, Lambeth K, Hanley KA. Asymmetric competitive suppression between strains of dengue virus. BMC Microbiol 2008; 8: 28.

Pongsiri P, Auksornkitti V, Theamboonlers A, Luplertlop N, Rianthavorn P, Poovorawan Y. Entire genome characterization of Chikungunya virus from the 2008-2009 outbreaks in Thailand. Trop Biomed 2010; 27: 167-76.

Porterfield JS. Antibody-dependent enhancement of viral infectivity. Adv Virus Res 1986; 31: 335–55.

Raekiansyah M, Pramesyanti A, Bela B, *et al*. Genetic variations and relationship among dengue virus type 3 strains isolated from patients with mild or severe form of dengue disease in Indonesia and Thailand. Southeast Asian J Trop Med Public Health 2005; 36: 1187-97.

Reiter P. Climate change and mosquito-borne disease. Environ Health Perspect 2001; 109 (Suppl 1): 141-61.

Rianthavorn P, Prianantathavorn K, Wuttirattanakowit N, Theamboonlers A, Poovorawan Y. An outbreak of chikungunya in southern Thailand from 2008 to 2009 caused by African strains with A226V mutation. Int J Infect Dis 2010; 14 Suppl 3: e161-5.

Rianthavorn P, Wuttirattanakowit N, Prianantathavorn K, Limpaphayom N, Theamboonlers A, Poovorawan Y. Evaluation of a rapid assay for detection of IgM antibodies to chikungunya. Southeast Asian J Trop Med Public Health 2010; 41: 92-6.

Richards SL, Anderson SL, Smartt CT. Vector competence of Florida mosquitoes for chikungunya virus. J Vector Ecol 2010; 35: 439-43.

Rohani A, Potiwat R, Zamree I, Lee HL. Refractoriness of Aedes aegypti (Linnaeus) to dual infection with dengue and chikungunya virus. Southeast Asian J Trop Med Public Health 2009; 40: 443-8.

Rosen LD, Shroyer RB, Tesh JE, *et al*. Transovarial transmission of dengue viruses by mosquitoes: *Aedes albopictus* and *Aedes aegypti*. Am J Trop Med Hyg 1983; 32: 1108-19.

Sakoonwatanyoo P, Boonsanay V, Smith DR. Growth and production of the dengue virus in C6/36 cells and identification of a laminin-binding protein as a candidate serotype 3 and 4 receptor protein. Intervirology 2006; 49: 161-72.

Schilling S, Emmerich P, Günther S, Schmidt-Chanasit J. Dengue and Chikungunya virus co-infection in a German traveller. Journal of Clinical Virology 2009; 45: 163-4.

Scott TW, Morrison AC, Lorenz LH, *et al*. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: population dynamics. J Med Entomol 2000; 37: 77-88.

Simon F, Parola P, Savini H. Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. Med Clin North Am 2008; 92: 1323-43.

Sriprom M, Chalvet-Monfray K, Chaimane T, Vongsawat K, Bicout DJ. Monthly district level risk of dengue occurrences in Sakon Nakhon Province, Thailand. Sci Total Environ 2010; 408: 5521-8.

Tang Y, Rodpradit P, Chinnawirotpisan P, *et al*. Comparative analysis of full-length genomic sequences of 10 dengue serotype 1 viruses associated with different genotypes, epidemics, and disease severity isolated in Thailand over 22 years. Am J Trop Med Hyg 2010; 83: 1156-65.

Thavara U, Siriyasatien P, Tawatsin A, *et al*. Double infection of heteroserotypes of dengue viruses in field populations of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) and serological features of dengue viruses found in patients in southern Thailand. Southeast Asian J Trop Med Public Health 2006; 37: 468-76.

Thavara U, Tawatsin A, Pengsakul T, *et al*. Outbreak of chikungunya fever in Thailand and virus detection in field population of vector mosquitoes, *Aedes aegypti* (L.) and *Aedes albopictus* Skuse (Diptera: Culicidae). Southeast Asian J Trop Med Public Health 2009; 40: 951-62.

Thongrungkiat S, Maneekan P, Wasinpiyamongkol L, *et al*. Prospective field study of transovarial dengue-virus transmission by two different forms of *Aedes aegypti* in an urban area of Bangkok, Thailand. J Vector Ecol 2001; 36: 147-52.

Tio PH, Jong WW, Cardosa MJ. Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. Virol J 2005; 2: 25.

Tuksinvaracharn R, Tanayapong P, Pongrattanaman S, *et al*. Prevalence of dengue virus in Aedes mosquitoes during dry season by semi-nested reverse transcriptase-polymerase chain reaction (semi-nested RT-PCR). J Med Assoc Thai 2004; 87 (suppl 2): 129-33.

Ungchusak K and Kunasol P. Dengue haemorrhagic fever in Thailand. Southeast Asian J Trop Med Public Health 1988; 19: 487-90.

Urdaneta L, Herrera F, Pernalete M, *et al*. Detection of dengue viruses in field-caught *Aedes aegypti* (Diptera: Culicidae) in Maracay, Aragua state, Venezuela by type-specific polymerase chain reaction. Infect Genet Evol 2005; 5: 177-84.

van den Hurk AF, Hall-Mendelin S, Pyke AT, Smith GA, Mackenzie JS. Vector competence of Australian mosquitoes for chikungunya virus. Vector Borne Zoonotic Dis 2010; 10: 489-95.

Vaughn DW, Green S, Kalayanarooj S, *et al*. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. j infect dis 2000; 181: 2-9.

Vazeille M, Mousson L, Martin E, Failloux A-B. Orally Co-Infected Aedes albopictus from La Reunion Island, Indian Ocean, Can Deliver Both Dengue and Chikungunya Infectious Viral Particles in Their Saliva. PLoS Negl Trop Dis 2010; 4: e706.

Wangroongsarb Y. Dengue through schoolchildren in Thailand. Dengue Bull 1997; 21: 52-62.

Watts DM, Watts DS, Burke BA, *et al*. Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus. Am J Trop Med Hyg 1987; 36(1): 143-52.

WHO (1997) Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva.

WHO (2000) Scientific working group on dengue. Meeting Report 3–5 April, 2000 [online]<http://apps.who.int/tdr/publications/tdr-research-publications/swg-dengue/pdf/dengue-swg.pdf >(WHO, Geneva, Switzerland).

WHO (1998) World Health Organization Information Dengue and Dengue hemorrhagic fever. Revised November 1998 Fact sheet No 117-Hyperlink [Cited 2005 Sept 14]. Available from: URL: http://www.who.int/inf-fs/en/fact - http:www.who.int/inf-fs/en/fact_117.html

WHO (2011) Impact of Dengue. http://www.who.int/csr/disease/dengue/impact/en/

WHO (2007) Scientific Working Group Report on Dengue [online] (WHO, Geneva, Switzerland).

Yamamoto K, Matumoto K, Lim CK, Moi ML, Kotaki A, Takasaki T. Chikungunya fever from Malaysia. Intern Med 2010; 49: 501-5.

Yasuno M and Pant CP. Seasonal changes in biting and larval infestation rates of Aedes aegypti in Bangkok, Thailand, in 1969. WHO/VBC/70.200: 5P. (DPMIAC LOC: WHO SHELF).

Yenchitsomanus P, Sricharoen P, Jaruthasana I, *et al*. Rapid detection and identification of dengue viruses by polymerase chain reaction (PCR). Southeast Asian J Trop Med Public Health 1996; 27: 228-36.

Zhao R, Chinnawirotpisan P, Klungthong C, Zhang C, Putnak R. Evidence for inter- and intra-genotypic variations in dengue serotype 4 viruses representing predominant and non-predominant genotypes co-circulating in Thailand from 1977 to 2001. Virus Genes 2010; 41: 5-13.

PUBLICATIONS

1. Potiwat R, Komalamisra N, Thavara U, Tawatsin A, Siriyasatien P. Competitive suppression between chikungunya and dengue virus in *Aedes albopictus* c6/36 cell line. Southeast Asian J Trop Med Public Health. 2011;42(6):1388-1394.
2. Chompoosri J, Thavara U, Tawatsin A, Anantapreecha S, Siriyasatien P. Seasonal Monitoring of Dengue Infection in *Aedes aegypti* and Serological Feature of Patients with Suspected Dengue in 4 Central Provinces of Thailand. Thai J Vet Med. 2012;42(2):185-193.