Genetic Diversity of Dengue Virus in *Aedes aegypti* Mosquito at Ban Phaeo District, Samut Sakhon Province, Thailand

Mr. Veerayuth Kittichai

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2014

ความหลากหลายทางพันธุกรรมของเชื้อไวรัสเดงกีในยุงลายบ้าน ณ อำเภอ บ้านแพ้ว จังหวัดสมุทรสาคร

นายวีรยุทธ กิตติชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	Genetic Diversity of Dengue Virus in *Aedes aegypti* Mosquito at Ban Phaeo District, Samut Sakhon Province, Thailand
By	Mr. Veerayuth Kittichai
Field of Study	Medical Science
Thesis Advisor	Associate Professor Padet Siriyasatien, M.D., Ph.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Medicine
(Associate Professor Sophon Napathorn, M.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Wilai Anomasiri, Ph.D.)

.....Thesis Advisor
(Associate Professor Padet Siriyasatien, M.D., Ph.D.)

.....Examiner
(Associate Professor Parvapan Bhattarakosol, Ph.D.)

.....Examiner
(Chalisa Louicharoen Cheepsunthorn, Ph.D.)

.....External Examiner
(Usavadee Thavara, Ph.D.)

วีรยุทธ กิตติชัย : ความหลากหลายทางพันธุกรรมของเชื้อไวรัสเดงกีในยุงลายบ้าน ณ อำเภอ บ้านแพ้ว จังหวัดสมุทรสาคร (Genetic Diversity of Dengue Virus in *Aedes aegypti* Mosquito at Ban Phaeo District, Samut Sakhon Province, Thailand) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.เผด็จ สิริยะเสถียร, 113 หน้า.

อุบติการณ์ของโรคไข้เลือดออกของแต่ละพื้นที่มีแนวโน้มสูงขึ้นเรื่อยๆทุกปี การสำรวจทางระบาดวิทยาที่อาศัยข้อมูลด้านพลวัติทางวิวัฒนาการของเชื้อไวรัสเดงกีรวมถึงอาศัยข้อมูลด้านสิ่งแวดล้อมนั้น สามารถนำไปใช้ในการศึกษาแนวโน้มของการระบาดของโรค อันจะนำไปสู่การพยากรณ์และวางแผนควบคุมการระบาดของโรคได้ในอนาคต งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาวิเคราะห์วิวัฒนาการของไวรัสที่ระบาดในพื้นที่ระบาดสูงตามฤดูกาลและการกลายพันธุ์ของเชื้อดังกล่าวร่วมด้วย ซึ่งศึกษาทั้งในยุงลายบ้านของแต่ละฤดูกาลและในประชากรท้องถิ่นที่ อำเภอบ้านแพ้ว จังหวัดสมุทรสาคร เนื่องจากเป็นพื้นที่เฝ้าระวังที่มีรายงานของโรคไข้เลือดออกในอัตราสูงเป็นประจำของทุกๆปี และยังอยู่ในระดับสิบจังหวัดแรกของประเทศไทย งานวิจัยนี้ได้เก็บตัวอย่างทางกีฏวิทยาจากทั้งสามฤดูกาลระหว่าง พ.ศ. 2555-2556 และเก็บตัวอย่างเลือด (ผสมสารกันเลือดแข็งชนิด K_2EDTA) จากอาสาสมัครในพื้นที่ที่ไม่มีอาการแสดงของโรค ในช่วงอายุระหว่าง 25-60 ปี ในต้นฤดูฝนประจำปี พ.ศ. 25556 และได้ศึกษาโดยอาศัยวิธีการเชิงอณูชีวิทยาโมเลกุลกับยีน Envelope เพื่อตรวจวิเคราะห์การติดเชื้อไวรัสเดงกี ผลการศึกษาพบว่า ในยุงพาหะมีอัตราการติดเชื้อสูงสุดในฤดูฝนปี 2555 คิดเป็น 63.10 % แต่อัตราการติดเชื้อดังกล่าวลดลงอย่างมีนัยสำคัญในฤดูหนาวและฤดูร้อน ซึ่งคิดเป็น 4.19% และ 4.35% ตามลำดับ ส่วนในฤดูฝนปี 2556 อัตราการติดเชื้อคิดเป็น 8.70% จากข้อมูลดังกล่าว การติดเชื้อไวรัสของยุงต่อหนึ่งตัวมีหลายแบบ (single infection หรือ multiple infection) ซึ่งสัดส่วนของการติดเชื้อนี้มีความสัมพันธ์เชิงบวกกับอัตราป่วยในฤดูฝนปี พ.ศ. 2555 จากผลการศึกษาด้านปัจจัยพันธุกรรม พบว่าค่าร้อยละ Mean diversity และค่า pairwise p-distance ของเชื้อไวรัสเดงกีทั้ง 4 สายพันธุ์ มีค่าแนวโน้มเปลี่ยนแปลงตามฤดูกาล เมื่อพิจารณาการรูปแบบการเปลี่ยนแปลงของค่า codon usage bias ที่ได้จากยุงพาหะพบว่าเฉพาะในไวรัสเดงกีสายพันธุ์ที่ 2 มีค่าเปลี่ยนแปลงตามฤดูกาลอย่างชัดเจน ยิ่งกว่านั้นอัตราการติดเชื้อไวรัสเดงกีจากตัวอย่างพลาสมามีค่าร้อยละ 9.62 ซึ่งประกอบด้วยเชื้อไวรัสเดงกีสายพันธุ์ที่ 1, 3 และ 4 จึงแสดงให้เห็นว่าในคนมีรูปแบบการติดเชื้อที่หลากหลายได้ ยิ่งกว่านั้นยังพบว่าเชื้อไวรัสเดงกีสายพันธุ์ที่ 1 และ 3 มีการกลายพันธุ์ในระดับกรดอะมิโนแบบจำเพาะต่อโฮสท์ ซึ่งพบใน fusion loop ของโดเมนที่ 3 ของโปรตีน envelope ในขณะเดียวกัน มีการกลายพันธุ์ที่บริเวณพื้นผิวของโครงสร้างทุติยภูมิชนิด B-strand เฉพาะเชื้อไวรัสเดงกีสายพันธุ์ที่ 3 และ 4 จากข้อมูลการศึกษานี้แสดงให้เห็นว่า ไวรัสเดงกีมีคุณลักษณะแบบ Quasispecies ดังนั้น ผลการศึกษาวิจัยนี้ จะมีประโยชน์ในการพยากรณ์อุบัติการณ์การเกิดโรคไข้เลือดออกในอนาคตและนำไปสู่การวางแผนควบคุมป้องกันด้วยวัคซีนเพื่อกำจัดไวรัสเดงกีได้อย่างมีประสิทธิภาพ

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5474919830 : MAJOR MEDICAL SCIENCE
KEYWORDS: BAN PHAEO DISTRICT, SAMUT SAKHON PROVINCE, 113 VIRUSES

VEERAYUTH KITTICHAI: Genetic Diversity of Dengue Virus in *Aedes aegypti* Mosquito at Ban Phaeo District, Samut Sakhon Province, Thailand. ADVISOR: ASSOC. PROF. PADET SIRIYASATIEN, M.D., Ph.D., pp.

Dengue incidence has been dramatically increasing in the recent years. Epidemiological surveillance based on the evolution dynamics of circulating-dengue viruses, including environmental condition, can be used to determine the trend in dengue transmission, which has led to the prediction and planning of effective control approach. The objective is to focus on the mutation and seasonal evolutionary analysis of dengue virus existing in the highly endemic area through mosquitoes and local residents. Samut-Sakhon province was selected since it is considered as one of the top-ten province of having the highest annual morbidity in Thailand. Entomological collection was undertaken over-three seasons, during 2012 to 2013 and in early rainy season in 2013. The EDTA-blood was drawn from local asymptomatic volunteers of age ranges 25-60 years old, once in rainy season in 2013. A molecular method targeted on an envelope gene was used to verify the dengue infection. During the study period, the dengue prevalence was highest in monsoon period and significantly declined in the winter and dry season. Infection rates in the vector of rainy, winter, dry of 2012 were 64.29%, 4.19%, 4.35% respectively and 8.70% in early rainy period of 2013. Various types (single- and multiple-) of infection within individual mosquito were positively associated with the increase of morbidity. Mean diversity and the evolutionary distance (p-distance) varied within all dengue serotypes by season. Considering to the biased-codon usage in the vector, only serotype-2 had varied by season. There is 9.62% of the rate found in plasma samples with the serotype-1, 3 and 4, which also showed various types of the infection. Host-specific mutation residues were mainly found in fusion loops in domain-III residues (serotype-1 and 2) of the envelope gene but only B-strand was found the mutation in serotype-3 and 4. All data revealed that the viral character considered in the structure of quasispecies. This data is significant for predicting further dengue situation for further surveillance and planning in order to eliminate the virus with effectively specific vaccination.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Associate Professor Padet Siriyasatien, M.D., Ph.D.; and my thesis committee, Associate Professor Wilai Anomasiri, Ph.D., Associate Professor Parvapan Bhattarakosol, Ph.D., Chalisa Louicharoen Cheepsunthorn., Ph.D., Usavadee Thavara, Ph.D. for their valuable advice. I would also like to express my appreciation to the staff members from the Medical Entomology Unit, Department of Medical Sciences, Ministry of Health, Thailand, for all their suggestions on the entomological sampling at Yokkrabat subdistrict, Ban Pheao district, Samut Sakhon province, Thailand. I also would like to thank the staff from the Medical Virology Unit, Department of Medical Sciences, Ministry of Health, Thailand, for kindly providing dengue virus stocks. I am deeply thankful to Assistant Professor Sunchai Payungporn, Ph.D., for his suggestion with bioinformatics analysis. I would like to give my sincerest gratitude to Assistant Professor Kiattawee Choowongkomon, Ph.D., Department of Biochemistry, Kasetsart University, Thailand, with his great commences and suggestions with molecular modeling.

I am particularly grateful to National Science and Technology Development Agency (Thailand) for Research Chair Grant; the Ratchadapiseksompotch Research Fund (RA 57/099) and the Chulalongkorn University's Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumiphol Adulyadej for financial support throughout my research project.

I am deeply thankful to all staff members at Thamnopphaeo Tambon Health Promoting Hospital, Yokkrabhat subdistrict, Ban Phaeo district, Samut Sakhon province, Thailand, for their suggestions with the entomological and blood sample collections.

Finally, I would like to thank my parents, my family, teacher, and my loved ones who encouraged and blessed me to have the power for completing my research.

CONTENTS

Page

LIST OF TABLES

Pages

LIST OF FIGURES

PAGE

LIST OF ABBREVIATIONS

A	= Absorbance
AAs	= Amino acids
am	= Ante Meridiem/ before midday
BHQ	= black hole quencher
bp	= Base pair
^{o}C	= Degree Celsius
C	= Capsid
DENV	= Dengue virus
DENV1-4	= Dengue virus type 1-4
df	= Degree of freedom
DF	= Dengue fever
DHF	= Dengue hemorrhagic fever
dNTPs	= Deoxyribonucleotide triphosphates
DSS	= Dengue shock syndrome
E	= Envelope
E. coli	= *Escherichia coli*
EDI	= Domain I of envelope gene
EDII	= Domain II of envelope gene
EDIII	= Domain III of envelope gene
EIP	= Extrinsic incubation period

LIST OF ABBREVIATIONS (cons)

ELISA	= Enzyme-linked immunosorbent assay
FETP	= Field epidemiology training-program
h	= Human
IgG	= Immunoglobulin G
IgM	= Immunoglobulin M
JEV	= Japanese encephalitis virus
km^2	= Square kilometer
m	= Mosquito
Mg^{2+}	= Magnesium ion
na	= Non-analysis
NS	= Non-synonymous substitution
NS1	= Non-structural subunit 1
NS2	= Non-structural subunit 2
NS3	= Non-structural subunit 3
NS4	= Non-structural subunit 4
NS5	= Non-structural subunit 5
nt´	= Nucleotide
UTR	= Untranslated region
p	= *p-value*
PDB	= Protein Data Bank

LIST OF ABBREVIATIONS (cons)

prM	= Pre-membrane
RH	= Relative humidity
RNA	= Ribonucleic Acid
rpm	= Revolutions per minute
RSCU	= Relative Synonymous Codon Usage
RT-PCR	= Reverse transcription polymerase chain reaction
S	= Synonymous substitution
Taq	= *Thermus aquaticus*
UV	= Ultra vilolet
WHO	= World Health Organization
WNV	= West Nile virus
X^2	= Chi-Square Statistics
YFV	= Yellow fever virus

CHAPTER I
INTRODUCTION

1. Background and rationale:

Human infected by dengue virus can be presented with classical dengue fevers (DF) and the severe form, namely, dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). Over a 50 year period (1), annual dengue cases 50-100 million (10-15%) out of 390 million infected patients were symptomatic, which led to 500,000 hospitalizations and eventually developed into severe form (2). This is especially true of the population who inhabit in the tropical and subtropical regions (1) where annual hospitalization and death rates of patients by the severe form is highest; most countries of Southeast Asia, southern and central America, and the Caribbean and south Pacific but with a lower rate of infection in Africa so reported possibly due to ineffective surveillance (3). This warning trend in various dengue epidemiological profiles is the intensive of its morbidity and mortality.

Dengue viruses (DENV) are positive-sense single-stranded RNA viruses composed of approximately 10,600 nucleotides (4). They are members of the Flavivirus genus of the *Flaviviridae* family and are classified into four serological serotypes (DENV1-4). They are mainly dominant in endemic zones with different distribution patterns (5-7). Dengue transmission to humans occurs through the bite of an infected-female mosquito vector and following saliva containing the virus spreading to the host. *Aedes aegypti* is mainly a domestic vector and easily adapts to new areas. Therefore the increase of dengue disease in America, Asia and western Africa is mainly due to the geographic expansion of the mosquito (8) (and a minor local dengue vector, *Aedes albopictus*). The viral spread occurs between a human-mosquito-human transmission cycle (epidemic cycle) more than via a sylvatic transmission cycle via primate and mosquito (enzootic cycle). Interestingly the extrinsic incubation time of the virus in the mosquito is especially responsive to temperature and relative humidity fluctuation (9). The mean global temperature has increased by roughly 0.74^{o}C +/- 0.18^{o}C in the past century. The weather-sensitivity of the dengue virus results in the globally-geographic distribution.

Co-circulating DENV viruses have been intensifying in various scales where had been referred as hyperendemicity within the relevant countries (10), even-though medical authorities are faced with various problems of further vaccine planning. Control efforts currently focused on the principal vector with limited resources and limited success in many endemic areas. However, the fundamental knowledge of the relationship between dengue-transmission cycle and the environment could effectively guide assessments of the scheduling, location, and magnitude of risk and then advise possible use of resources.

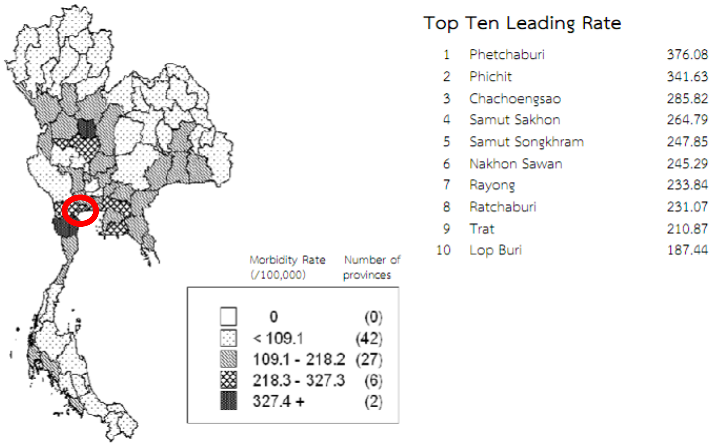


Figure 1 Reported Cases of D.H.F in Thailand in 2011, total per 100,000 populations by province, Thailand, 2011 (http://boe.moph.go.th/)

Thailand experiences hyper-endemic dengue spread with seasonal phases of disease that unpredictably diverge in degree across provinces (each year-round) (11). All four dengue viruses (DENV-1 to 4) have been circulating in the area attributed for various risk factors (12). These were considerably contributed the introduction of all four DENV viruses to the population of the area (13). However, the alteration causes difficulty prediction and control of a changing target, which are focused on dengue spread-regulating factors (mosquito and human host). Therefore, using the potential surveillance by narrowing into each small scale of the endemic zones should enable recognition of the interaction between weather-based and non-weather-based regulation of transmission.

Samut Sakhon province is located along the Gulf of Thailand at the mouth of the Tha Chin River and in south-central region of Thailand. Annual estimates have reported an increase of infection rate even where the vector is controlled by local government. Moreover, this area is the number fourth of morbidity rate of Thailand in the year 2011 with 264.79 per 100,000 (Fig.1). Between 2006 and 2010, a retro-perspective study indicated that the rate of the disease was approximately 355.66 per 100,000 in an age range between 5 and 24. In year 2012, the incidence rate of DHF was found to be 252.26/100,000, which was classified as a severe epidemic year of dengue disease with the 90^{th} percentile (14). In addition, Ban Phaeo district residents were reported most infected with 286.57 per 100,000 people and Krathum Baen and Maung districts are the least with 142.03 and 137.05 per 100,000 people infected, respectively. The highest rates of the infection were related to the occupation mainly in students, contract workers, agriculture workers, housekeepers, and traders of which leads to a high number of hospitalizations as monthly reported.(15).

An understanding of the seasonal transmission of dengue serotype virus which takes into account the geographical, demographical and variation of the virus is essential. The evolutionary epidemiology data of the circulating dengue population is a key to comprehend the relation between disease and risk factors, the amount of population growth and the emergence of new viral lineage. Additionally, phylodynamic analysis can reveal the signature of a pathogen which is shaped by the interacting effects of viral genome diversity, selection, transmission and unstable evolution (16). The rising knowledge of variation interaction among sites may provide indispensable insight into the key structures and functional sites of protein, which is the opportunity for designing the appropriate tools of diagnostic and vaccine development. More study of dengue phylogeny in urban and rural endemic areas has been proposed (17-19), but with no supporting data about the transmission cycles of the viruses each year-round.

The proposed study was mainly considered the role of genetic factor/ weather conditions in dengue cycles. The objectives of this study are to determine the rates of seasonal DENV serotypes and analyze the sequences in *Ae. aegypti* mosquitoes and asymptomatic subjects who inhabit in the highly endemic areas. Investigation of seasonal transmission cycle, the genetic variation and evolutionary phylogeny of the

dengue virus were undertaken in Yokkrabat sub-district, Ban Phaeo district, Samut Sakhon province. Comparison of dengue detection and sequences will be made between viruses obtained from field-capture *Ae. aegypti* mosquitoes with that obtained from local human. The benefit of this study could be an early key and the knowledge gained for designing effective diagnostic tools, drugs, vaccine production and then use in decision about whether to vaccinate at particular site in the future.

2. Research question:

- Does viral-genetic regulate the seasonal transmission cycles of DENV serotype viruses circulating in Ban Phaeo district, Samut Sakhon province in Thailand?

3. Objectives:

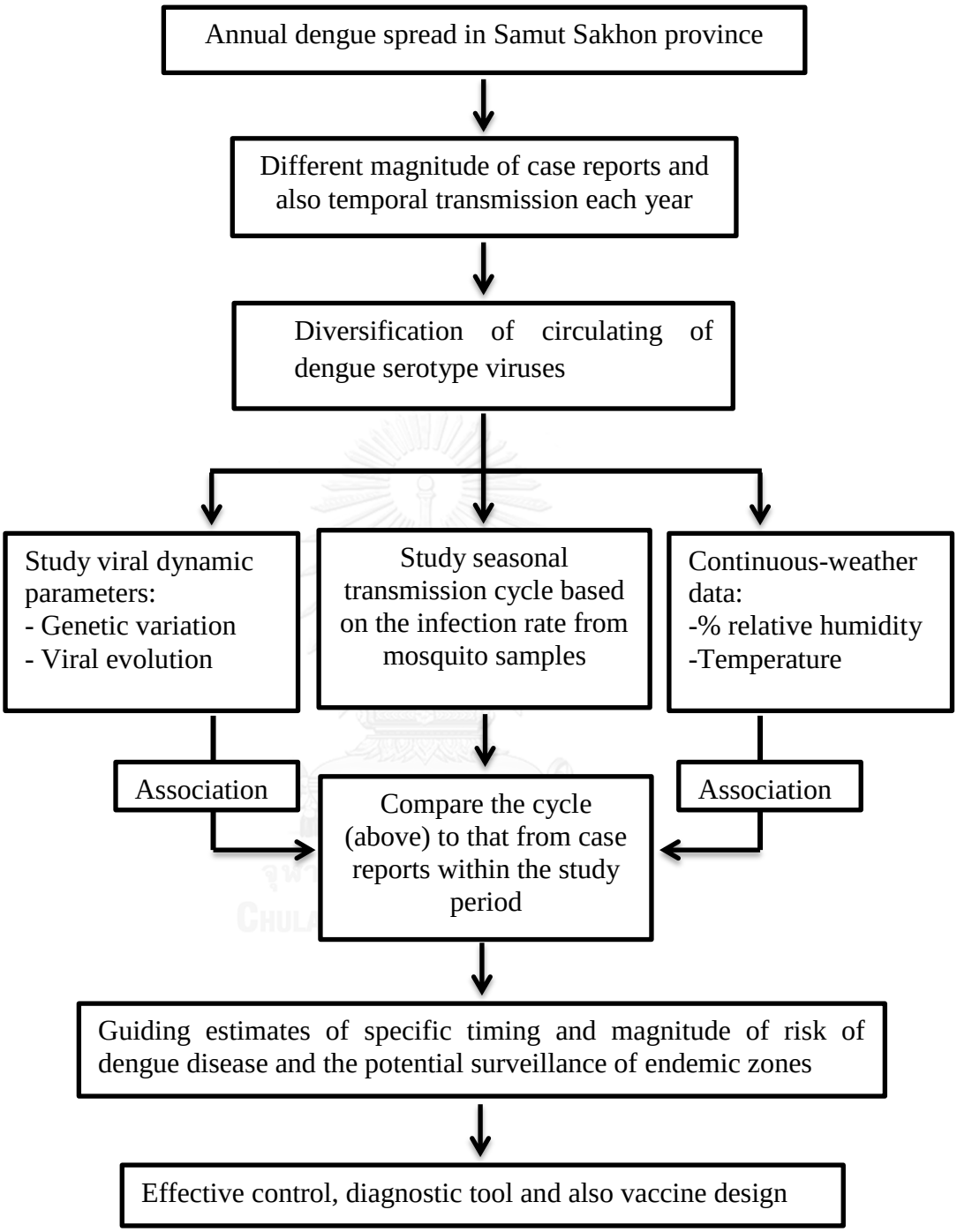
1) To study the association between genetic variations, evolutionary dynamic and also continuous data of weather conditions with the dengue transmission cycle.

2) To compare the dengue transmission cycles which assessed from the infection rates in the mosquito and that from case reports within the study-period in Ban Phaeo district, Samut Sakhon province.

4. Key words:

Dengue viruses
Ban Phaeo district, Samut Sakhon province, Thailand
Aedes aegypti mosquito
Evolutionary dynamics
Seasonal transmission cycle
Quasispesies
Positive selection

5. Conceptual framework:



6. Beneficial of the study

The study was performed to analyze and define the relationship between dengue-transmission spread and genetic variation of the virus and the environment which could effectively guide essential knowledge and estimates of specific timing and magnitude of risk. Then, this was contributed to the potential surveillance of endemic zones. Moreover, the study of evolution and also protein structure could provide the knowledge required for designing effective diagnostic tools, drugs and vaccine development and then be used in decisions about whether to vaccinate at particular sites in the future.

CHAPTER II
REVIEW OF RELATED LITERATURE

1. Dengue viruses

Dengue viruses are members of the virus family found in humans with yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) all of which belong to the *Flaviviridae* family. The DENV virus is serologically classified into four antigenic serotypes (DENV1-4) (4) and each of these contains 4-6 genotypes based on geographical and genetic variations. All four serotypes can cause the dengue disease in endemic zones and emerged in the tropics and subtropics as long as a half-decade ago. The viruses are spherical and the enveloped viruses [which can be captured by electron microscopy and X-ray crystallography (20)] are approximately 50 nm in diameter (21), and contain a positive sense single strand of viral RNA with 10,600 nucleotides of a genome.

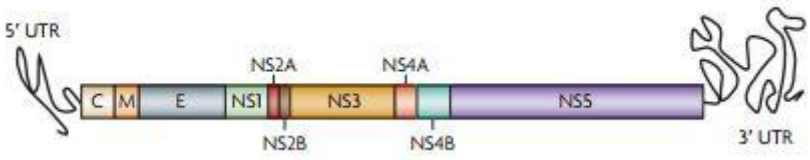


Figure 2 Dengue virus genome including the single ORF encodes 5´-UTR; 3´-UTR; three structural proteins (C, M and E) and seven non-structural proteins, namely, NS1, NS2A, NS2B, NS3, NS4A, NS4B and N55 (2).

The viral genome contains structural and non-structural encoding genes. It is capped by 5´-type structure (m´G5´ppp5A) and by 3´-tail (without a poly-adenylated region), plays a role in the translation regulation (4). The seven non-structural proteins are named NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Fig. 2). The three structural encoding genes are capsid (C) surrounding the viral genome, linked by a membrane (M) where the membrane precursor (prM) controls the folding of the envelope (E) protein in the *trans*-Golgi network which forms the structure protruding through the two trans-membrane proteins (lipid bilayer) and both (M+E proteins) surrounding a nucleocapsid and exposed to the environment surrounding the particles.

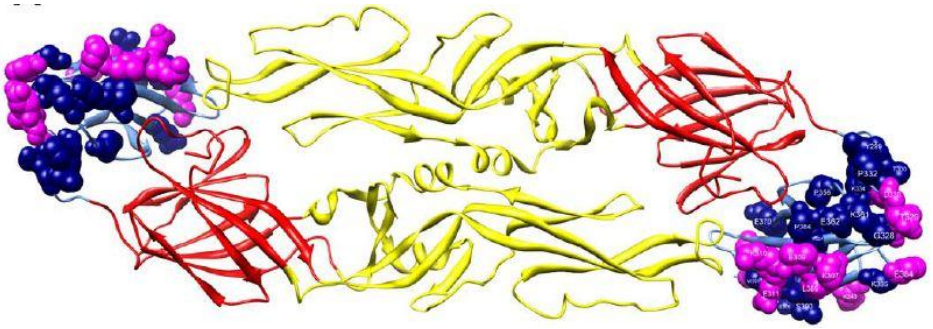


Figure 3 E-glycoprotein dimers contains distinctly three domains which are labeled with red color for EDI, yellow color for EDII and blue color for EDIII covered by dark-blue and pink-colored valence electron (22).

The *E* glycoprotein mediates the attachment and entry to the host cell and is the target of neutralizing antibodies. Each protein monomer of ninety dimers can be separated into three functional domains with EDI: the central domain, EDII: the dimerization protein represents the fusion peptide which functions by fusing into the host cellular membrane, and EDIII: a receptor binding domains which contribute the contact between the virus and host cell (Fig. 3). The viral entry occurs via envelope receptor-mediated endocytosis after the fusion via intra-membrane (23). Disassociation of the viral genome in the cytoplasm prepares the mRNA for translation, assembles them in the endoplasmic reticulum, and conducts the structure in the Golgi apparatus before leaving the cell. The mutation of domain-III sequences (Fig. 4) is involved with the virulence of the dengue virus (24) and leads to the thermodynamic stability change and protein folding of the domain in DENV-3 under as shown in experiment (25). Moreover, the highly specific monoclonal antibody (mAb) against the domain affects the viral attachment and infection both in the mice model and in human (26). However, Beltramello and *et al* (2010) proposed that the dengue virus can be neutralized by human mAb acting against dengue EDIII (27).

Except for EDIII domains (as has ELISA) have been useful for serologic diagnostics to detect both IgM and IgG because the EDI and EDII can be play an important role in antigenicity with less cross-reaction when compared to EDIII (28, 29). Interestingly, a partial or recombinant protein can be produced from the EDIII domain (30). This is protective against flaviviruses, and was accepted as the best immunogenic for producing a potential vaccine. This vaccine which can be either

monovalent or multivalent also stimulates the neutralizing antibodies to protect at mice host (31).

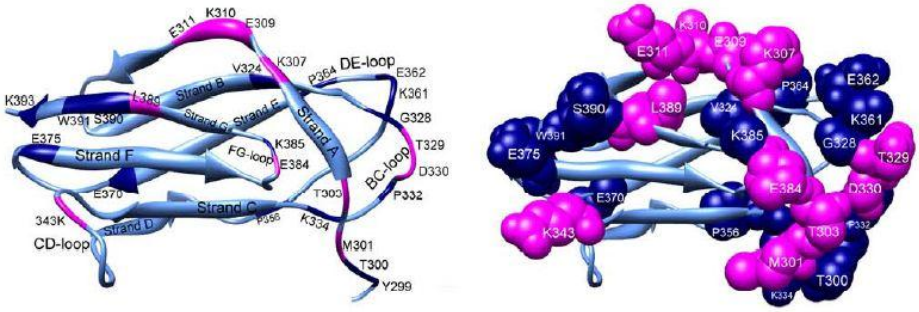


Figure 4 Domain III of the *E* gene with reported several-mutation positions. It were coded by amino acid codes and follow in Arabic number in the beta-strands and in fusion loops such as E311, K393 and E362 (22).

2. Dengue infection and surveillance

The virus infections have been annually distributed globally over 50 years both DF (by infection with single or repeat homogeneous strains) and the severe form such as DHF and/or DSS (initiate by infection with heterogeneous strains). By definition in DF the incubation period ranges from 3 to 14 days (commonly ranging from 4 to 7 days) (32), followed by high titer (around 10^5-10^6 infectious unit per ml) of viral replication in specialized cells such as dendritic cells and blood stream cells such as macrophages and lymphocytes (4) and the patient will recovered by the end of that period. Fever onset is associated with headache, pain around the eyes, myalgias and arthralgias, anorexia, abdominal pain and nausea, polymorphic appearances of rash (macular, maculopapular, morbilliform, intense pruritus and desquamation, scarlatiniform or petechial the latter two which are commonly seen in arms and thighs) which are observed in >80% of DF cases. After the incubation period patients can display symptoms or remain asymptomatic. The asymptomatic form can persist or eventually can be reverted to symptomatic DF. Symptoms manifestation of either DHF or DSS or both can be appeared together in the same patient. These various of the clinical manifestations can be treated differentially. Increased serum transaminases accompanied with leukopenia and thrombocytopenia is commonly indicated in the symptomatic condition (33, 34). DHF and DSS which are the severe

disease forms display the symptoms listed above but also are accompanied by fever, the presence of hemorrhage, bleeding and fluid loss by vascular leakage resulting in hypervolemia, ascites (35, 36). It is well known that dengue viruses can be serologically classified into four strains by using specific antibodies that were developed in the laboratory (37). But, the above method cannot be the only approach to interpret the viruses isolated from patients as viruses can be more virulent or less. This is because we lack a suitable surrogate model of dengue disease to study; even when a rat or primate is infected the subjects do not show the hemorrhagic rash but appeared to have just the transient viremia (38). Further issues arise from the hypothesis of the immune enhanced phenomenon as more evidence has accumulated that is involved in the disease severity and the pathogenesis of multiple dengue strain infection (39), however, this is still controversial. The relative association between genotyping (within serotypes) and clinical appearances has played an important role to date in evaluating and monitoring the epidemiological and evolutionary phylogeny of the virus in outbreaks and in the hyperendemic area.

Figure 5 Endemic areas/ countries in the tropical and subtropical region where colored-coded area are at high risk of dengue spread (15).

More than 50 million infections and 100,000 case of the severe form occur annually for both DHF and DSS (15, 40, 41). By contrast, Bhatt and et al (2013)

proposed a different rate of infection (390 millions) and manifestation (96 million with all levels of dengue severity) which is higher than the reports from WHO (2007) by as much as three times (42). This is because the WHO reports may lack of real distribution information and underestimate the risk of dengue infection and its burden.

A comprehensive measure of dengue distribution and disease burden would contribute to efforts to determine how to intervene with the limited tools available for controlling the disease. The number of victims who have been hospitalized and recovered have died and reported by the relevant countries are as follows (Fig. 5 and Fig. 6), South East Asia (66.8 million), Southern and Central America (13.3 million), Oceania (0.18 million), and the Caribbean and South Pacific (specific numbers not reported). The reports of fewer cases derive from Africa than one would expect (15.7 million) may be because of ineffective surveillance (3, 42).

In 2012, in St. Croix, a US Island a higher amount of DENV-1 and DENV-4 were found and the outbreak affected children school. Incidence determination was by the IgM anti-dengue positive and molecular diagnostic test (43). Even Europeans countries must not ignore dengue infection as shown by the importation of the dengue virus from an outbreak on the Island of Madiera, Portugal (44) to cold Finland where the strain was classified as DENV-1 (45). In Caribbean countries such as Barbados, there is poor identification and characterization of epidemiology and low clinical presentation. It has also emerged that dengue disease in children resulting between 0.29 and 2.92 cases/1000 children with classical dengue fever (225/545, 41%), dengue hemorrhagic fever (15/545, 3%) and a mortality rate is 0.3% (46). More focused and enhanced weekly surveillance was undertaken in Puerto Rico and results showed that 41% of the population was infected and either in the acute or convalescent phase of the illness (47). There was also the multiple introduction of dengue into spread to Latin American countries for example in Venezuela where it was found DENV2 caused the severe disease (48). In Peru the American and American/Asian genotypes of DENV2 have been circulating for more than a decade and have present commonly between October and January. So in Latin America as a who the lineage-II of American/Asian DENV2 has been more virulent in the countries that presented the specific transmission pattern of only one dengue serotype but

within that serotype there has been increased variation as the virus has adapted to the region (49).

DENV-2 (Asian1 genotype) was reported in Lao PDR after detection by RT-PCR and was cladded by a phylogenetic tree from a Chinese patient's serum (50). In Cambodia Wilson (2013) found that dengue seemed to be active in the central nervous system of children where only the dengue virus was detected (by RT-PCR) and its role confirmed by the fact that no other pathogen was found (51). The situation in Saudi Arabia indicates that the virus is likely to spread throughout the Middle East. Surveillance in Saudi began in 2006 in order to record outbreaks of the disease for spatial and temporal clustering for effective control (52).

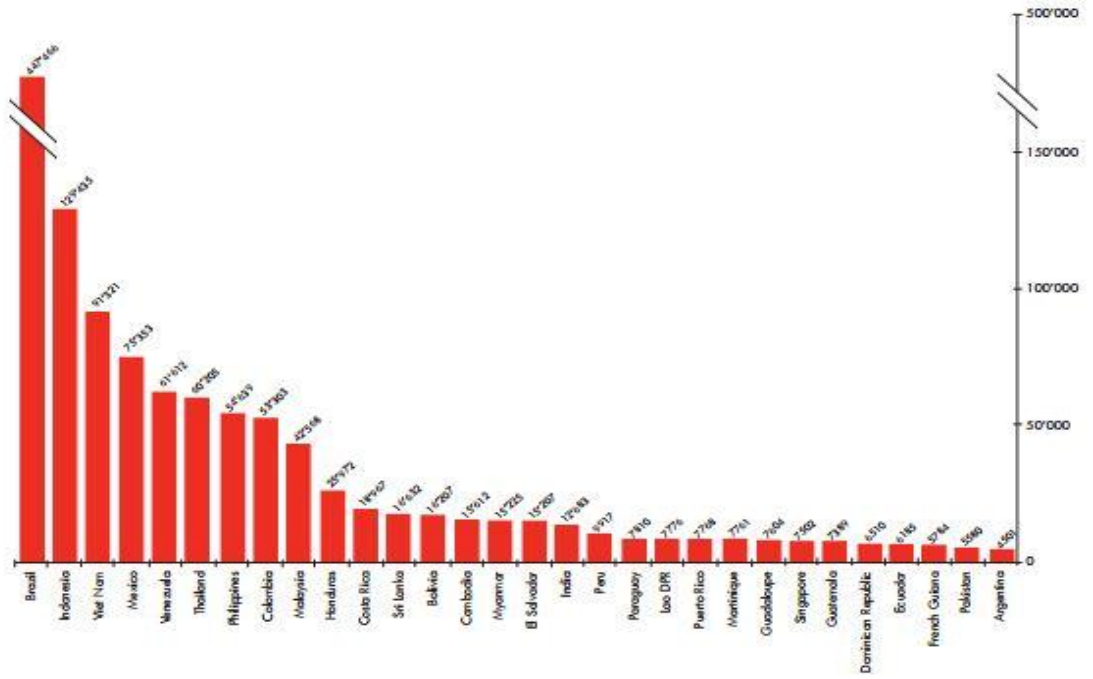


Figure 6 Global dengue infections on average, in the highly endemic areas reported to WHO, 2004–2010 (43).

It was found that all groups irrespective of age, gender or nationality were equally at risk. As with Saudi Arabia more frequent detailed surveillance should be undertaken season by season in all affected countries since this can assist to predict the risk and spreading factors something which is urgently needed (53). Between 2007-2009 DENV-1 (genotype V) was the predominant strain in New Delhi which replaced the earlier circulating genotype III & I as shown by evidence based on the

phylogeny of the E-NS1 gene (54). Thus the replacement of a dominant genotype must be carefully monitored in case the new dominant strain has greater virulence which may affect the health of the local residents.

3. Genetic diversity and evolution

Comprehension of dengue genetic diversity is critical and it is also important to study the evolution and epidemiology, which are affected by host (probably from human susceptibility). The genetic variation and fitness of arboviruses such as dengue viruses have been studied and it was shown that there were fewer mutations after replication in invertebrate than were found in vertebrate hosts (55-57). The lack of 3´-proofreading of the viral RNA polymerase results in more varied population (found in multiple or even single hosts) which have the potential to increase the virulence of the virus (58).

The viral population may contain both homogeneous and heterogeneous groups, which are referred as quasispecies but only when found in human host (56, 59). However, the quasispecies is transferred from the human host to the mosquitoes and back to another human host is unclear. Lin and et al (2004) proposed that the vector may contribute to stabilize the evolutionary RNA sequences of a major population/ dominant strain/ homogeneous population but the minor and/or defective groups may be found in a human host and this is relevant to the pathogenesis in a patient (55). The virulence of the virus has been hard to measure and there has been difficulty in differentiating one form of the virus to another due to the lacking both in vivo and in vitro models of the disease. However, the mutation rate of the virus is a possible relevant determinant of disease. For example the variations of the E-gene sequence have been proposed as candidates affecting the RNA structure and enveloped protein properties of the virus and this mutation has increased the level of disease in mice (60). Moreover, this high rate of mutation has implications when a new form of virus is introduced to an area and displaces a previous form resulting in new outbreaks of the disease (Fig. 7).

Thus carefully monitoring of alterations in the virus to the accurate genotyping within serotypes is essential in making predictions and in determination of the geographical epidemiology and human pathogenesis of the disease.

Rico-Hess (1990) proposed a cutoff value of 6% divergence before a genotype was considered outside the boundary of DENV-1 (38). They were six genotypes within DENV-1 (61), namely: sylvatic genotype, genotype-I (South East Asia), genotype-II (Thailand), genotype-III (Malaysia), genotype-IV (South Pacific) and genotype-V (Americas/Africa) (62, 63). DENV-1 could be considered to be the strain with the greatest epidemiological spread as it is found in many countries such as Brazil, Venezuela, Columbia, Peru, Myanmar (61), India (64), and Taiwan (65). This strain is the locally emergent strain in the Asian-Pacific region and ranges from Myanmar to sites up to 10,000 km distant. Genotype-1 is considered the most significant in spread and epidemiological impact with II and III ranking second and third respectively (61). When Ke and et al (2013) investigated the DENV1 outbreak and long term transmission in Myanmar 2001 they found the co-transmission of both defective virus (containing the deletion and /or stop codon in genome sequences) and functional virus was the cause of the outbreak (66). Moreover, they were surprised that their experimental results showed that the mechanism (of co-transmission) increased the potential transmission compared to that by the functional virus only.

DENV-2 found to be involved in the first ever reports of epidemic DHF. The cut-off value is 2% divergence that led to differential classification of the strains found in: sylvatic/west Africa, the Americas, Southeast Asia, and the Malaysia/Indian subcontinent (38). The Asian genotype was found to have high epidemiological impact which was associated with severity of impact in many countries such as in Peru, Venezuela and Mexico. In Guangdong province in 2010 the Asian 1 genotype of DENV-2 was displaced by the Cosmopolitan genotype (97.8% amino acid homology) increase of secondary infections (68).

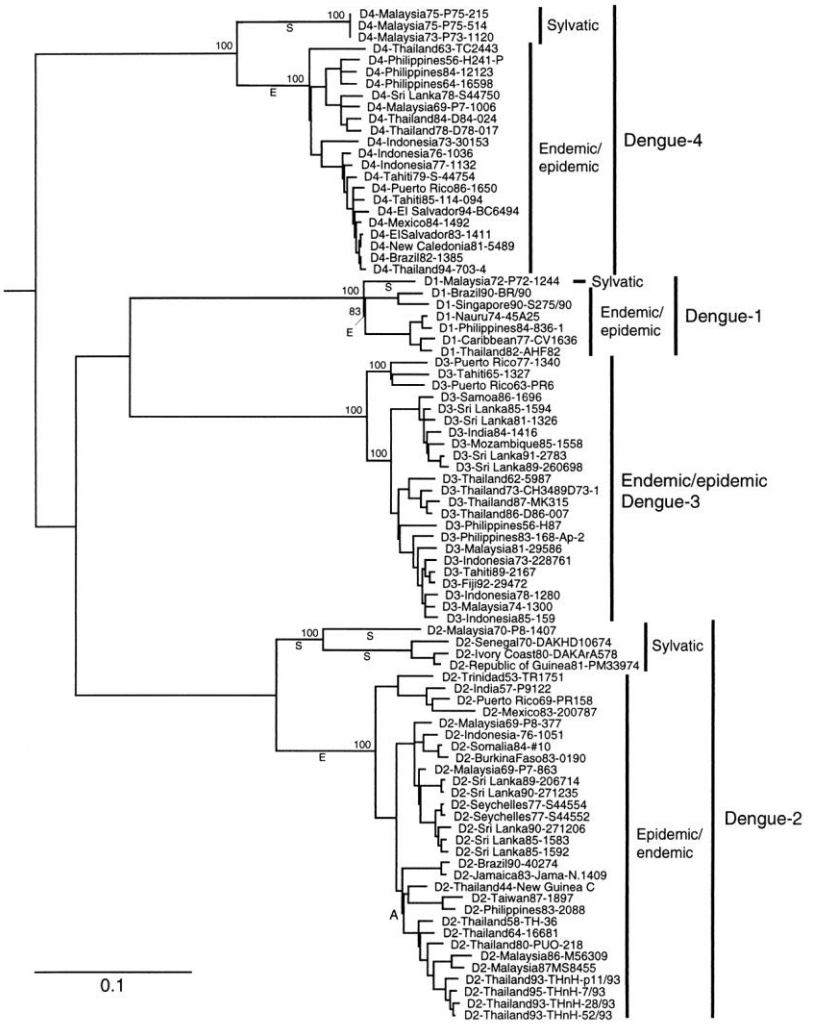


Figure 7 Phylogenetic tree derived from nucleotide sequences of *E* protein gene. The tree represented that endemic/epidemic DENV strains have emerged from the sylvatic (67).

The first phylogenetic classification of DENV-3 was through the envelope gene sequence (68), and led to the conclusion that there were four geographic original genotypes, namely, the Americas, Indian subcontinent, Thailand, and southeast Asia/South Pacific, but no sylvatic genotype. The disease severity (DHF) of DENV-3 was reported in many countries such as Sri Lanka, Thailand (69), and Singapore (17), where in each country DENV-3 was found to be co-circulating with DENV-1.

DENV-4 viruses were classified into the Malaysian, Southeast Asian, and Indonesian genotypes based on 6% divergence (38). The strain was found to be

continuing to spread in many endemic countries, particularly in America. This information has revealed a formerly hidden aspect way of viral emergence occurs within many genotypes. The dynamic of dengue spreading might be occurred by spatial more than that by temporal (within the one area). This is because there has been shown to be more geographical spread than alteration in the one area over time through mutation. This fact was uncovered through investigation of the disease in a school in Kamphaeng Phet, Thailand (70).

4. Effect of environmental factor against dengue transmission in *Ae. aegypti* mosquito

Ae. aegypti mosquitoes belong to the family Culicidae and are small fragile insects, which invade in the tropics and subtropics (71, 72). The mosquito is the main vector which is commonly accepted to be the vector for dengue fever and yellow fever epidemics in urbanized areas (73, 74). Dengue disease is a medically important mosquito-borne disease both in classical and severe forms, and caused by the dengue virus which is transmitted between humans and *Ae. aegypti* (predominant vector) and *Ae. albopictus* mosquitoes, the potential vectors of the disease. The mosquito life cycle is divided into four stages: egg, larva, pupa and adult and the length of the life cycle will vary by the temperature and humidity of the season. Both males and females of all mosquito species require nectar, and only the females suck the blood as a protein source for their egg production.

The virus transmission is associated with the infected blood being transferred from one victim to the next through the bite of the mosquito. During the extrinsic incubation period (EIP) of approximately 14 days inside the mosquito, the virus titer will be increased in the mosquito's midgut (in around 7-10 days) and then spread to the mosquito salivary glands to wait for transmission (Fig. 8). Watts and et al 1987 report that in the laboratory the EIP will be shortened above 26^{o}C and that shortening will be accelerated until temperature rise to 30^{o}C (75). On average the estimation of EIP at 25^{o}C and 30^{o}C were 5-33 (average, 15 days) and 2-15 (average, 6.5) days, respectively (76). This correlated to mean the intrinsic incubation period (IIP) at 5.9 days (95% CI) assumed among 3-10 days.

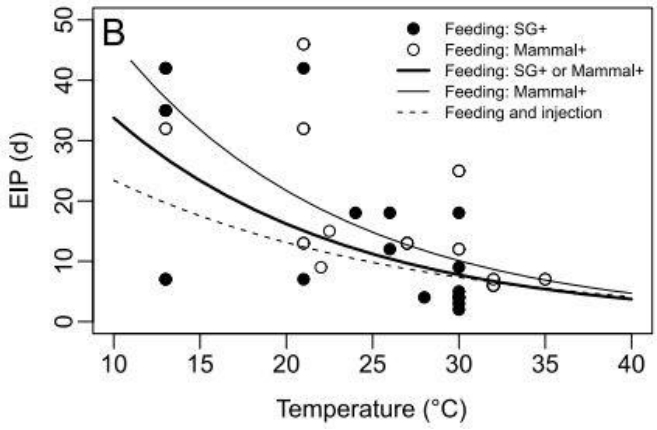


Figure 8 Estimation time of temperature-depend extrinsic incubation period (EIP). Average EIP of the virus collected from salivary gland (SG) of the mosquito and from fed-mice (mammal-blood) inversely varied by the ranges of temperature (78).

Additionally, the residual pollution of the chemicals will eventually be a harmful factor for the health of the human population, and cause environmental, economic and social problems. Moreover, several factors which affect the spread of dengue include the immune status of hosts (77), their age levels (78), the virus traits (69), the mosquito vector, and the environment involved with the reemergence and precipitation of the disease. Currently the lack specific treatment and need effective control reducing the incidence of dengue. Therefore it is necessary to develop new efforts to monitor the spatial and temporal evolution and epidemiology of the virus, an efforts that will be a central to an approach to design a vaccination plan for the future.

In summary, dengue viruses therefore exist as a quasispecies that is a population sharing a closely related genetic sequence. This fact is believed to play an important role in the evolution and virulence of the major variant group of the virus as well as in the pathogenesis of human disease (56) and in transmission cycle via the mosquito vector (55). From the above, the dengue virus circulation and evolution in the endemic area will be investigated through weather, labor-movement effects and genetic sequence analysis which provide the parameters defining the transmission cycle of the virus. Our objective in this analysis is to determine the transmission cycle of dengue serotype viruses and whether the relevant parameters motivate the dengue spreads in one particularly endemic area.

CHAPTER III
MATERIALS AND METHODS

1. Study site

The study sites selected for investigation are in Ban Phaeo district, Samut Sakhon province, in central Thailand. The province (latitude 13.48-13.69° and longitude 100.02-100.27°) locates in the central of Thailand where around 29 km in distance from the capital city, Bangkok is.

The province has annually reported an increase of infection rate of DF and DHF even where the vector for the disease is controlled by local government. A retro-perspective study indicated that the rate of dengue fever (between 2001 and 2010) was approximately 167.15 per 100,000 people (Fig. 9). The rate of the infection not occurs only between June and November of each year, but also found in winter and dry season. This leads to remain a number of hospitalizations as monthly report (15).

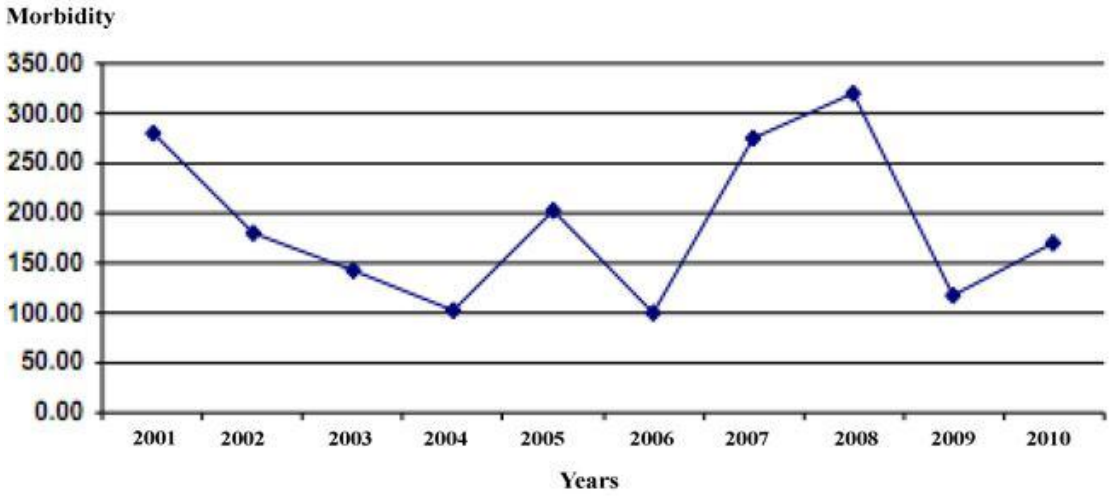


Figure 9 DHF per 100,000 people of Samut Sakhon province between 2001 and 2010 (http://boe.moph.go.th/)

The Field Epidemiology Training-Program (FETP) of Thailand reported that Samuth Sakhon province was in the top-ten of having the highest morbidity rate of the country during the year 2007-2012. It is clear from the rate that the bulk of the infections occurred in an age range between 5 and 24 where the infection rate is

355.66 per 100,000. The highest infection rate involved children 10-14 years of age, namely 448.87 per 100,000, while the lowest infection rate was found in the elderly aged >65. That rate was 26.08 per 100,000. Seven villages in Ban Phaeo district of the area were the sites chosen for study as whole provinces are classified as being endemic areas of dengue.

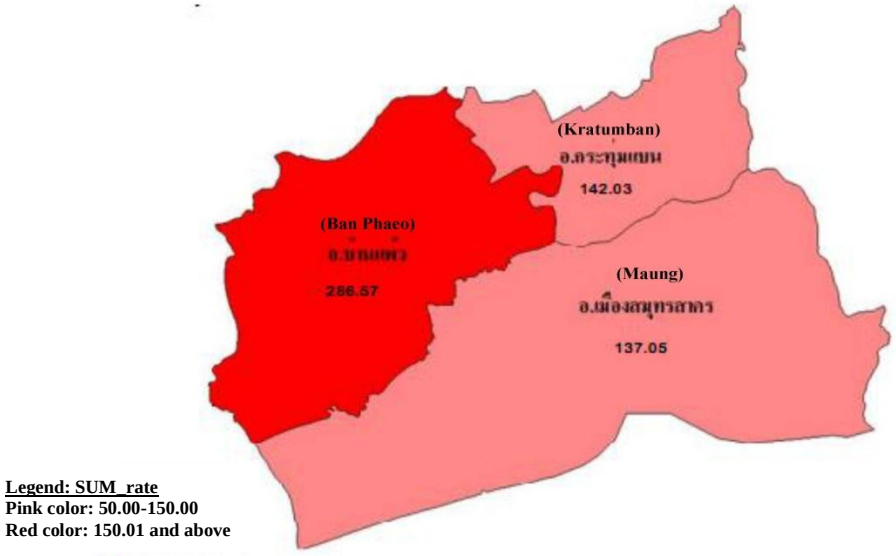


Figure 10 DHF per 100,000 people which was classified by the districts of Samutsakhon province, 2010.

Of the districts of Samut Sakorn province Ban Phaeo district is a relatively populous region with 94,278 residents and also containing 23,530 housing structures all located in an area of 245.031 km^2 (derived Department of Provincial Administration, Ministry of Interior, Statistical Forecasting Bureau, National Statistical Office, Thailand). Residents were reported most infected with 286.57 per 100,000 people (severe endemics) (14), and Krathum Baen and Maung districts were 142.03 and 137.05 per 100,000 people infected, respectively (Fig. 10). DHF incidence tends to dramatically increase seasonally (79-81) and global warming promoting the circulation of both vector and viruses. As Ban- Phaeo district is the endemic area with the highest rate of infection it is suitable for the appropriate model for seasonal surveillance in the present study.

2. Sample collection

2.1) Entomological sample collection

Entomological sample collection inside and around the resident's houses (whose permission us by their written consent) was randomized (79). Seven villages of Yokkrabat sub-districts are the targets of the collection and were selected for collection of larvae, pupae, males and females of *Ae. aegypti* mosquitoes, and approved with respect to the life-stage by a professional entomologist in the laboratory. The field captured mosquito was done after obtaining written permission from the residents. Human baiting (82) was performed by officers with highly experience from National Institute of Health, Thailand. The study was undertaken in all Thai seasons comprising rainy, winter and dry seasons from 2012 to 2013. The living individual samples were collected into vial and collection was occurred between 8.00 am and 11.00 am (based on the suitable period of host-blood seeking) once or twice each season. The mosquito sample was collected from approximately 10% (150 samples) of the study site per season. All insect stages were immediately transferred to the Entomology Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University.

2.2) Sample size of human blood collection

The 3 ml of each K_2EDTA-blood sample were randomly collected from local subjects (during having the monthly checked-up at the Health Promoting Hospital (HPH)) aged 26-60 years old, who all gave written consent, early in rainy season of the year 2013. Individual samples were recorded with basic information: age, village cluster and/or current sign and symptom if possible. Preservation was immediately done on ice until transfer to the laboratory within 24 hours. Plasma and buffy coat was prepared by centrifugation (3,000 rpm, 10 minute in room temperature) and both components stored by deep freezing at -80oC for the next experiment. Total samples will number approximately 250 samples in accordance with the sample size calculation specified in the equation (below) in accordance with the equation to estimate prevalence which was approved by a professional statistician, Department of Chula Research Center, Faculty of medicine, Chulalongkorn University. The study

was approved by the Ethical Committees of Research Affairs Department, Faculty of Medicine, Chulalongkorn University (COA No. 328/2014).

$$\text{Sample size (S)} = \frac{n}{1 + \frac{n}{population}}$$

$$\text{In which; } n = ZxZ\left\{P\frac{(1-P)}{exe}\right\}$$

Population = 94,278 residents

P = an expected frequency value as 0.2

e = expected Frequency Value minus (-) Worst Acceptable Value as 0.05

Z = area under normal curve corresponding to the desired confidence level, confidence Level as 95% / 1.960

2.3) Dengue cases and Environmental data

The dengue case reports and environmental data consist of the weather from 2011-2013. Weather data of max/mean/minimum-value of temperature, relative humidity and rainfall derived from Asian Start Regional Center. The monthly data of dengue cases (both DF & DHF/DSS) derived from Samut Sakhon provincial Department of Disease Control, Ministry of Public Health of Thailand.

3. Viral RNA extraction

The viral RNA was extracted and purified from all stages of the mosquitoes: larva, pupa, and adult, and plasma and/or buffy coat following the manufacturer's instruction Invisorb®Spin Virus RNA Mini kit (Invitek Gmbh, Germany) by mixing the approximate samples in a 1.5 ml eppendorf tube with 600 µl lysis buffer RV, 20 µl carrier RNA and 20 µl proteinase K. After incubating for 10 minutes at 65^0C in a heat box the 400 µl binding solution was added to the sample which is transferred to the membrane filter tube. Washing by wash buffer R1 and R2 was performed by centrifugation at 8,000 rpm 30 sec. Forty µl viral RNA was eluted to be the template for experimental assay. RNA concentration and purity are quantified by Nano Drop 2000c spectrophotometer (Thermo scientific, USA) as shown by an A260/A280 ratio equal to 1.8-2.0. An RNA sample from the plasma and mosquito was divided into two

equal aliquot volumes, the screening dengue infection and serotyping and sequencing by targeting the *E* gene fragment. The RNA was kept at -80^{o}C for long term storage or at 4^{o}C for ready use.

4. Screening the dengue infection

Dengue serotype infection of *Ae. aegypti* mosquitoes was detected by using TaqMan probe based multiplex real time PCR. Dengue serotypes-specific primers and fluorogenic probe set were designed on the 3'NTR of the dengue virus (AITbiotech Pte Ltd, Singapore). All experiments were tested by using unrevealed highly specific primer pairs caused by commercial negotiated condition based on double-dye hydrolysis probes and using standard protocol from abTES™ Den 4 qPCR I Kit (AITbiotech Pte Ltd, Singapore) for simultaneous detection of dengue serotype 1-4 (DENV-1, DENV-2, DENV-3 and DENV-4) within a single tube without cross reactivity.

The DENV-1 probe was labeled with Cy5® at 5´end as the reporter dye and with a black hole quencher (BHQ) at 3´end, DENV-2 probe was labeled with FAM® and BHQ, DENV-3 probe was labelled with Texas Red® and BHQ, DENV-4 probe was labeled with Quasar 705® and BHQ, and CHIKV probe was labelled with HEX and BHQ. PCR cycling condition was established and validated on Bio-rad CFX96 and the result were analyzed with CFX manager software.

The infection of plasma samples was well-defined as molecular technique targeted *C/PrM* gene using the serotype-specific oligonucleotide primer with sensitivities of 94% (DENV-1), 93% (DENV-2) and 100% (DENV-3 and 4), compared with virus isolation (68). Nested RT-PCR were done using the instruction of QIAGEN® OneStep RT-PCR Kit (QIAGEN GmbH, Germany) for 1st PCR and followed by BIOTAQ™ PCR Kit (Qiagen, Germany) for nested PCR. An optimized condition was done following the condition as Table. 1. Amplicon sizes for 1st PCR was 511 bp by all strains and for nested PCR provided serotypes 1-4 were 482, 119, 290, 392 bp, respectively.

5. Dengue classification based on *E* protein gene

The purified RNA sample from the mosquito and human plasma were amplified for dengue infection by RT-PCR using the instruction of QIAGEN® OneStep RT-PCR Kit (QIAGEN GmbH, Germany) and nested PCR using the primer pairs specified in the conserve region of all dengue viruses. Each RT-PCR reaction mix containing 1x; 2.5mM $MgCl_2$ of 5x QIAGEN OneStep RT-PCR Buffer, 1x of 5x Q-Solution, 2 µl of QIAGEN OneStep RT-PCR Enzyme Mix, 400 µM of 10mM dNTP, 5 µl of 1 pg-2 µg RNA template each reaction, 0.4 µM E-gene specific DEUL-DEUR primer which are specified amplification DEN1-4. RNase free water was adjusted to be 25 µl in total. The optimized condition obtained within a thermal cycler (Eppendorf, USA) with 30 min of cDNA production at 50^{o}C, followed by 2 min for initial denature at 94^{o}C, 40 cycles for primer probing at 98^{o}C for 10 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec, 7 min for final extension at 72^{o}C (Table. 1). The amplicon size by RT-PCR is 641 bp. Nest-PCR was used to amplify the sequences following fermentas *Taq* DNA polymerase (Fermentas, Lithuania). The PCR reaction mixes comprise: one µl of diluted PCR product diluted 1:10 with PCR grade water as template, 1x PCR (NH_4Cl) buffer, 1.25 mM $Mgcl_2$, 0.2 mM dNTPs, 0.4 µM of DENUL-DENUR primer pairs as Table. 2 (Yenchitsomanus *et al*, 1996) and the total volume as 25 µl by PCR-grade water.

The cycling program for amplification is 3 min for initial denature at 95^{o}C, the cycling at 94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec, this cycle was repeated 40 times and then 7 min for final extension at 72^{o}C. The PCR product was run on 2% agarose gel (w/v), stained by ethidium bromide and a specific band of DEN1-4 (433 bp) and was visualized by gel based UV-visualization.

To study the prevalence of any dengue serotype viruses and the extent of variation sequences of the envelope gene in the sample from plasma and mosquito, RT-PCR and nested-PCR was undertaken. Each positive sample from the screening section was amplified again according to QIAGEN® OneStep RT-PCR Kit (QIAGEN GmbH, Germany) as described below. The *E* gene was amplified by RT-PCR (which produces 641 bp in size) using first primer pairs specific to the conserve region of the envelope gene of all dengue strains, namely DEUL-DEUR as in Table. 2 (85).

Table 1 Nested RT-PCR was done following the conditions for RT-PCR and PCR. Using for amplifying and classifying of dengue viruses based on E and C/PrM genes (68, 83).

PCR steps	RT-PCR conditions	PCR condition
Reverse Transcription	55^{0}C for 30 min	-
Initial denaturation	94°C for 3 min	94°C for 3 min
Denature	94°C for 30 sec	94°C for 30 sec
Anneal	50°C for 30 sec	55°C for 30 sec
Extend	72°C for 45 sec	72°C for 45 sec
	40x	35x
Final extension	72°C for 7 min	72°C for 10 min

The optimized condition was used within a thermal cycler (Eppendorf) with 30 min of cDNA production at 50^{0}C, followed by 2 min for initial denature at 94^{0}C, 40 cycles for primer probing as 98^{0}C for 10 sec, 50^{0}C for 30 sec and 72^{0}C for 45 sec, 10 min for final extension at 72^{0}C. Specific amplicons were visualized by 2% gel based UV-translumination.

Tenfold dilution of 1st PCR-product was used as the template of dengue serotyping by nested PCR. The PCR was conducted following Fermentas Taq DNA polymerase (Fermentas, Lithuania).

In brief, reaction mix comprise: One µl of diluted PCR product was diluted 1:10 with PCR grade water as template, 1x PCR (NH4Cl) buffer, 1.25 mM Mgcl2, 0.2 mM dNTPs, 0.4 µM of D1L-D1R for DEN1, D2L-D2R for DEN2, D3L-D3R for DEN3 and D4L-D4R for DEN4 detection as Table.1 (85). The cycling program for amplification is 3 min for initial denature at 950C, the cycling is 940C for 30 sec, 550C for 30 sec and 720C for 45 sec, this cycle was repeated 40 times and then 7 min for final extension at 720C. The PCR product was run on 2% agarose gel (w/v), stained by ethidium bromide and a specific band of DEN1 (504 bp), DEN2 (346 bp), DEN3 (198 bp) and DEN4 (143 bp) which were visualized by gel based UV-visualization as in Table. 2. The reliability of the amplification of all dengue viruses was confirmed by comparison with positive dengue strains, namely: AF180817 for DEN1, JF812114 for DEN2, JQ922554 for DEN3 and U18429 for DEN4.

Table 2 The oligonucleotide primers for detection on E gene fragments of dengue virus **(**83**)**.

Primers	Primer sequences (5'-3')	Products (bp)
E region		
DEUL	5'-TGGCTGGTGCACAGACAATGGTT-3'	641
DEUR	5'-GCTGTGTCACCCAGAATGGCCAT-3'	
DENUL	5'-GATCTCAAGAAGGAGCCATGCA-3'	433
DENUR	5'-ATGGAACTTCCCTTCTTGAACCA-3'	
Serotype specific primers in *E* region		
D1L	5'-GGGGCTTCAACATCCCAAGAG-3'	504
D1R	5'-GCTTAGTTTCAAAGCTTTTTCAC-3'	
D2L	5'-ATCCAGATGTCATCAGGAAAC-3'	346
D2R	5'-CCGGCTCTACTCCTATGATG-3'	
D3L	5'-CAATGTGCTTGAATACCTTTGT-3'	198
D3R	5'-GGACAGGCTCCTCCTTCTTG-3'	
D4L	5'-GGACAACAGTGGTGAAAGTCA-3'	143
D4R	5'-GGTTACACTGTTGGTATTCTCA-3'	

6. Cloning based on the *E* gene of the dengue virus for sequencing

The PCR product was ligated into pGEM T-easy vector plasmid (Promega, USA) with optimal conditions and transformed into competent cell (*E. coli* strain DH5α) to propagate the vector quantity (Fig. 12).

The ligation mixture include 2x rapid ligation buffer with 5 µl, pGEM T-easy vector for 1 µl, T4 ligase enzyme for µl, PCR product for 1.5 µl and the total volume adjusted up to 10 µl per reaction with deionized water. The 100 µg/ml ampicillin-contained Luria-Bertani (LB)-plates was used to culture the transformed-bacteria by incubation, overnight at 37^{o}C. 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) (Thermo Scientific Inc., USA) was supplemented into the plate for energy

source of the bacteria, which was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) (Thermo Scientific Inc., USA).

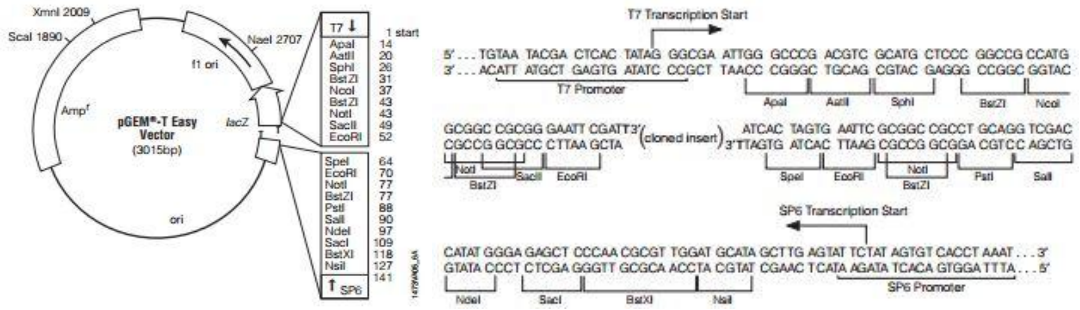


Figure 11 Map and sequence with reference points of pGEM®-T and pGEM®-T Easy Vector Systems (84).

Pure-white colonies expected that its plasmid containing the targeted gene fragment were selected to culture in LB broth under shaking at 200 rpm at 37^{o}C, overnight. Purified plasmid DNA was done following the procedure of purified kit (Invitek GmbH, Germany). For sequencing based on cycle sequencing method the plasmid DNA sample was submitted to 1st-based company (Malaysia).

7. Data analysis

Transmission cycle of dengue viruses by season was estimated by highest frequency of each strain of dengue infection within the area of interest. This rate was calculated and analyzed from the clusters of positive samples in both plasma and mosquitoes. Then the rate was used to construct the graphs which are correlated with Thai season during the year of 2012-2013.

7.1) Relationship between dengue transmission cycles and environmental factors

Relation between dengue cycles (in mosquito) as above, dengue case report during year of studied periods, maximum/mean/minimum values of weather (temperature, relative humidity) was studied to show the monthly relative graphs.

7.2) Sequence analysis

7.2.1) Sequence identity

The high-quality sequences were checked by choosing the region with good signal of histogram and cutting the primer sequences. Consensus sequences were tested with their homology sequence in Gen-Bank nucleotide database using the basic alignment program of the National Center for Biotechnology Information (NCBI) Gen-Bank database webserver (http://blast.ncbi.nlm.nih.gov/Blast). All sequences were submitted in Gen-Bank and were assigned for accession number as KM003974-KM003970 for DENV1, KM003971-KM003982 for DENV2 and KM003983-KM004010 for DENV4 except for the sequences of DENV3, because it was less than 200 bp in size. However, all DENV serotypes were confirmed and also genotype by using dengue genotype determination and online webserver; http://www.denguedb.org/viewGenotypeResult.aspx (Fig. 14).

7.2.2) Infection rate and Dengue virus serotype

The prevalence rate prioritized the frequency of each strain of dengue infection which is relevant to the area it was obtained from. This rate was analyzed and calculated from cluster positive samples in both plasma and mosquito. Seasonal infection types of dengue viruses amplified from the infected mosquito and plasma were determined to be single-, double-, triple-, and quadruple infection by manual analysis. The type was studied and compared by season. Dengue proportion differences of infection types each season (not by comparing of mean difference) in the *Ae. aegypti* mosquito vector and human within serotype from the year of 2012 to 2013 was statistical performed by X^2 test (using SPSS v.20.0). This is because the most percent of the infection rate each season were greater than an expected value as 20%. The statistical significance could be defined if the p-value less than 0.05.

7.2.3) Diversity and pairwise p-distance

The comparative sequences and the substitution of nucleotide and amino acid was determined using the ClustalW function of the BioEdit software version 7.1.3.0. The silent/non-silent amino acid ratio and mean diversity was determined manually along with the alignment sequences. The sequence was identified for the relative organism by pubmed online blast: http://blast.ncbi.nlm.nih.gov/Blast.cgi. The genetic variation analysis the alignment sequence was done by Bioedit 2.0. The viral diversity of nucleotide (nt´) and amino acid (AA) was calculated by the number of nt´/AAs substitutions divided by the total number of nt´/AAs of all sequences (18). The parameter was studied and paralleled with pairwise p-distance value, the mean and ranges of which were measured by using MEGA 6 (85).

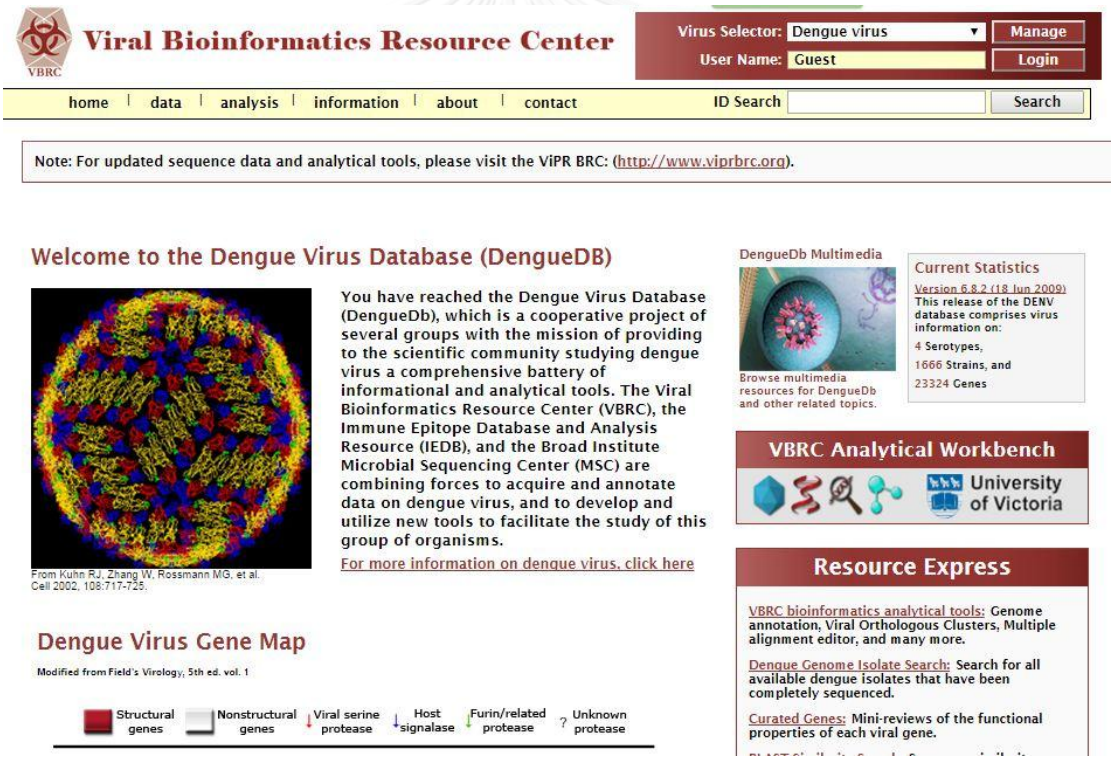


Figure 12 Online website of Viral Bioinformatics Resource Center using for determine dengue genotype (86).

7.2.4) Mutation position expose on surface of *E* protein structure

Significant mutation position exposed on the surface of *E* protein structure was demonstrated by using comparative protein structure and viewing the 3D-structure by using the SWISS-MODEL (Fig.15) (87). The comparative protein comprises: 4GSX, 3UZV_A, 3UZE_A and 3WE1 to be protein templates for studying the models of DENV1-4, respectively. Mutation position on the surface-expose protein structure could be affected on the potent of the epitope for escaping from human neutralizing antibodies.

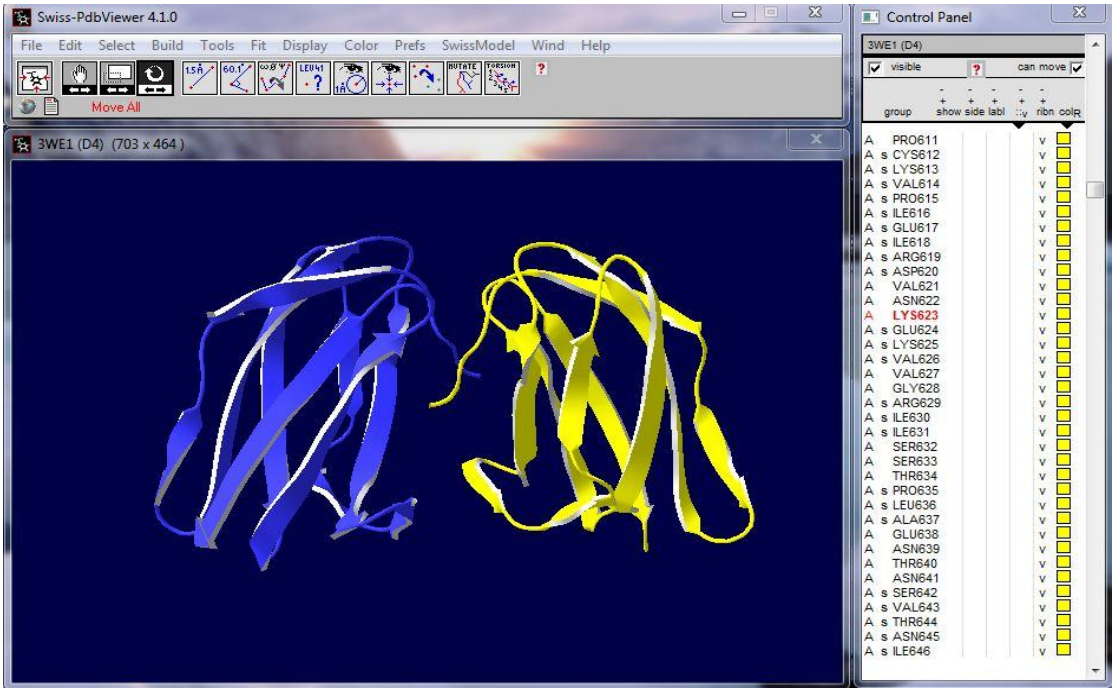


Figure 13 SWISS-MODEL using to identify mutation positions each dengue serotype based on E-gene (87).

7.2.5) Codon usage bias

Analysis of codon usage was studied based on the relative synonymous codon usage (RSCU) indices of individual codon in each serotype were calculated by MEGA6 (85).

Figure 14 Datamonkey website using for analyzing the selection pressure (88).

7.2.6) Selective pressure analysis

The selective pressure analysis in the dengue sequences data was performed by using the online adaptive evolution server http://www.datamonkey.org/ (Fig. 16). Datamonkey tools three corresponding methods for identifying locations under selection (88). *Single likelihood ancestor counting (SLAC)* method process an alignment with >100 sequences and codons per minute, by means of likelihood-based branch lengths, nucleotide and codon substitution parameters and ancestral sequence reconstructions, non-neutral evolution in >50 sequences alignments. *Fixed effects likelihood (FEL)* method use to fit an independent d*N* and d*S* to every site in the context of codon substitution models and test whether d*N* = d*S*. *Random effects likelihood (REL)* method is somewhat susceptible to Type 1 errors, especially for small datasets, where parameter estimates are likely to have large associated errors. The positive selection based on the non-synonymous to synonymous substitution ratio ($\omega = dN/dS$) will be executed by employing any method in the server.

If the ω value < 1 then purifying (stabilizing) selection, ω value = 1; neutral (i.e. no) selection and ω-value > 1; positive selection.

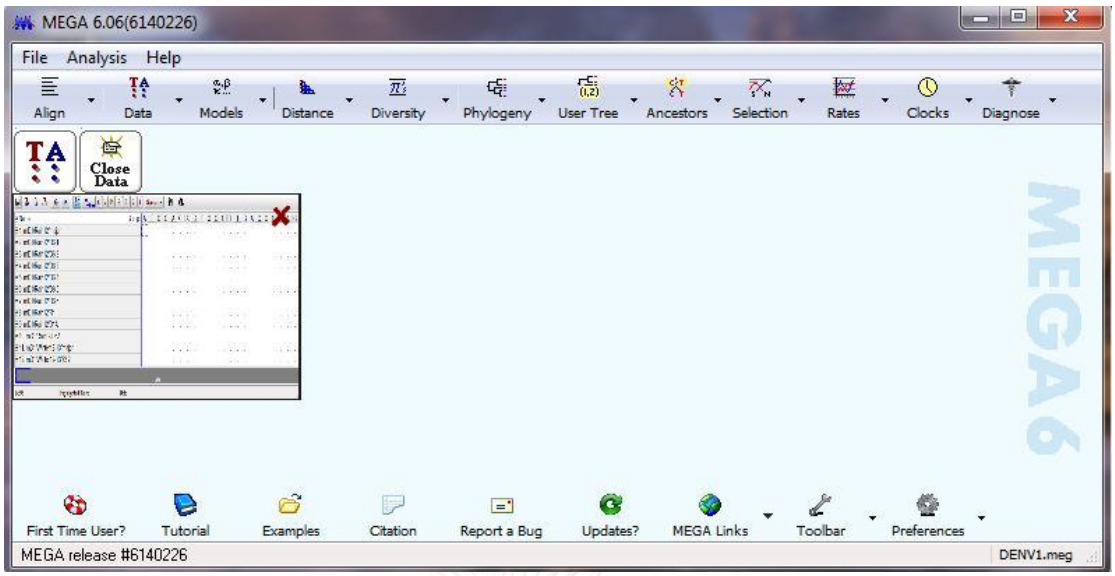


Figure 15 MEGA 6 using for construct the evolutionary tree of all dengue serotype (86).

7.2.7) Phylogenetic construction

To study the epidemiology and evolution of the virus the Neighbour-joining (NJ) method was used to construct tree using MEGA 6 (85)(Fig. 17), representing the partial E gene of all DENV serotypes. The trees of DENV-1, DENV-2 and DENV-3 were computed by determining the transitions and transversions using Kimura's 2-parameter distance model, except for DENV-4 Maximum Composite Likelihood model which was used instead. Bootstrap statistics was performed to support the individual node by 1,000 replicates. The trees of DENV-1 to 4 were constructed based on nucleotides sequences under the accession no. mentioned above. Out-group of each tree we used DENV-2 (JF967989) and DENV-3 (JF968088) for DENV-1; DENV-1 (D00502), DENV-3 (JF968088) and DENV-4 (U18429) for DENV-2; DENV-1 (D00502), DENV-2 (JF968989) and DENV-3 (JF968088) for DENV-4.

CHAPTER IV
RESULTS

1. Sample collection data

The mosquito samples were individually collected by season during the study period. There are 129 mosquitoes in rainy season 2012, 119 mosquitoes in winter season 2013, 116 mosquitoes in dry season 2013 and 58 mosquitoes (collected from the home where the resident having dengue positive) in rainy season 2013. Blood samples were collected in rainy season 2013, of which there are 52 samples. In addition, raw data of the number of monthly dengue cases (during 2008-2013) derived from Samut Sakhon provincial Health Office, Ministry of Health, Thailand. Continuous data of temperature and relative humidity (during 2008-2013) within the area derived from the Southeast Asia START Regional Center.

2. Dengue detection and typing

In order to determine the prevalence of the currently circulating virus and its transmission through the dengue cycle during 2012-2013, the primary vector (*Ae. aegypti*) collected by season was used to qualitatively test for the viral RNA by TaqMan probe with high sensitivity (Table.3 and Fig. 16).

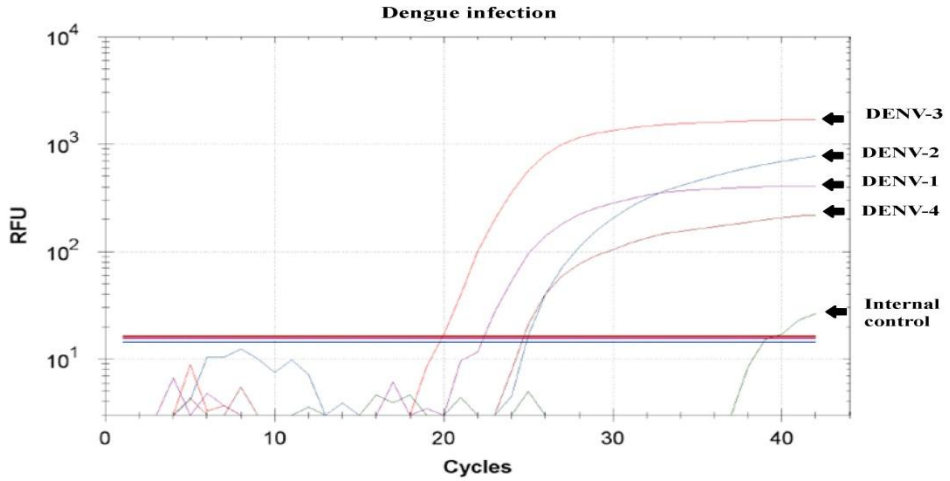


Figure 16 Amplification (log-graphs) results of dengue viruses by using TaqMan probe based-multiplex real time PCR. Individual-mosquito sample infected with all

four serotypes. If it showed negative for dengue infection, only internal control (IC) signal was amplified and IC also representative for high quality RNA samples.

The significantly highest rate was shown in rainy season (collected the sample during August-November in 2012) with 63.1% (81/129) when compared to the rate of other seasons, 4.24% (5/119) in February 2013 of winter season in 2013, 4.35% (5/116) in April 2013 of dry season and 8.7% (5/58) in July 2013 of rainy season (Fig. 17). In addition, the DENV1-4 from the insect sample discriminated by nested RT-PCR were used to study the prevalence based on infection rate by season (Fig. 18). Although the infection rate between rainy and others referred to as a decline phase, the trend of infection between winter and early-rainy (2013) season increased.

Table 3 Specific channels of CFX96 software represented DENV1-4 positive with different sensitivity.

Targets	CFX96 channel (color)	Sensitivity
DENV1	Cys5 (Dark-brown)	10.30 copies/µl
DENV2	FAM (Blue)	2.82 copies/µl
DENV3	Texas Red (RED)	23.77 copies/µl
DENV4	Quasar705 (Purple)	3.22 copies/µl
Internal Control	HEX (Green)	-

The trend above seems to correspond with provincial-DHF situation which also occurred in the same period (data not shown). Additionally, plasma samples of asymptomatic local subjects of the studied site which collected in May 2013 showed dengue positive with 9.62% (5/52). 2-samples were found co-infection or double infections (DENV-1 & 4 and DENV-3 & 4) and 3-samples were single infection with DENV-4.

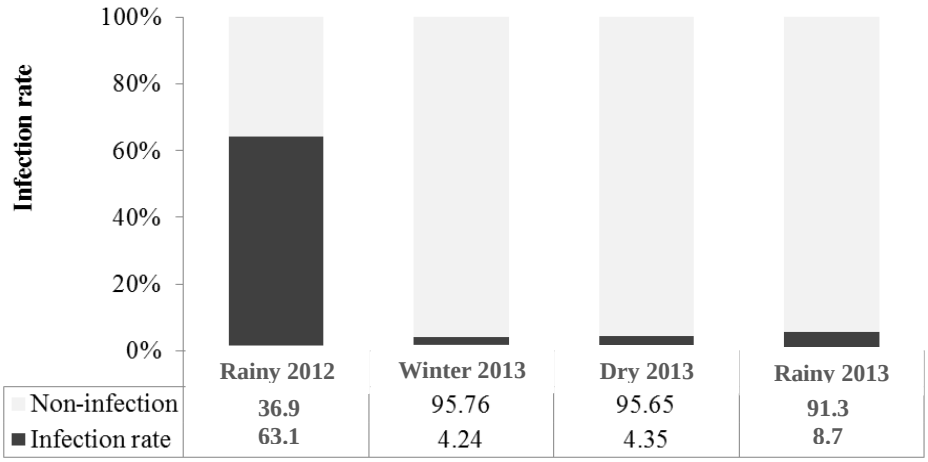


Figure 17 Dengue infection rate in the mosquito vectors (*Ae. aegypti*) during 2012-2013.

Interestingly, these positive samples were not detected the infection based on nested RT-PCR targeted on C/prM gene region. Since the low sensitivity of the method based on the oligonucleotide primers mentioned as above is not enough to amplify the targeted-gene in the case with the low viral load. However, the sample can be amplified by the same technique as mention above by using different oligonucleotide primers targeting on *E* gene region.

3. Transmission cycle and proportions of dengue infection in vector and human host

Dengue infection in the mosquito and the volunteer was determined as percentage of infection based on high sensitivity technique, nested RT-PCR. Rate of infection in the mosquito was demonstrated by total and serotypes within season (Fig. 19A).

In mid-rainy season of 2012, we found 63.1% of total infection which were 29.41% for DENV-1, 27.06% for DENV-2, 48.24% for DENV-3 and 20% for DENV-4.The percent of infection was calculated from number of serotype infections divided by total samples by each season. Next consecutive season in 2013, 4.24% of total infection in winter season was demonstrated as 1.69%, 3.39%, 4.24% and 3.39% of DENV1-4 respectively. In dry season, 4.35% of total infection was only DENV-4.

There were 8.7% of total infection in rainy season which was 2.17% and 4.35% of DENV-1 and 3. In human host 9.62% of total infection was found, which is 1.62%, 1.62% and 9.62% of DENV-1, 3 and 4 respectively.

All four serotypes of the dengue virus were detected in the mosquito samples by four types of infection; single, double, triple and quadruple infection (Fig. 19B). There are also found DENV-1, 3 and 4, except DENV-2, in asymptomatic human host. The single and double infections were surprisingly found in their plasma samples. Determining the proportion of infection types in each season as above, there are at least one infection type (single infection) in rainy season of 2012 which showed statistically important differ from others as p= 0.001, df= 3. In 2013, only single infection was found in winter and dry season. By contrast, no statistically difference in proportion of the infection types in both the mosquito from the winter season (p= 0.819, df =2) and in the human (p= 0.655, df = 1) in rainy 2013.

4. Correlation between dengue transmission cycles and environmental factors

Recent dengue incidence which increases both morbidity and mortality rates was associated with the potential of the viral transmission. The relevant factor that is possible regulation the disease spread through seasonal bottleneck was significantly offered, mainly; weather (76, 89). The geographical pattern of dengue epidemiology in Thailand is hardly differently occurred by time to time or year to year (36, 43, 79, 90). This might be the significant evidence to push and potentially develop the dengue dynamics within the countries and area of each other. Vector control could be possible to succeed more than vaccine production which has been developing. Only effective method in surveillance would contribute the control approach later.

Vector competence has been reported that depended on an infective including the longer mosquito survival with shortening incubation period of viral replication (75, 91-93). In addition, the length of each development stage of the mosquito also controlling the length of the life cycle will vary with the temperature (Temp) and relative humidity (RH) of the season (89, 94, 95). Especially, optimized-temperature reduced the length of viral EIP in the mosquito (76). Each cycle a year was characterized into four-phases; L: latent/quiet phase, I: increase phase, P: plateau phase and D: decline phase (89).

The dynamic spread of the dengue within each phase seemed likely to associate with both Temp and RH significantly (89). Lowest number of cases found in L-phase matched with wide-range of RH (50-85%) and mean-Temp was 28^{o}C-35^{o}C. I-phase happened with highest-RH (70%-90%) and mean Temp range 27 ^{o}C-30^{o}C, P-phase with declining RH from 90% to 40% and mean-Temp range 30-32^{o}C, and D-phase with highly fluctuation of both RH (40%-85%) and Temp (22 ^{o}C-40^{o}C). Trend in number of dengue cases of I and P-phases mentioned above was clearly depended on the moment-temperature within the phase and it was regulated by highest-RH at previous-phase (Fig.20). This humidity might be the important factor that contributed the vector density, which also increase the future risk of dengue infection and spread the disease later (14).

However, only the weather condition may not be enough information for determining the trend of both viral transmission and also the disease prevalence. Further novel surveillance focusing on the principal dengue vector must be advanced to access an effective tool which uses to reduce the emergence of dengue disease within an endemic and/or new areas. Therefore, focusing on the genetic variation and evolutionary dynamics of the co-circulating viruses might be answered an aspect of the relationships between the morbidity rates and the viral transmission cycle within the same period.

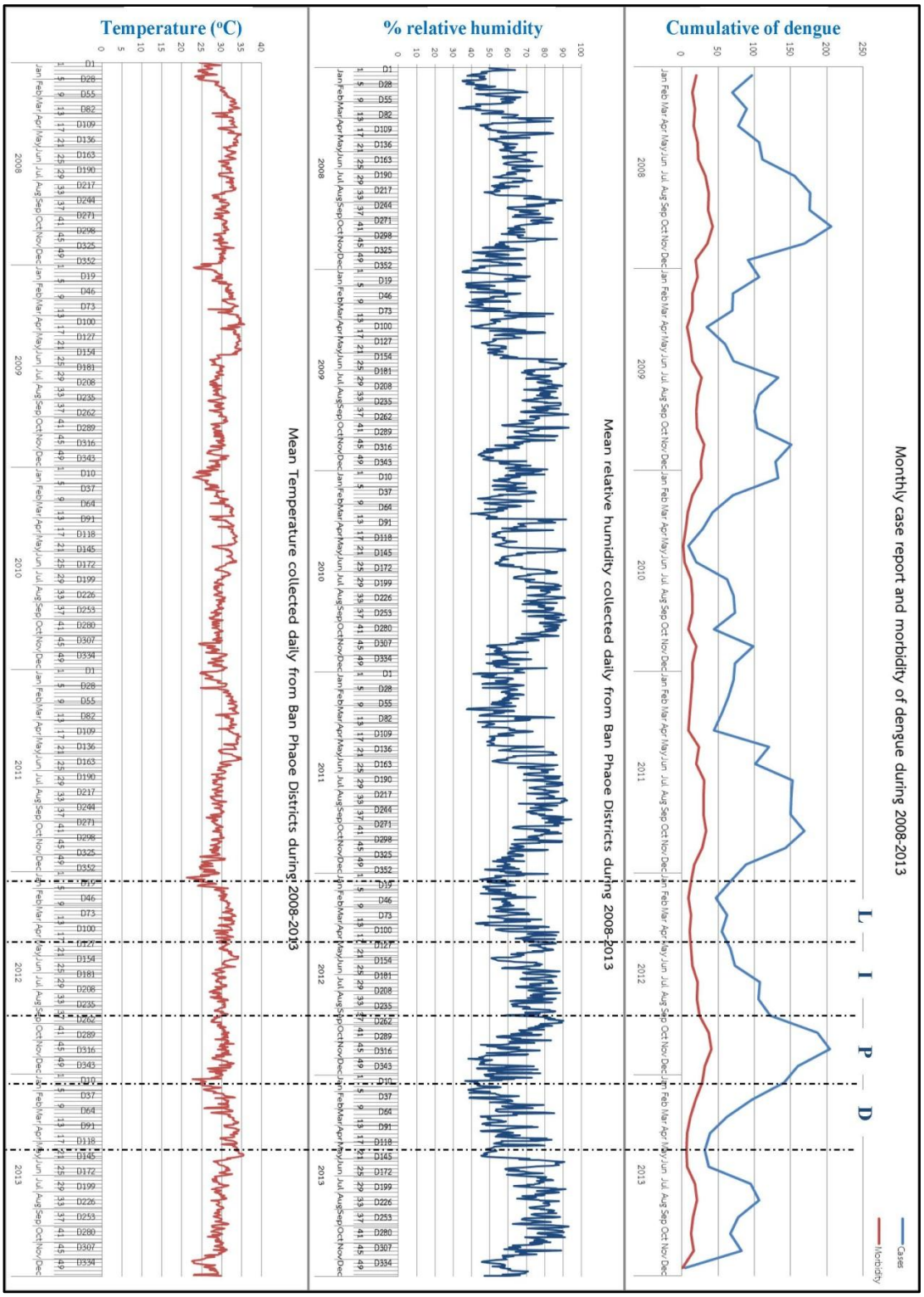


Figure 18 Correlation between dengue cycles and weathers (temperature and relative humidity) during 2008-2013. Transmission dynamics were represented in four-time points; L: latent phase, I: increase phase, P: plateau phase and D: decline phase (89).

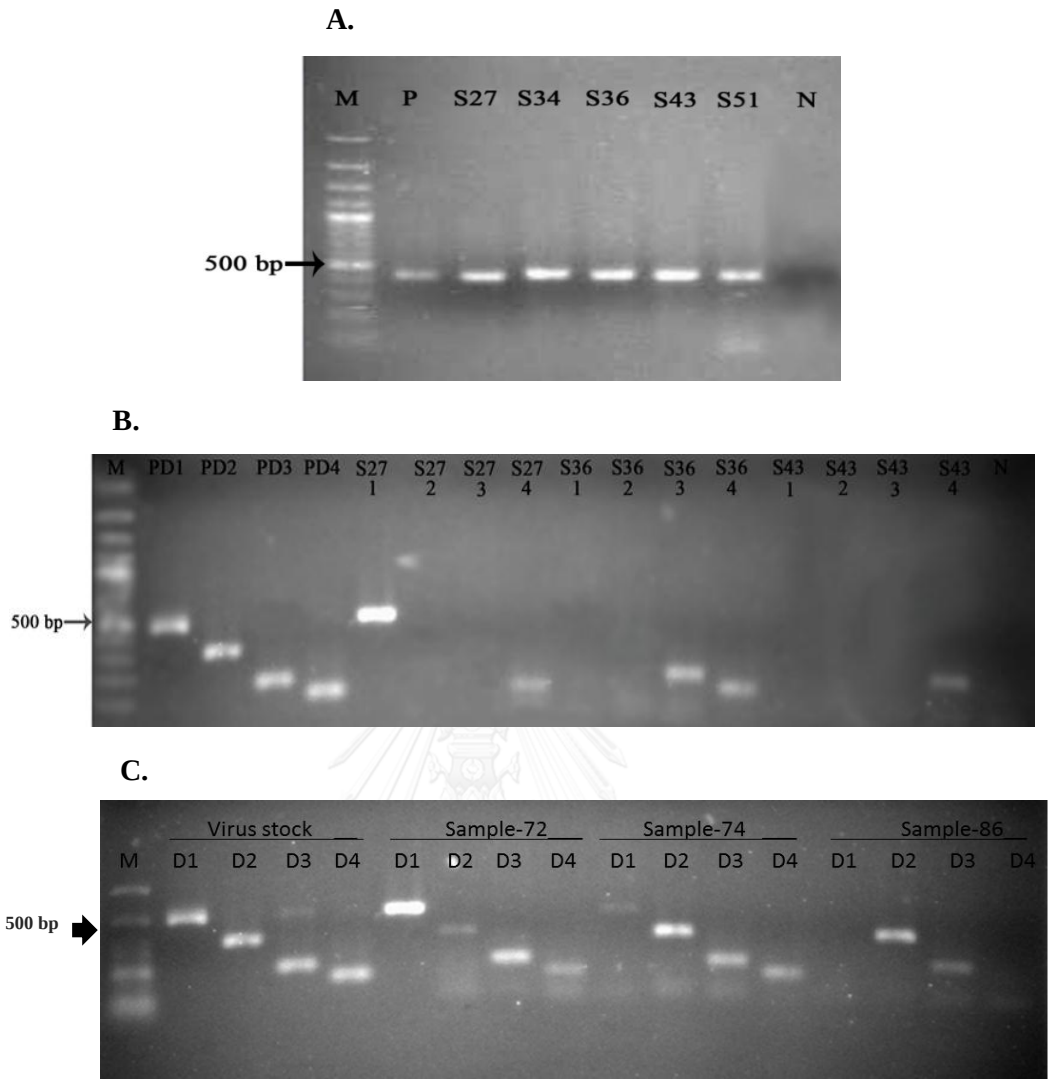


Figure 19 Dengue serotype identification by nested RT-PCR based on the *E* gene region. A) 1st PCR product approximately 433 bp. Lane of P: positive, S27: sample-27, S34: sample-34, S36: sample-36, S43: sample-43, S51: sample-51 and N: negative control.

B) Detection of dengue virus in plasma samples. Lane of M: ladder, M: ladder, PD1-4: positive for DENV1-4 respectively, S27-1 to 4: typing of DENV1-4 respectively in sample-27, S36-1 to 4: typing of DENV1-4 respectively in sample-36, S43-1 to 4: typing of DENV1-4 respectively in sample-43 and N: negative control. C) Detection of dengue virus in individual mosquito. Lane of M: ladder of which, M: ladder, PD1-4: positive for DENV1-4 respectively, S72 and S74 positive for DENV1-4, respectively, S86 positive for DENV-2 and 3.

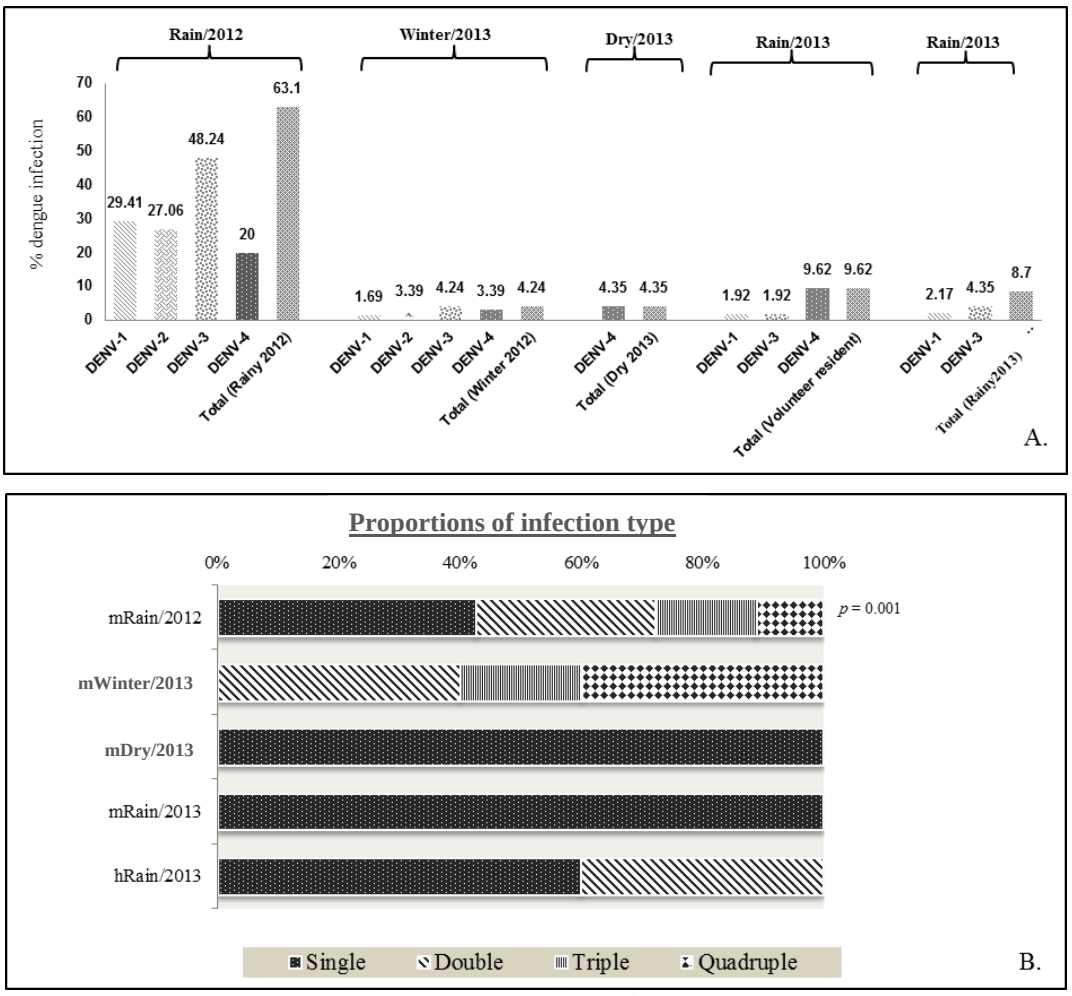


Figure 20 Percent of infection and dengue serotypes in mosquitoes and human samples. A) Dengue infection rate in *Ae. aegypti* mosquitoes by season and in human. B) Proportions of dengue infection in *Ae. aegypti* mosquito vector and human within serotype. “m” and “h” refers to mosquito and human host respectively. mRainy season in 2012; single, double, triple and quadruple infection 42.59, 29.64, 16.66 and 11.11% respectively; mWinter during 2012 and 2013; double, triple and quadruple infection; 39.95, 20.09 and 39.95% respectively; only single infection found in mDry season in 2012-2013 and mRainy season in 2013. Both single and double infection status found in local human host were 59.98 and 40.02%. There is statistically significant differ by proportion of infection types in the rainy season 2012, $p < 0.05$.

5. Genetic diversity study and pairwise-distance within dengue serotype

The diversity of the pairwise genetic of dengue virus targeted on envelope gene fragment was studied with respect to mean diversity and pairwise distance. In addition, the codon usage pattern within individual synonymous substitution site was calculated by season. DENV-1, the mean diversity of nucleotide (nt´) from the mosquito captured during rainy and winter seasons of the year 2012 and the rainy season of the year 2013 were 0.59%, 0.21% and 0.21% respectively, and 1.15%, 0.42% and 0.49% calculated from amino acid (AAs) sequences (Table. 4). On average, mean diversity (nt´/AAs) between mosquitoes were 0.30% / 0.64% and that from human host as 0.36%/ 0.67%.

The p-distance range of nt´ was from a mean of 0.89 to 1.36% (range: 0.25% - 2.03%) and of AAs had a mean 0.02 to 0.03% (range: 0.01 - 0.04%). There is a different p-distance values of nt´ derived from vectors and humans but the p-distance value from AAs is not differ. In DENV-2, the sequence was only derived from the vector and the mean diversity of nucleotide and amino acid was 0.24% and 0.41%. The p-distance value was 0.03% (range: 0-0.01%) and 0.13% (range: 0-0.02%) respectively. In DENV-3, mean diversity varied by season which was 0.26% calculated from nt´ and 0.40% AAs. The sequence from human host was 1.90 % calculated from the nt´ sequence and 1.68% from the AAs sequence. The p-distance value calculated from nt´ and AAs were 0.02% (range: 0.01-0.04%) in the vector and 0.06% (range: 0.03-0.09%) and 00.07% (range: 0.02-0.14%) in the human host. In DENV4, the mean diversity calculated from nt´ and AAs was 0.80% and 1.19% from the vector and 1.76% and 2.94% from the host. The p-distance value calculated from nt´ and AAs were 0.02% (range: 0-0.03%) and 0.03% (range: 0.01-0.06%) from the vector and 0.04% (range: 0.02-0.09%) and 0.09% (range: 0.03-0.19%) from the human host.

Table 4 Mean diversity, pairwise p-distance and selective pressures of all dengue viruses (DENV1-4) were shown as Table A-D, respectively. The symbol "na" is indicated that non-analysis due to the alignment sequences must include less than

A. (DENV-1)

Season (Samples)	**No. clones**	**Substitution NS/S**	**Mean diversity of nt´ & AA**	**p-distance (%) of nt´ & AA**		**Selective pressure dN/dS (ω)**
				Mean	Ranges	
mRainy/2012 (SMK-RD)	6	9/5	0.59 & 1.15	1.20 & 0.02	(0.25-2.03), (0.00-0.02)	**1.243**
mWinter/2012 (SMK-W2)	11	6/5	0.21 & 0.42	0.89 & 0.02	(0.25-1.53), (0.01-0.03)	0.859
mRainy/2013 (SMK-MRv)	11	7/2	0.21 & 0.49	1.36 & 0.03	(0.76-1.78), (0.02-0.04)	**1.032**
mTotal			**0.30 & 0.64**	**0.86 & 0.02**	**(0.25-2.03), (0.00-0.04)**	
hRainy/2013 (SMK-HP27)	8	7/3	**0.36 & 0.67**	**0.01 & 0.02**	**(0.00-0.01), (0.00-0.03)**	**1.080**

B. (DENV-2)

Season (Samples)	**No. clones**	**Substitution NS/S**	**Mean diversity of nt' & AA**	**p-distance (%) of nt' & AA**		**Selective pressure dN/dS (ω)**
				Mean	**Ranges**	
mRainy/2012 (SMK-R2)	8	1/0	0.12 & 0.37	0.00 & 0.01	(0.00), (0.00-0.01)	**na**
mWinter/2012 (SMK-W72)	8	2/0	0.08 & 0.25	0.00 & 0.01	(0.00-0.01), (0.01-0.02)	1.000
(SMK-W74)	8	3/10	0.53 & 0.61	0.01 & 0.02	(0.00-0.01), (0.01-0.02)	**0.190**
mTotal			**0.24 & 0.41**	**0.003 & 0.013**	**(0.00-0.01), (0.00-0.02)**	

C. (DENV-3)

Season (Samples)	No. clones	Substitution NS/S	Mean diversity of nt´ & AA	p-distance (%) of nt´ & AA		Selective pressure dN/dS (ω)
				Mean	Ranges	
mRainy/2012 (SMK-RL6)	10	1/0	0.07 & 0.21	0.01 & 0.01	(0.00-0.01), (0.01-0.02)	0.497
mWinter/2012 (SMK-W72)	6	3/4	0.74 & 0.94	0.03 & 0.04	(0.01-0.04), (0.02-0.06)	0.309
(SMK-W74)	9	1/0	0.07 & 0.24	0.01 & 0.02	(0.00-0.01), (0.01-0.03)	Na
mRainy/2013 (SMK-MRu)	9	1/0	0.07 & 0.21	0.01 & 0.01	(0.00-0.01), (0.00-0.02)	0.497
mTotal			**0.26 & 0.40**	**0.02 & 0.02**	**(0.00-0.04), (0.00-0.06)**	
hRainy/2013 (SMK-HP36)	**9**	**8/19**	**1.90 & 1.68**	**0.06 & 0.07**	**(0.03-0.09), (0.02-0.14)**	0.284

D. (DENV-4)

Season (Samples)	No. clones	Substitution NS/S	Mean diversity of nt´ & AA	p-distance (%) of nt´ & AA		Selective pressure dN/dS (ω)
				Mean	Ranges	
mRainy/2012 (SMK-RD9)	7	1/1	0.28 & 0.42	0.01 & 0.01	(0.00-0.02), (0.01-0.03)	0.407
mDry/2013 (SMK-D46)	3	2/2	1.31 & 1.96	0.03 & 0.04	(0.02-0.03), (0.03-0.06)	0.426
mTotal			**0.80 & 1.19**	**0.02 & 0.03**	**(0.02-0.03), (0.03-0.06)**	
hRainy/2013 (SMK-**HP36)**	10	14/4	**1.76 & 2.94**	**0.04 & 0.09**	**(0.02-0.09), (0.03-0.19)**	**1.080**

6. Protein Structure-Based Homology Modeling Analysis of the *E* gene

To understand the mutational effects against transmission, virulence and DHF incidence, conservation and mutation analysis of the gene enabled us to define specific-functional site of amino acid sequence in the structural protein (96). The information of the study will provide an essential key for determining the appropriate epitope in diagnostic approach and effective drug.

The study focused on amino acid variation in ED-I and III of the E glycoprotein gene (Fig. 21), which mediated to viral entry via host cell receptor. The aligned sequence was compared with those derived from the mosquito collected by season and from human subjects during 2012-2013. More variation each serotype was clearly shown. In addition, highly conservative sequences from both host type, which the virus used to maintain the viral structure from season to season were significant.

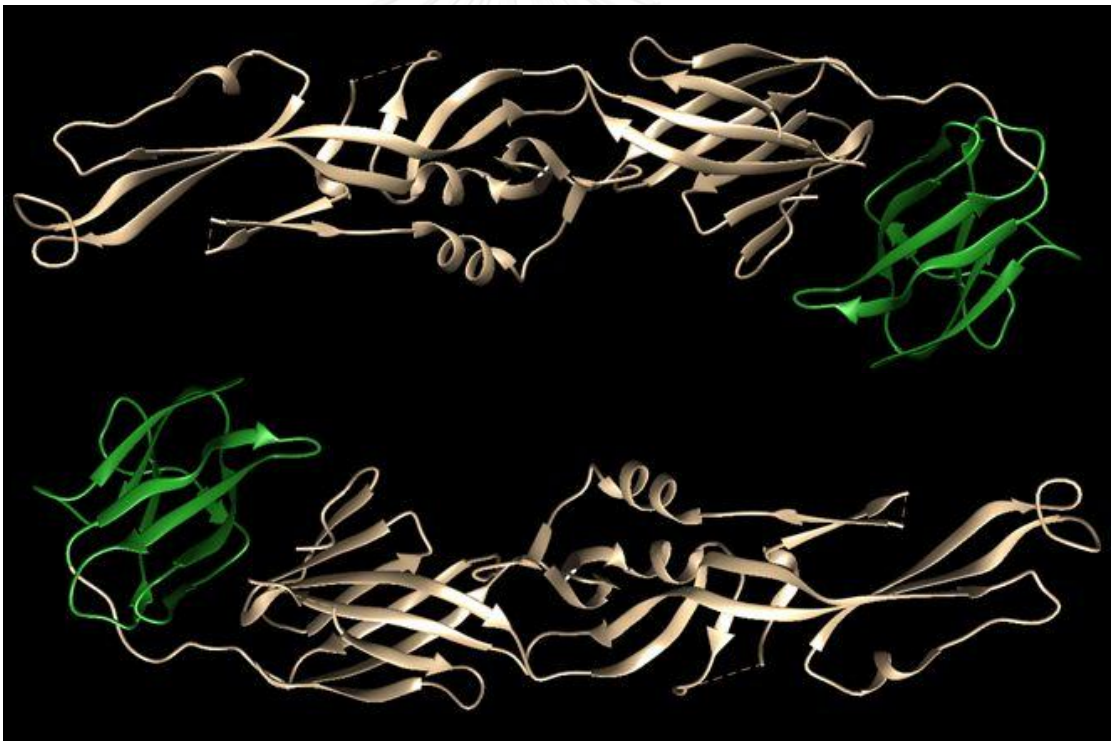


Figure 21 3D structure of dengue E-gene including EDI-II (grey color) and EDIII (green color), which is immunological-like protein.

In DENV-1, there are several positions of amino acid substitution found in the alignment sequence of interest (Fig. 22), which was separated by three groups based

on specific-host types (molecular signature of the virus) or not. Only the virus from the mosquito was studied the mutation by season. Two positions defined to be the signature of EDIII at V324I in B-strand and A345V in CD-loop (Table. 5).

In the mosquito during rainy season in 2012, there are 7 mutation positions in EDI; I270T, Q271R, S273P, H282R, K288E and stop codon mentioned the defective genome virus was shown in the position of K291*. Seven positions in EDIII; K310E (A-strand), T315I (AB-loop), D330G and K334E (BC-loop), E375K and I380V (F-strand) and L387S/* (G-strand). During winter in 2013, only one mutation was found in the connecting region between EDI and EDIII; S298P. EDIII were also found the mutation; S305P (A-strand), F337S (C-strand), T353A and S376C (DE-loop).

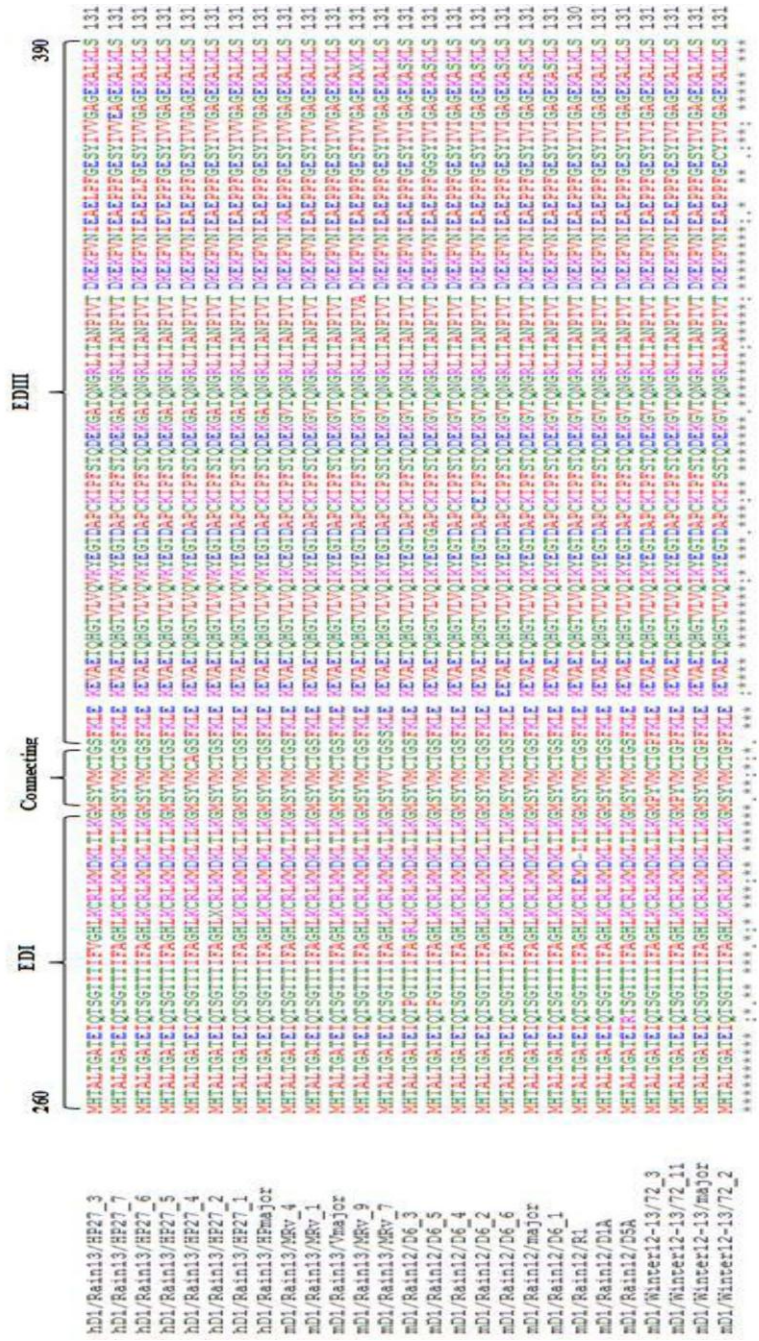


Figure 22 Extend sequence analysis of DENV-1 (from ClustalW) showed substitution positions and conserved regions of amino acid sequences corresponding to regions of E genes. The viral sequence (from the mosquito and human) covered at positions 260 to 390 of EDI-connecting-EDIII were shown. (*) is identical amino acid,
(space, : and .) referred to different amino acid in the column.

Table 5 Substitution positions of amino acid sequences corresponding to regions of E-gene of DENV-1. The mutation positions were determined by respected to the basis 3D structure of the PDB: 4GSX.

Season/ years	Substitution positions respecting to E gene in the						Host specific
	Mosquito			Human subjects			
	EDI	Connecting	EDIII	EDI	Connecting	EDIII	Human-Mosquito
Rainy /2012	I270T Q271R S273P H282R K288E K291*	-	K310E (A-strand) T315I (AB-loop) D330G K334E (BC-loop) E375K I380V (F-strand) L387S/* (G-strand)	-	-	-	V324I (B-strand) A345V (CD-loop)
Winter/2013	-	S298P	S305P (A-strand) F337S (C-strand) T353A S376C (DE-loop)	-	-	-	V324I (B-strand) A345V (CD-loop)
Rainy/2013	-	M301V	F306S (A-strand) Y326C (BC-loop) F337S (C-strand) T353A (D-strand) T359A (DE-loop) E368K (E-strand)	T276I A280V K284*	T303A	A369V (E-strand) P371L P372L G381E (EF-loop) I380V G381E (F-strand)	V324I (B-strand) A345V (CD-loop)

(Continued table)

Season/ years	Substitution positions respecting to E gene in the						Molecular signature
	Mosquito			Human subjects			
	EDI	Connecting	EDIII	EDI	Connecting	EDIII	H-position-M
			Y377F I380V (F-strand)				

(*); represented for stop-codon on the codon-frame. (ǂ); Amino acid mutation of predicted surface-exposed E residues and show 5Ao. -; not found for mutation. (_) ; the mutation is similar position with Lin et al (2012) (analysis of epitope with potent neutralizing mAbs) which generated a panel of 67 alanine-substitution mutants covering the surface-exposed E residues on DENV1-construct.

Early rainy season in 2013, no mutation was found in EDI but in the connecting region (M310V) and EDIII still shown by many mutations; F306S (A-strand), Y326C (BC-loop), F337S (C-strand), T353A (D-strand), T359A (DE-loop), E368K (E-strand), Y377F and I380V (F-strand).

In human subject (Table. 5), the mutation was found by all regions. T276I, A280V and K284* were found in EDI, T303A in the connecting region and A369V (E-strand), P371L-P372L- G381E (EF-loop), I380V and G381E (F-strand).

To clarify whether the mutation position effects on the antigenicity of the protein for binding the target receptor, the surface exposed-3D structure of the protein was labeled. The most significant mutations of the protein were; mF337S (Fig. 25); hT303A (Fig. 24); and A345V (Fig. 23) which might reduce the binding properties of neutralizing antibodies.

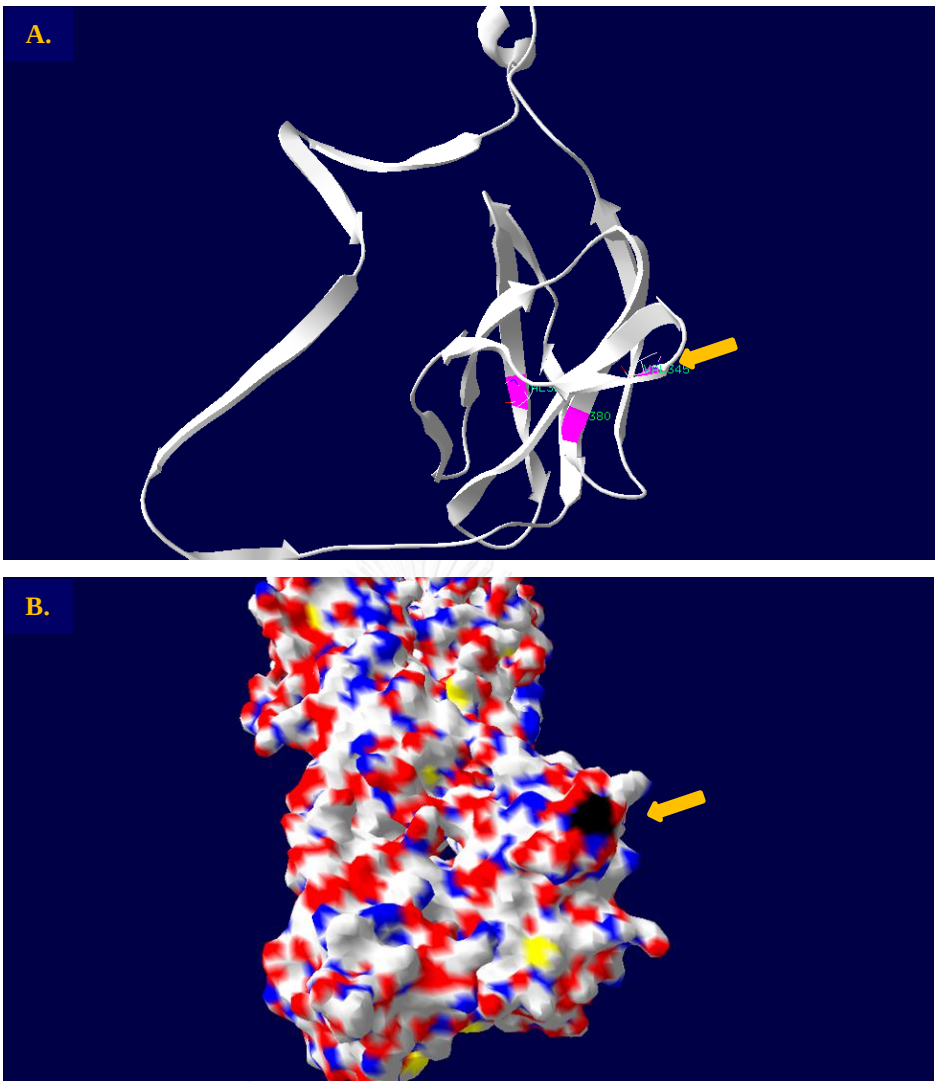


Figure 23 Three mutation position found in both human and mosquito's viruses which are representative as pink color (A), mentioned as molecular signature of DENV-1 in the area. Green arrow pointed to the position of D1EDIII-A345V mutation (black color) exposed to 3D-protein's surface (B).

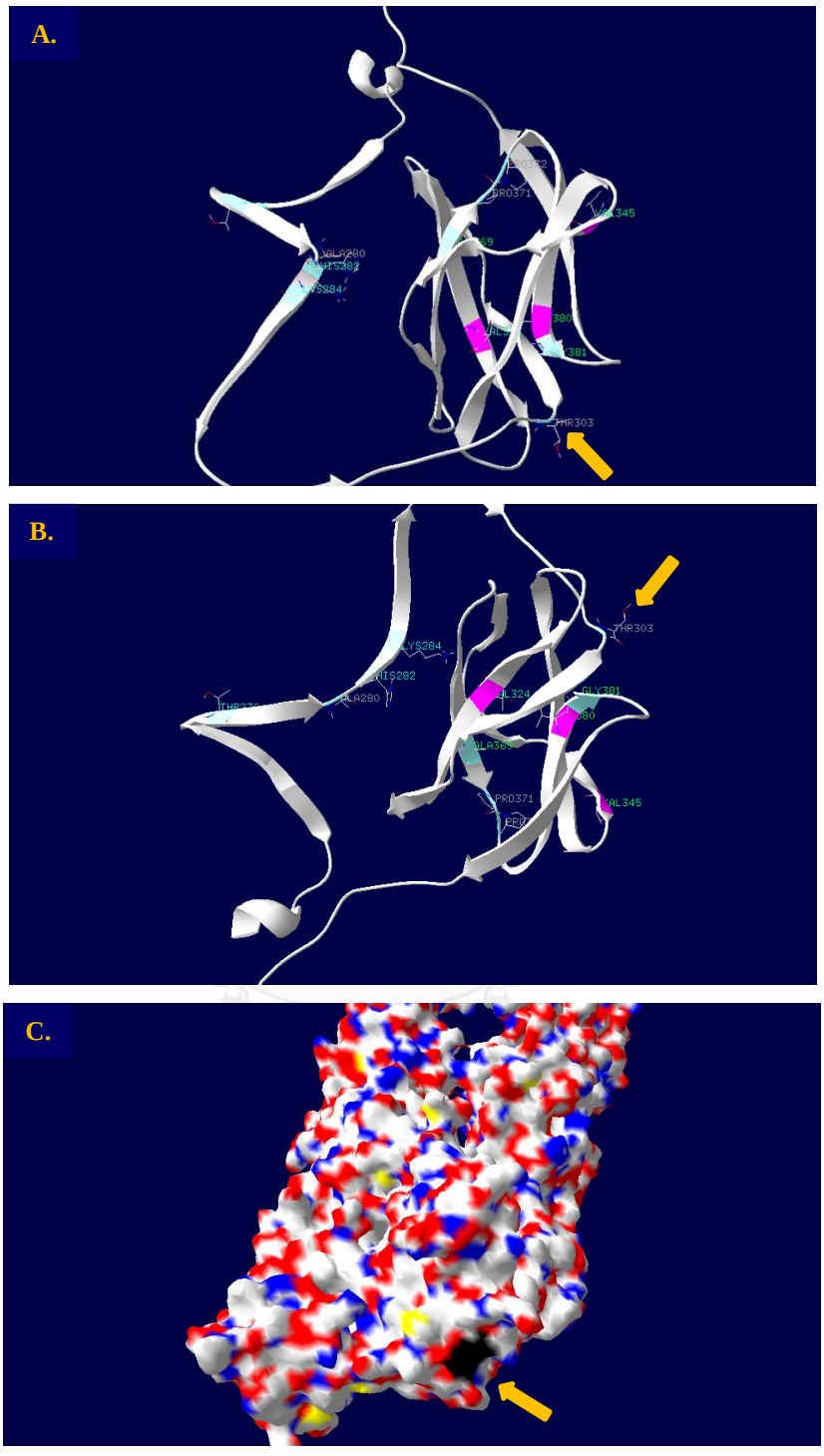


Figure 24 Mutation positions in human's virus which is representative as light-blue color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-hT303A (black color) mutation exposed to 3D-protein's surface (C).

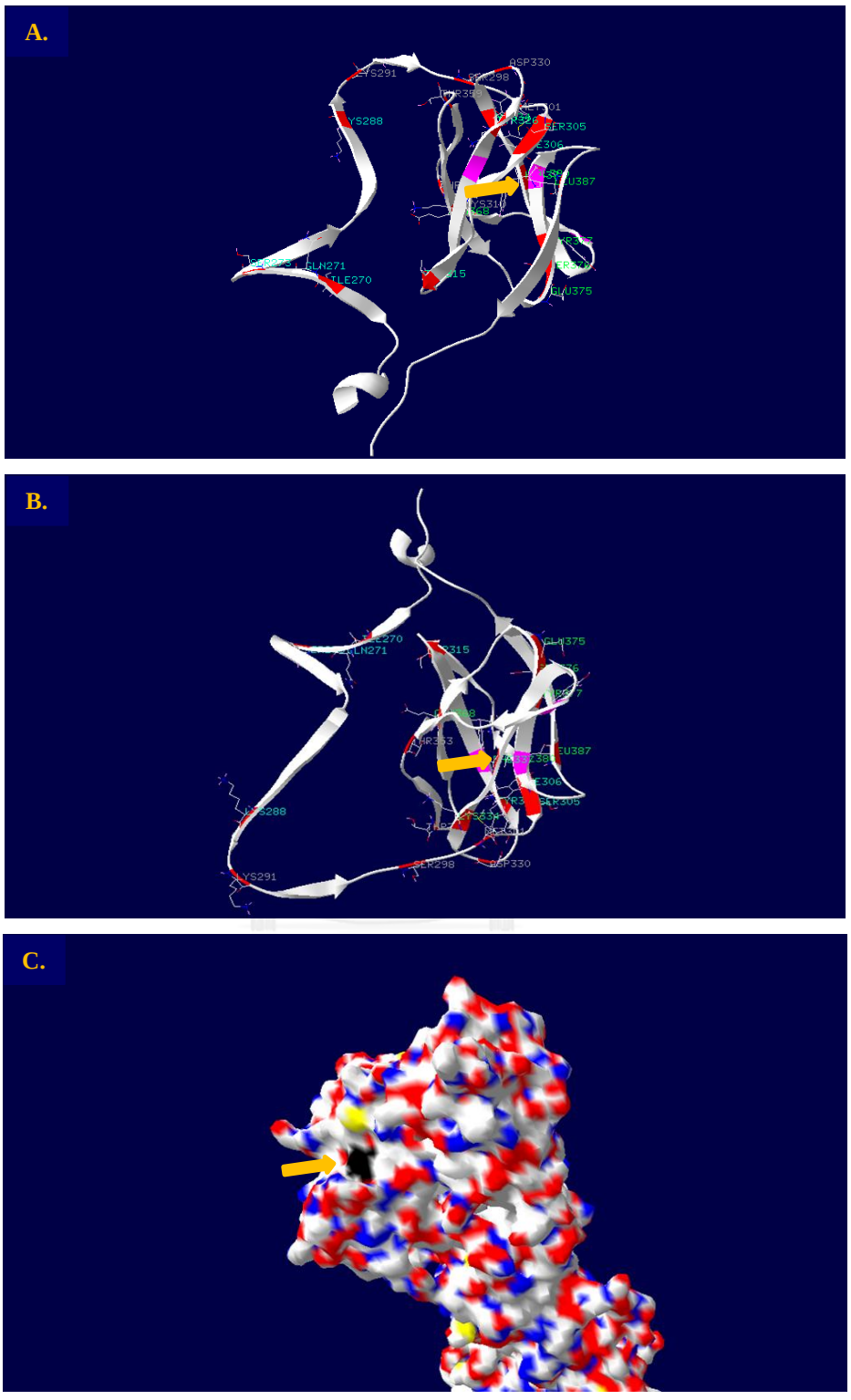


Figure 25 Mutation positions in the mosquito's virus which is representative as red color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-mF337S (black color) mutation exposed to 3D-protein's surface (C).

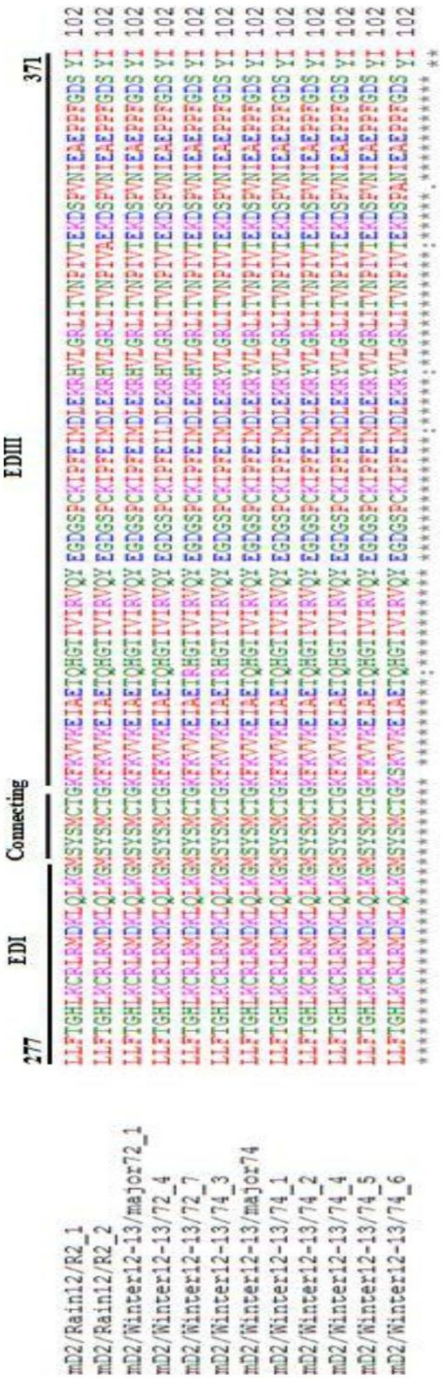


Figure 26 Extend sequence analysis of DENV-2 showed substitution positions of amino acid sequences corresponding to regions of E genes. The viral sequence (from the mosquito and human) covered at positions 277 to 371 of EDI-connecting-EDIII were shown. (*) is identical amino acid, (space, : and .) referred to different amino acid in the column.

In DENV-2, there are several positions of amino acid substitution found in the alignment sequence of interest (Fig. 26), which was separated by three criteria which was similar to those for DENV-1. Only the viral sequence from the mosquito was studied. During rainy season in 2012, there are 2 mutation positions in EDIII; Y339H (C-strand) and T352A (DE-loop).

In addition, there are four positions in EDIII; F306S (A-strand), Q316R (AB-loop), M333L (BC-loop) and V358A (DE-loop) during winter in early 2013 (Table. 6). No mutation was found in EDI and the connecting region. The most significant mutation of the protein was T352A (Fig. 27).

Table 6 Substitution positions of amino acid sequences corresponding to regions of E-gene of DENV-2. The mutation positions were determined by respected to the basis 3D structure of the PDB: 3UZV_A.

Season/years	**EDI**	**Connecting**	**EDIII**
Rainy /2012	-	-	Y339H (C-strand) T352A (DE-loop)
Winter/2013	-	-	F306S (A-strand) Q316R (AB-loop) M333L (BC-loop) V358A (DE-loop)

* ; represented for stop-codon on the codon-frame. ǂ ; Amino acid mutation of predicted surface-exposed E residues and show 5A^{o}. (-) ; not found the amino acid mutation. (_); the mutation is similar position with Lin et al (2012) (analysis of epitope) which generated a panel of 67 alanine-substitution mutants covering the predicted surface-exposed E residues on DENV1 prM/E expression construct.

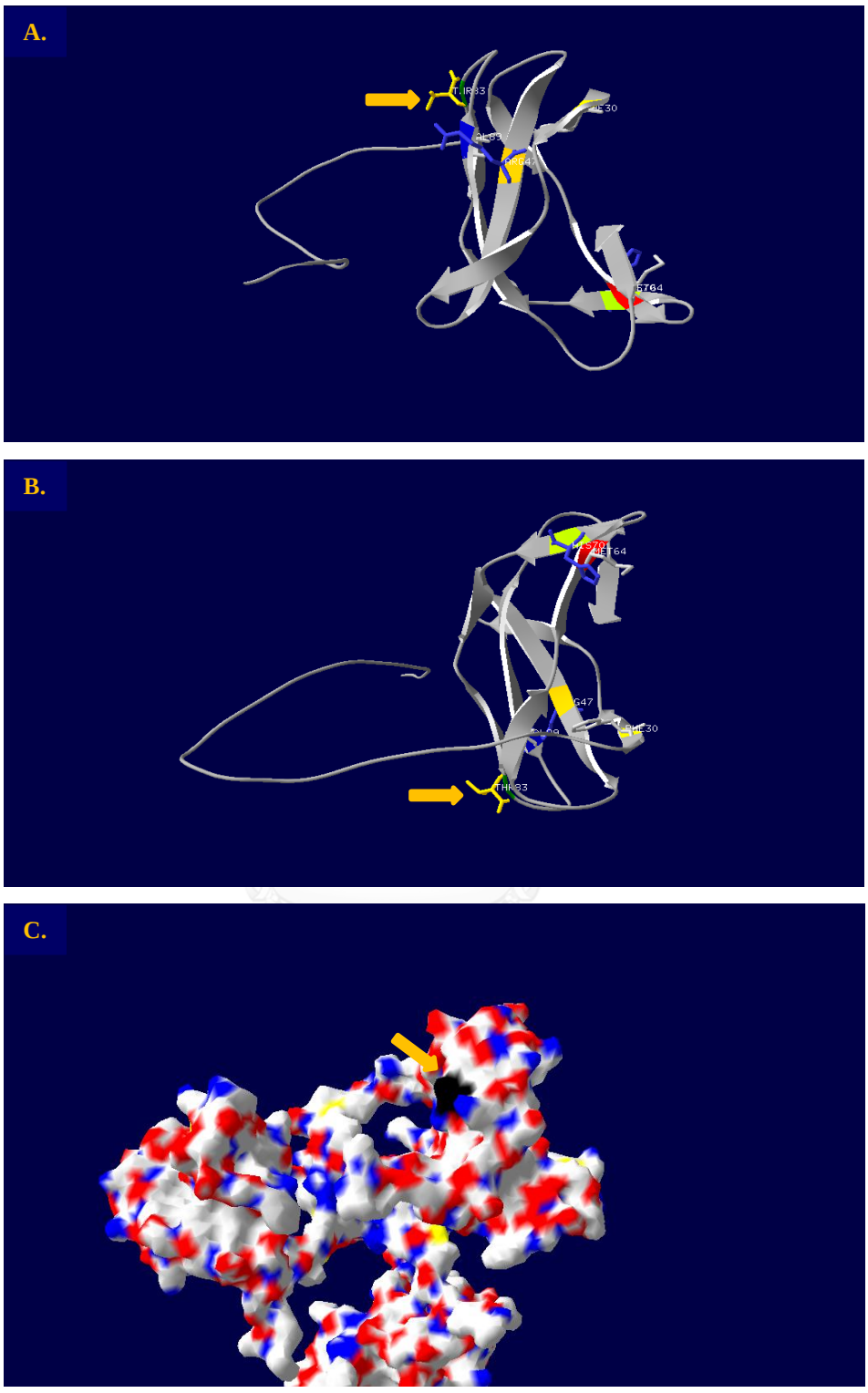


Figure 27 Mutation positions in the mosquito's virus which is representative as red-yellow-blue-green color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-mT352A (black color) mutation exposed to 3D-protein's surface (C).

In DENV-3, there are several positions of amino acid substitution found in the alignment sequence of interest (Fig. 28), which was separated by three criteria which was similar to those for DENV-1. Only the virus from the mosquito was studied the mutation by season. Two positions could be the signature of EDIII was H343L in CD loop and K323L in B-strand (Table. 7).

In the mosquito during rainy season in 2012, there are no mutation positions in the EDI and the connecting. One position in EDIII; T315A in A-strand was found. During rainy season in early 2013, only one mutation was found in the EDIII; T315A (A-strand), F333S (BC-loop) and D337N (C-strand). In human subject (Table. 7), the mutation in the EDIII were S309A (A-strand), E312H (A-strand), D326N (B-strand), E336K (C-strand) and R346K (CD-loop).The most significant mutations of the protein were; F333S in the mosquito (Fig. 31); D326N in human (Fig. 30); and H343L and K323L as signature (Fig. 29) which might affect the binding properties of neutralizing antibodies.

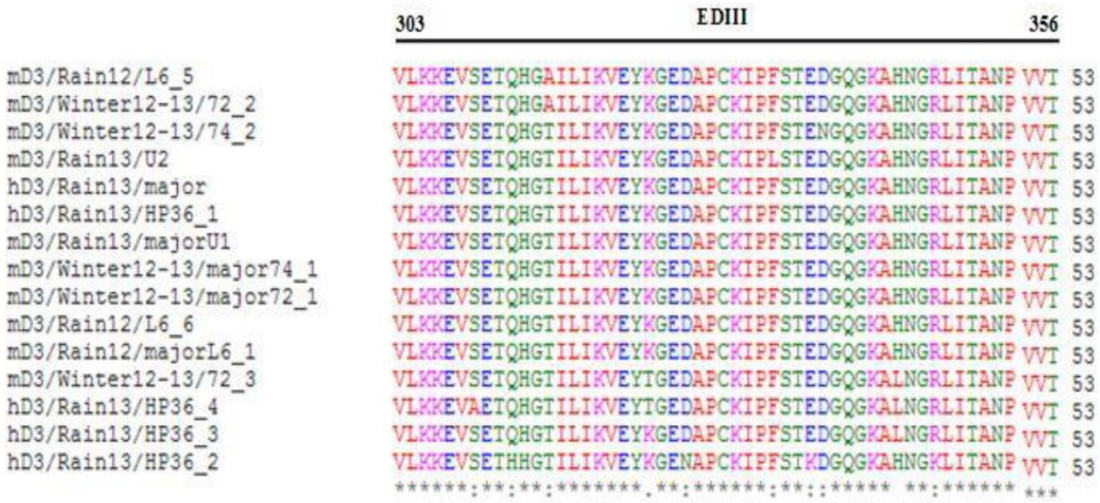


Figure 28 Extend sequence analysis of DENV-3 showed substitution positions of amino acid sequences corresponding to regions of E genes. The viral sequence (from the mosquito and human) covered at positions 277 to 371 of EDI-connecting-EDIII were shown. (*) is identical amino acid, (space, : and .) referred to different amino acid in the column.

Table 7 Substitution positions of amino acid sequences corresponding to regions of E-gene of DENV-3. The mutation positions were determined by respected to the basis 3D structure of the PDB: 3UZE_A.

Season/ years	**Substitution positions respecting to E gene in the**						**Host specific**
	Mosquito			**Human subjects**			
	EDI	**Connecting**	**EDIII**	**EDI**	**Connecting**	**EDIII**	**Human-Mosquito**
Rainy /2012	Na	Na	T315A (A-strand)	na	Na	Na	H343L K323L
Rainy/2013	-	-	T315A (A-strand) F333S (BC-loop) D337N (C-strand)	na	Na	S309A (A-strand) E312H (A-strand D326N (CD-loop) E336K (C-strand) R346K (CD-loop)	H343L (CD loop) K323L (B-strand)

* ; represented for stop-codon on the codon-frame. ǂ ; Amino acid mutation of predicted surface-exposed E residues and show $5A^{o}$. (-) ; not found the amino acid mutation. (na); non-analyses. (_); the mutation is similar position with Lin et al (2012) (analysis of epitope) which generated a panel of 67 alanine-substitution mutants covering the predicted surface-exposed E residues on DENV1 prM/E expression construct. The position was compared to those from DENV1 and 2.

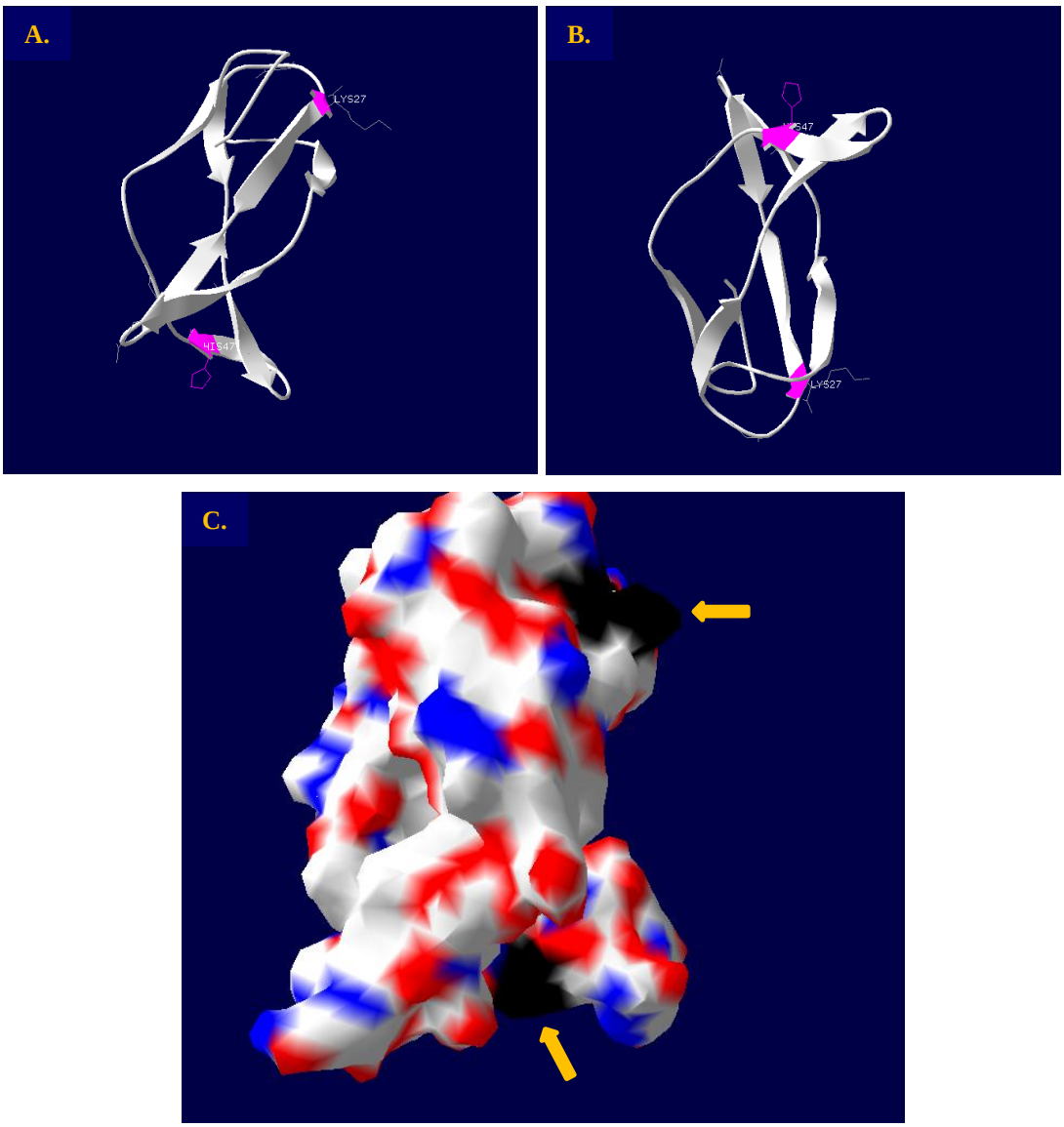


Figure 29 Mutation positions in both the mosquito and human's viruses which is representative as pink color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII- H343L & K323L (black color) mutation exposed to 3D-protein's surface (C).

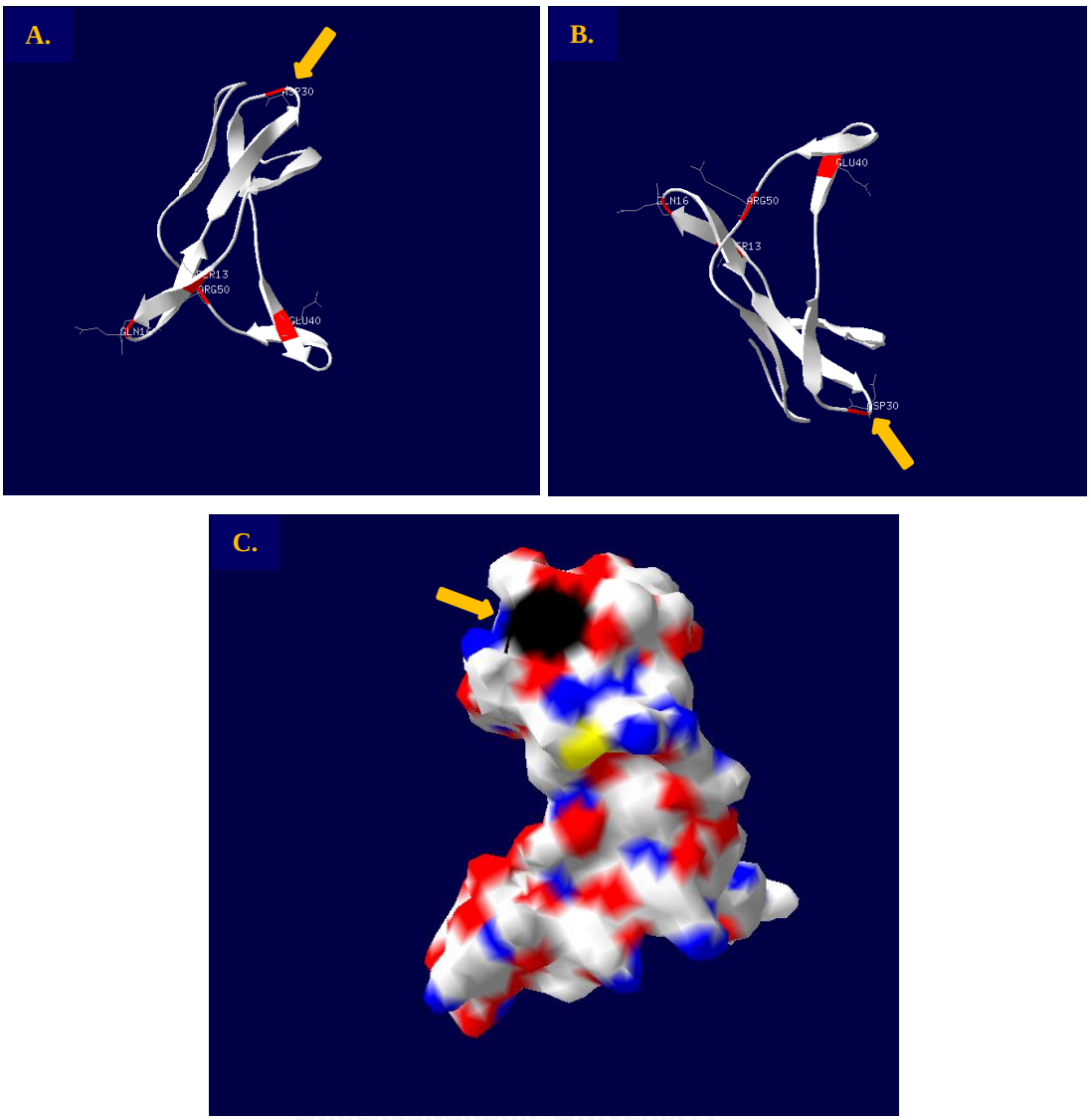


Figure 30 Mutation positions in the human's virus which is representative as red color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-hD326N (black color) mutation exposed to 3D-protein's surface (C).

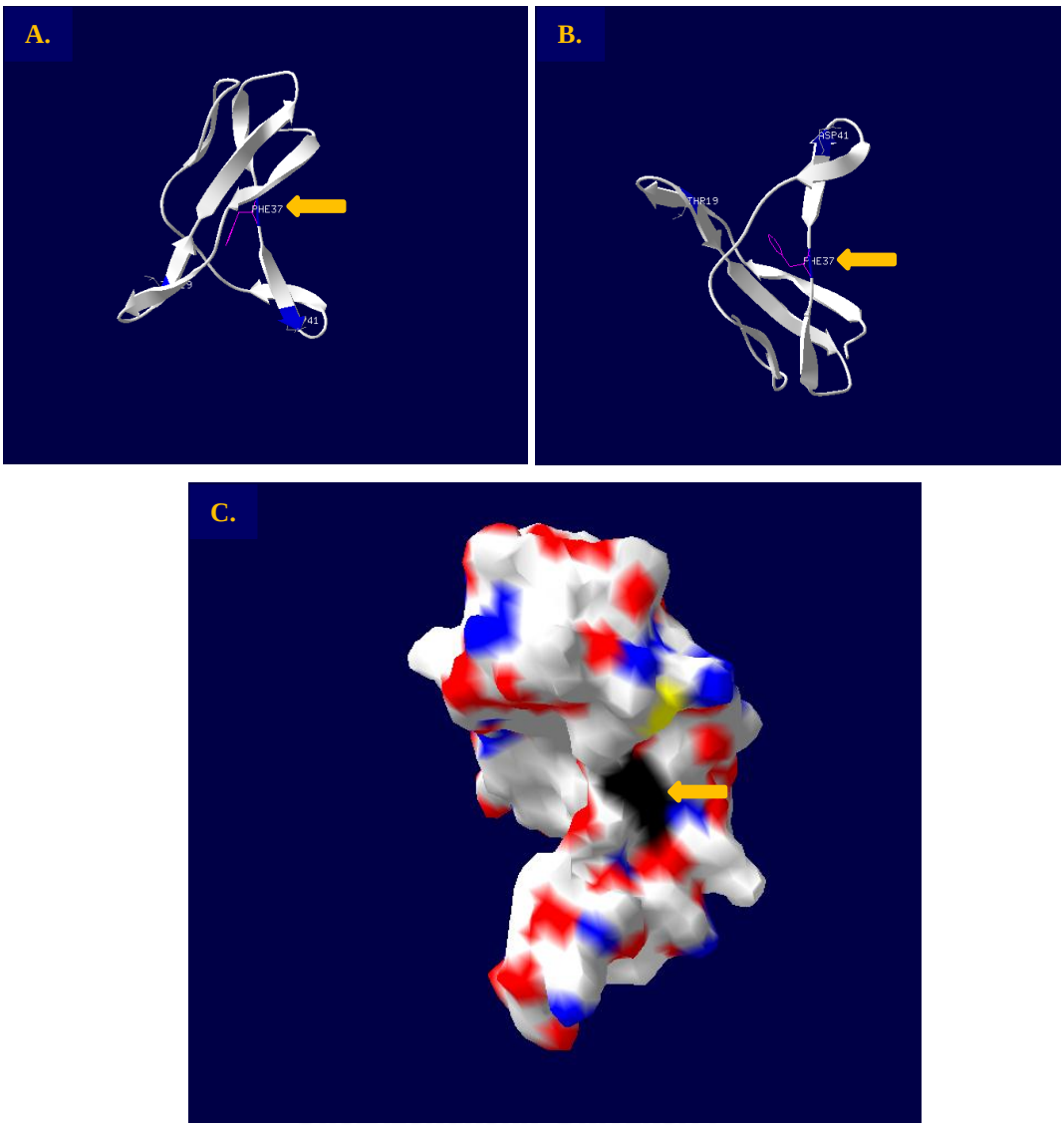


Figure 31 Mutation positions in the mosquito's virus which is representative as red color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-mF333S (black color) mutation exposed to 3D-protein's surface (C).

In DENV-4, there are several positions of amino acid substitution found in the alignment sequence of interest (Fig. 32), which was separated by three group and that similar to those for DENV-1. The mutation in the virus from the mosquito was studied by season. Four positions defined to as the signature of EDIII was A329T (BC-loop), L357F and A364V (DE-loop) and M383D (FG-loop) (Table. 8).

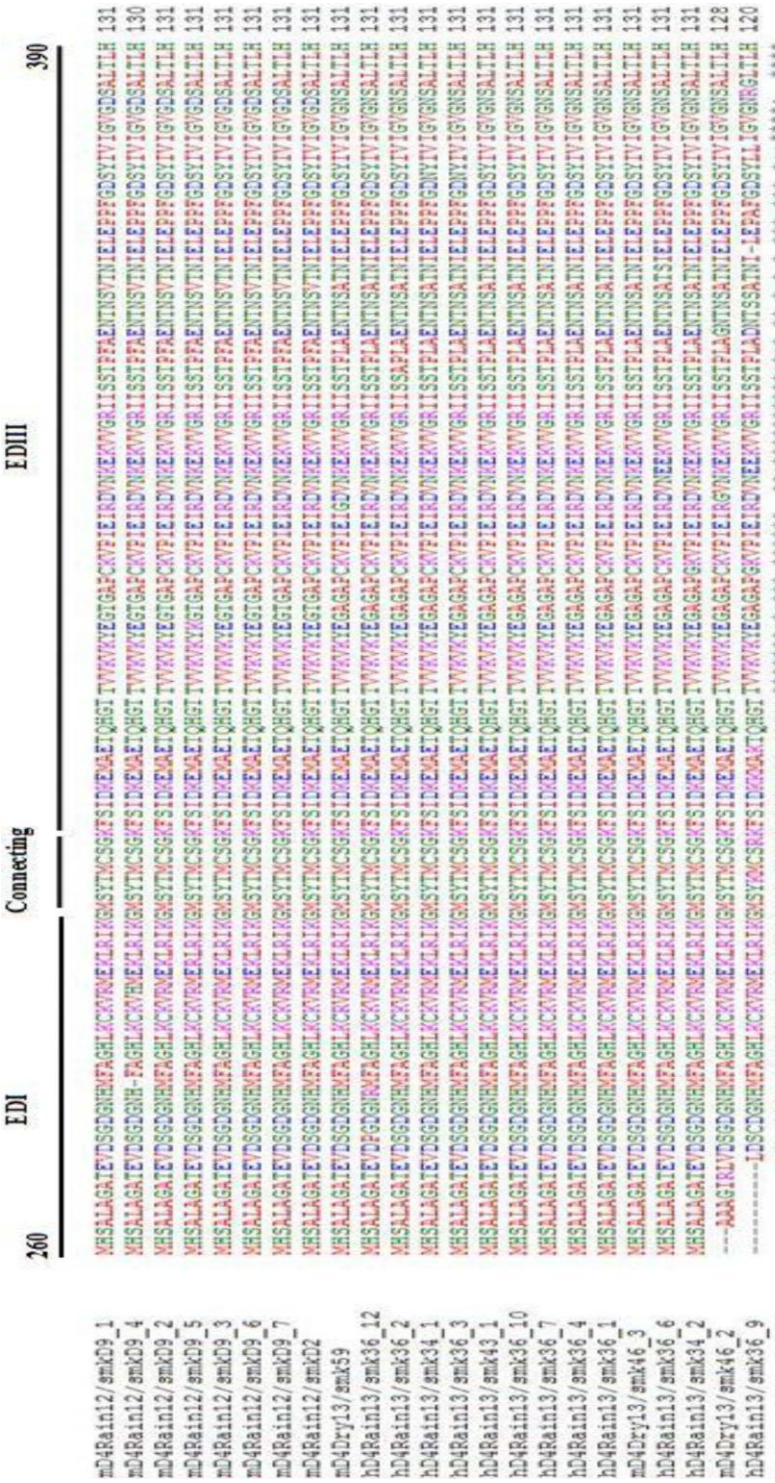


Figure 32 Extend sequence analysis of DENV-4 showed substitution positions of amino acid sequences corresponding to regions of E genes. The viral sequence (from the mosquito and human) covered at positions 260 to 390 of EDI-connecting-EDIII were shown. (*) is identical amino acid, (space, : and .) referred to different amino acid in the column.

Table 8 Substitution positions of amino acid sequences corresponding to regions of E-gene of DENV-4. The mutation positions were determined by respected to the basis 3D structure of the PDB: 3WE1.

Season/ years	Substitution positions respecting to E gene in the						Host specific
	Mosquito			Human subjects			
	EDI	Connecting	EDIII	EDI	Connecting	EDIII	Human-Mosquito
Rainy /2012	M278* R288H K291R	-	E327K (BC-loop) R340G (C-strand)	Na	na	Na	A329T (BC-loop) L357F A364V (DE-loop) M383D (FG-loop)
Dry/2013	K286R	-	-	Na	na	Na	A364V (DE-loop) M383D (FG-loop)
Rainy/2013	-	-	-	-	T300K	G304R (A-strand) A386G (FG-loop) D341G (CD-loop) E359G, D N362S T355A (DE-loop) N366S (E-strand) I377L S375N S385R (F-strand)	A329T (BC-loop) L357F A364V (DE-loop) M383D (FG-loop)

* ; represented for stop-codon on the codon-frame. ǂ ; Amino acid mutation of predicted surface-exposed E residues and show 5A^{o}. (-) ; not found the amino acid mutation. (na); non-analyses. (_); the mutation is similar position with Lin et al (2012) (analysis of epitope) which generated a panel of 67 alanine-substitution mutants covering the predicted surface-exposed E residues on DENV1 prM/E expression construct. The position was compared to those from DENV1 and 2.

In the mosquito during rainy season in 2012, there are three mutation positions in the EDI; M278*, R288H and K291R. Genome defective virus still was shown. Two positions in EDIII; E327K in BC-loop and R340G in C-strand were found. During rainy season in early 2013, only one mutation was found in the EDI; K286R.

In human subject (Table. 8) in 2013, the mutation in the EDI; T300K and nine-mutation position in the EDIII were G304R in A-strand, A386G in FG-loop, D341G in CD-loop, E359G&D, N362S & T355A in DE-loop, N366S in E-strand, I377L, S375N & S385R in F-strand. The most significant mutations of the protein were; R340G in the mosquito (Fig. 35); S385R in human (Fig. 34); and M383D in both host types as signature (Fig. 33) which might reduce the binding properties of neutralizing antibodies. Other mutations were not show the protein surface might affect with the structure and also the protein activity.

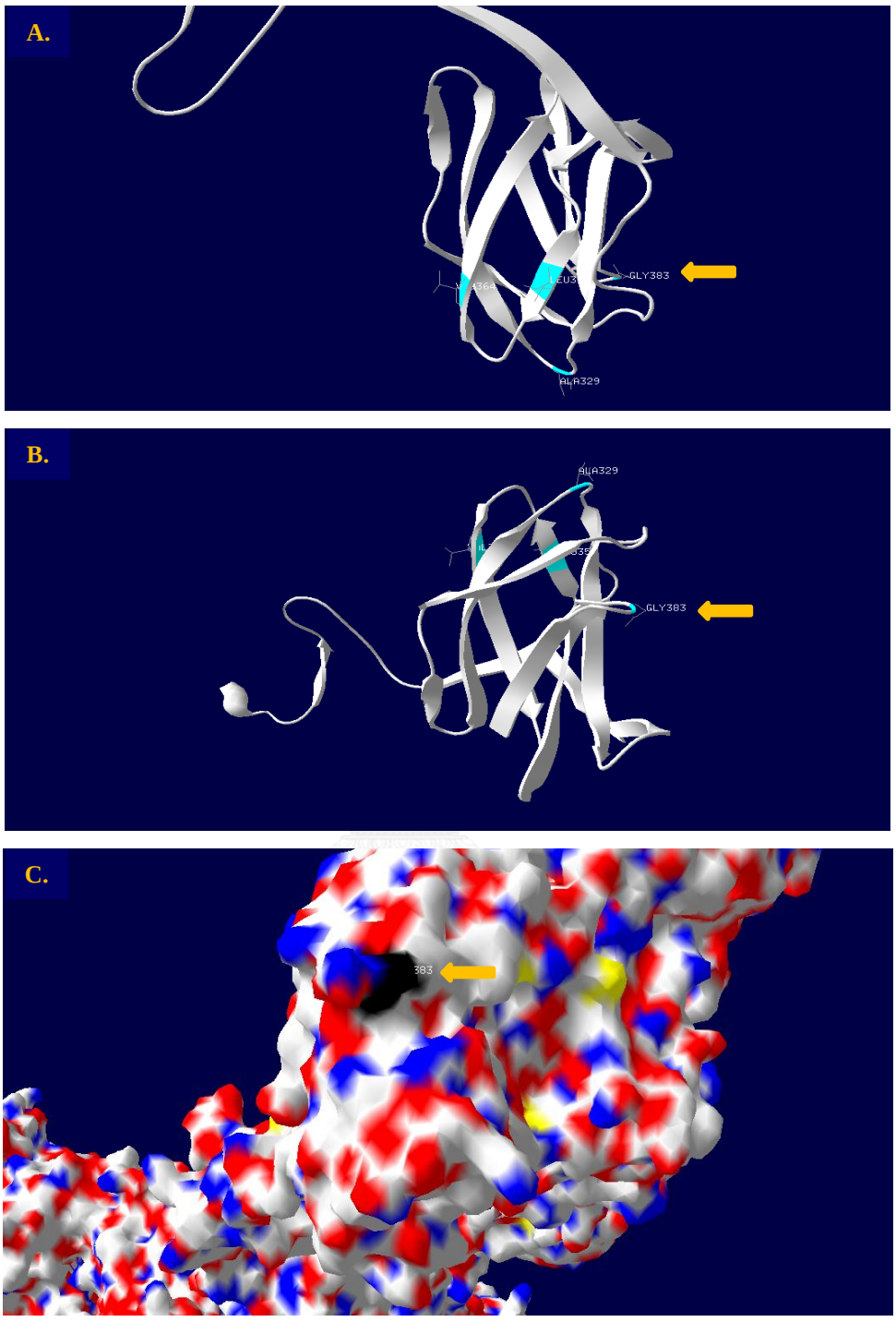


Figure 33 Mutation positions in both the mosquito and human's viruses which are shown in light-blue color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-M383D (black color) mutation exposed to 3D-protein's surface (C).

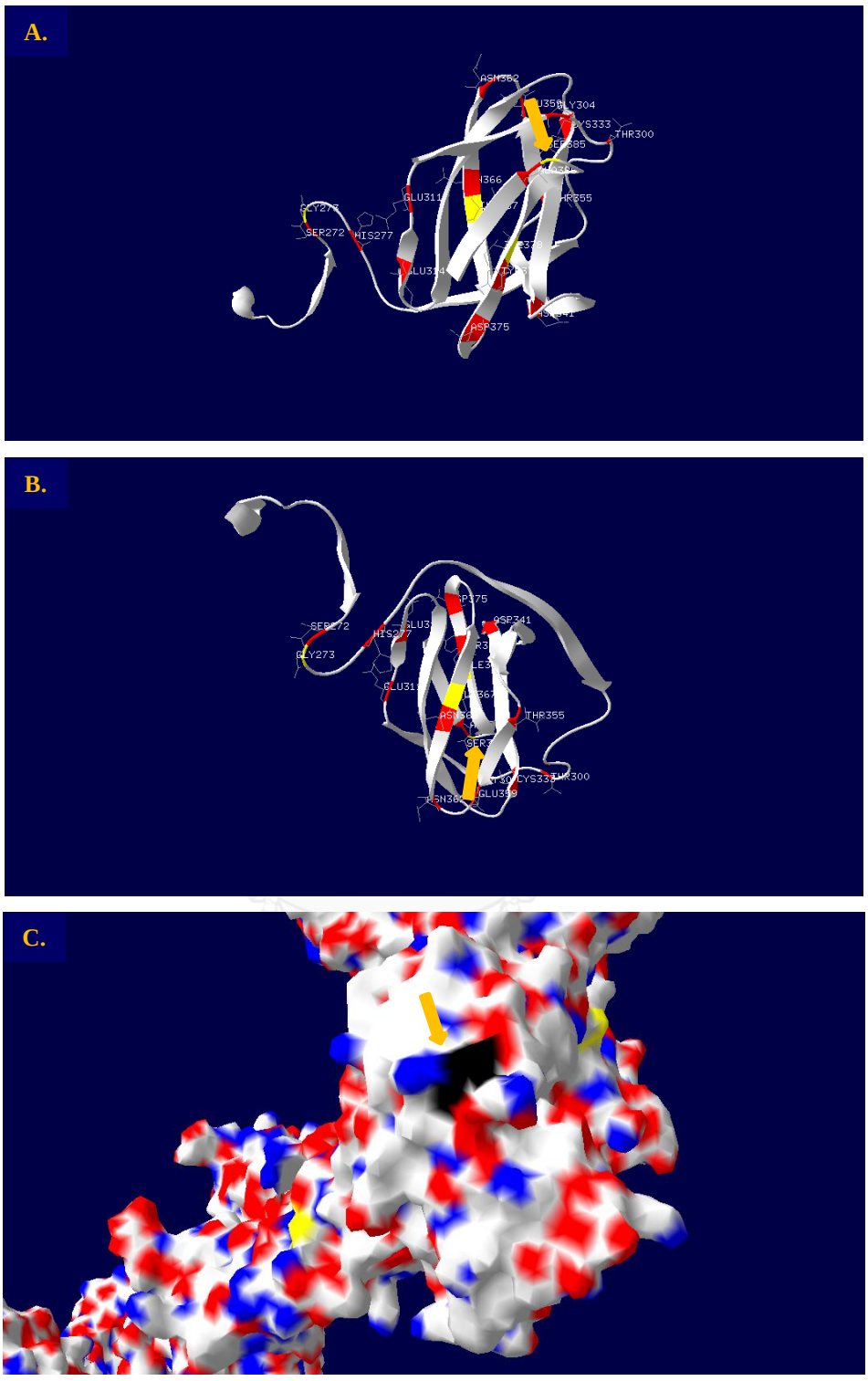


Figure 34 Mutation positions in the human's virus which is representative as yellow-red color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-hS385R (black color) mutation exposed to 3D-protein's surface (C).

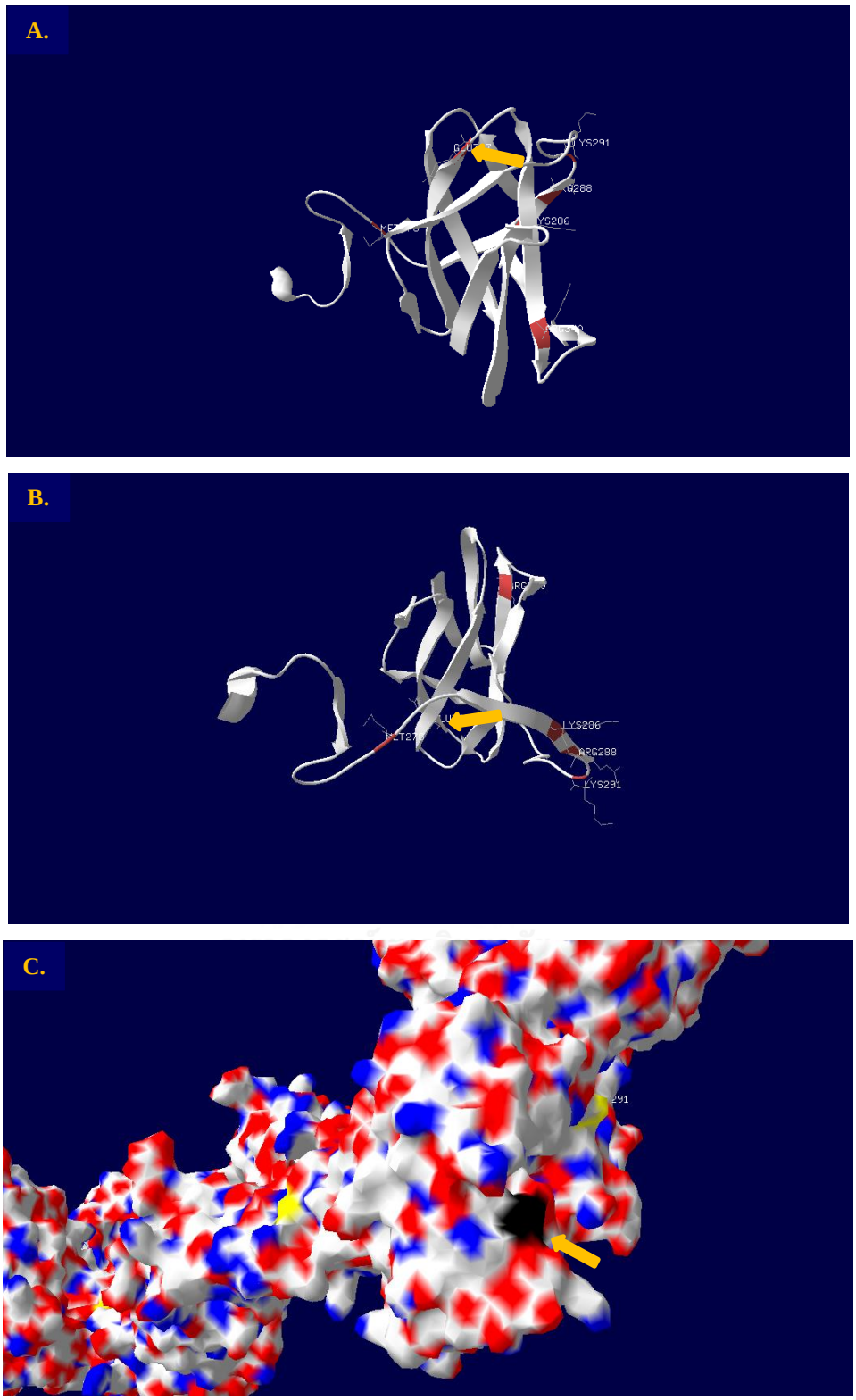
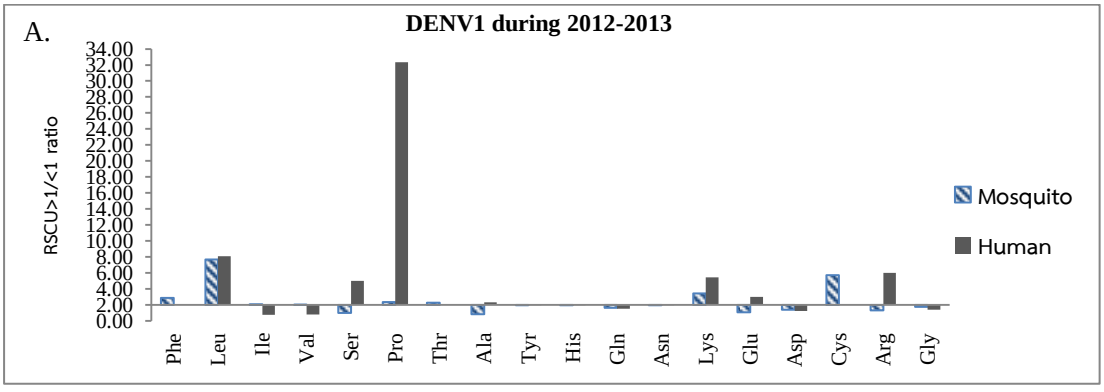


Figure 35 Mutation positions in the mosquito's virus which are shown in red-brown color (A) and inverted-picture of A (B). Green arrow pointed to the position of EDIII-mR340G (black color) mutation exposed to 3D-protein's surface (C).

7. **Codon usage bias**

The biased-codon usage was implied the viral genome fixation and also affected evolutionary changes (97). The degrees of biased usage were represented by the relative synonymous codon usage (RSCU), which was calculated from MEGA6. Moreover, the degree of the biased-value might be associated with the viral fitness by having the host selection with high infectivity. We found the seasonal-specific pattern of codon preference and non-preference within all four serotypes (Fig. 36 and Fig. 37). When considering the pattern of DENV1 (from vector) the most biased codon substitution site was associated with the codon group of Phe, Leu, Pro, Thr, Lys and Arg. The most codon usage bias from human host was associated with the codon group of Leu, Ser, Pro, Thr, Tyr, His, Ala, Lys, Asp, Cys and Arg. In DENV2 the codon bias derived from the vector was Leu, Ile, Val, Ser, Pro Thr, Ala, His, Asn, Glu, Cys and Arg. In DENV3 the codon bias derived from the vector was Phe, Leu, Ser, Pro, Ala, Tyr, Asp, Cys and Arg and from human host Phe, Ser, Pro, Thr, Ala, Tyr, Asn, Asp, Cys, Arg and Gly. In DENV4 the codon bias was Leu, Ile, Val, Ser, Pro, Thr, Ala, Tyr, Asn, Asp, Cys, Arg and Gly and that from human host was Lue, Ile,Val, Ser, Pro, Thr, Ala, Tyr, Asn, Asp, Cys, Arg and Gly. According to the pattern above, we can conclude that the viral structure was differed slightly by season. However, the amino acid pattern of the virus from the human host-can be definitely determined but a few amino acids could affect the structure (97).



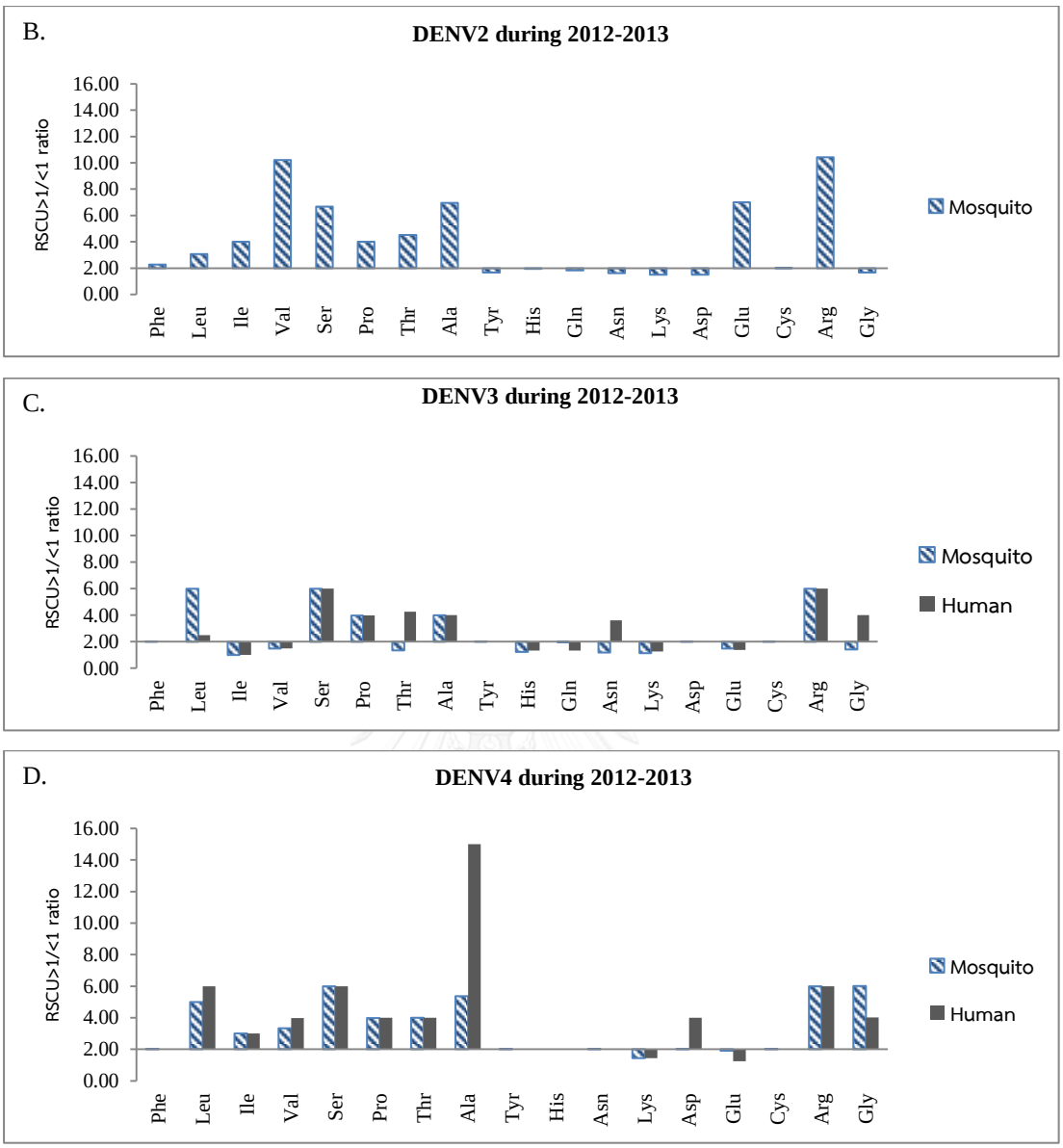


Figure 36 Patterns of codon usage bias of each dengue viruses (serotype1-4) were measured and the RSCU values were compared between *Ae. aegypti* mosquito vector and human host. Only serotype 2 was not found in the human host. The biased degree of codon usage were indicated to the RSCU (>1 divided by <1) ratio is greater than or equal to 2.00. The bias was the most found in human's virus (DENV-1 & 4) which contributed to survive them in the host.

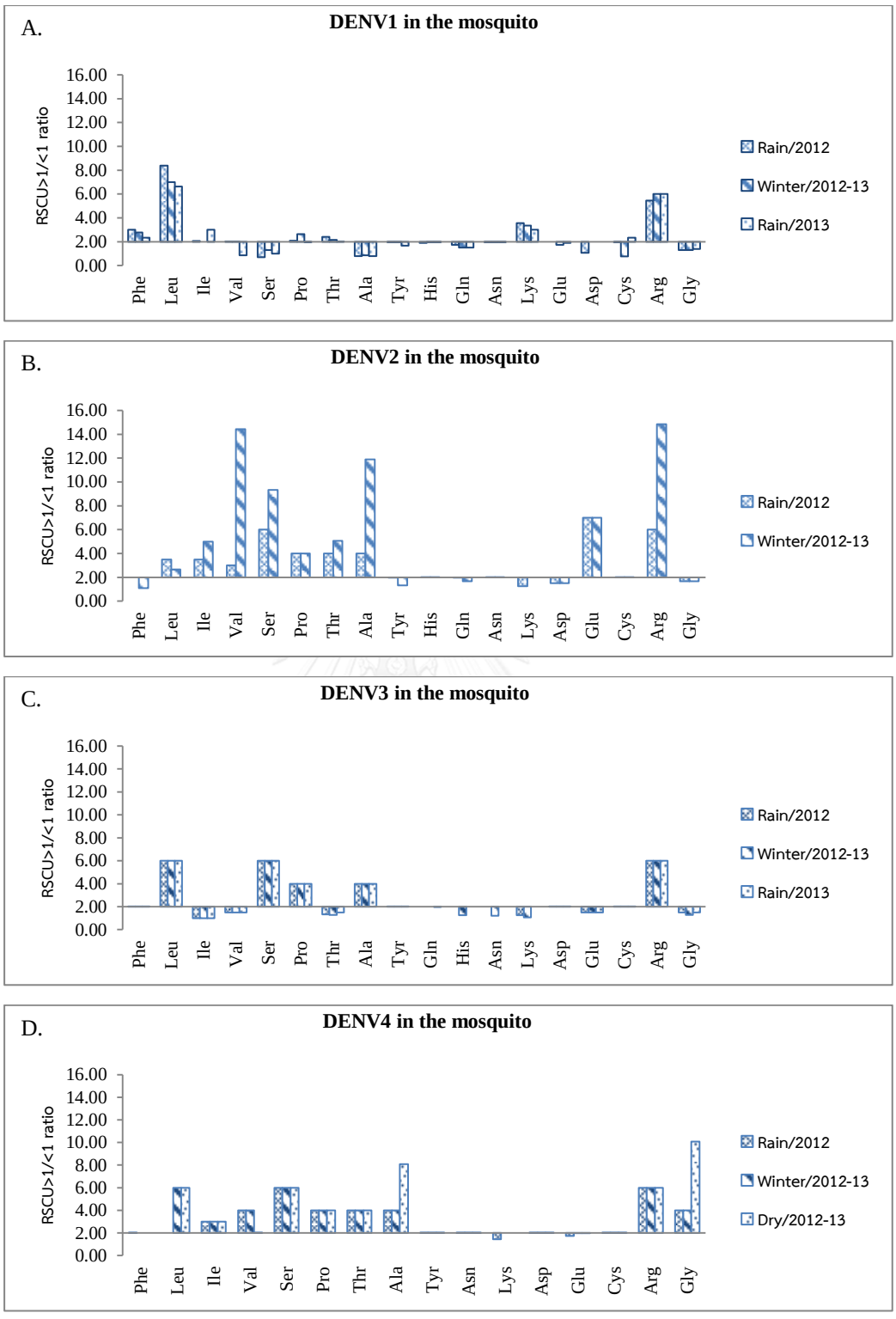


Figure 37 Seasonal patterns of codon usage bias of each dengue viruses (serotype1-4) amplified from *Ae. aegypti* mosquito vector were showed. DENV-2 and a few in DENV-4 was the most fixations their genome more than the rest-two serotypes. The event could contribute the viral survival through seasons.

8. Selective pressure analysis

We observed the selection within the E gene sequence of all four serotype by season with samples derived from both the vector and the human host (Table. 4). In DENV-1 we found the positive selection from the sample in the vector of rainy season in both years 2012 (ω=1.243) and 2013 (ω=1.032). This positive of the virus derived from the vector was firstly shown and no one has been reported. However, the mosquito might derive the virus with positive selection from the local resident who have infected. Moreover, this case might represent that the mosquito could be the competent vector. The sequence from human host in the year 2013 (ω=1.080) showed positive selection as well. The positive selection represented that the virus have exposed to protective & cross-reactive neutralizing antibodies. This led to adapt and also escape for survival in the human host. Only the sequence in winter season was negative for selection. No positive selection was found within the sequence of DENV-2 which was collected from rainy (ω=1.000; neutrality) and winter season (ω=0.190; negative). Although the sequence of DENV-3 was amplified by almost all the period of the study no positive selection on E-protein was found i.e. in the rainy season of the year 2012: (ω=0.497), winter season during the year 2012 and 2013: (ω=0.309), rainy season of the year 2013: (ω=0.497) and that from human in the year 2013: (ω=0.284). The negative selection could be represented that the virus was attenuate compared with DENV-1. On the other hand, short-sequences of the virus might result in the negative. For DENV-4 only the sequence derived from the human host in the year 2013 showed positive selection (ω=1.187). All virus samples derived from the mosquito showed negative selection, for rainy season in the year 2012 (ω=0.407) and dry season during the year 2012 and 2013 (ω=0.426).

9. Phylogenetic analysis of DENV serotypes based on *E* protein gene

To study the evolutionary relationships of DENV serotypes circulating in the existing strains or exportation and importation near of the studied site was done. Phylogenetic trees were constructed using the E-gene data set including the sequences derived from the study and reference sequences available from the GenBank database. The separation of the endemic genotypes of the virus was confirmed by high

bootstrap support values. Additionally, genotypes (Table. 9) classified into each dengue tree were confirmed by concurrently measure with the denguedb online webserver (86).

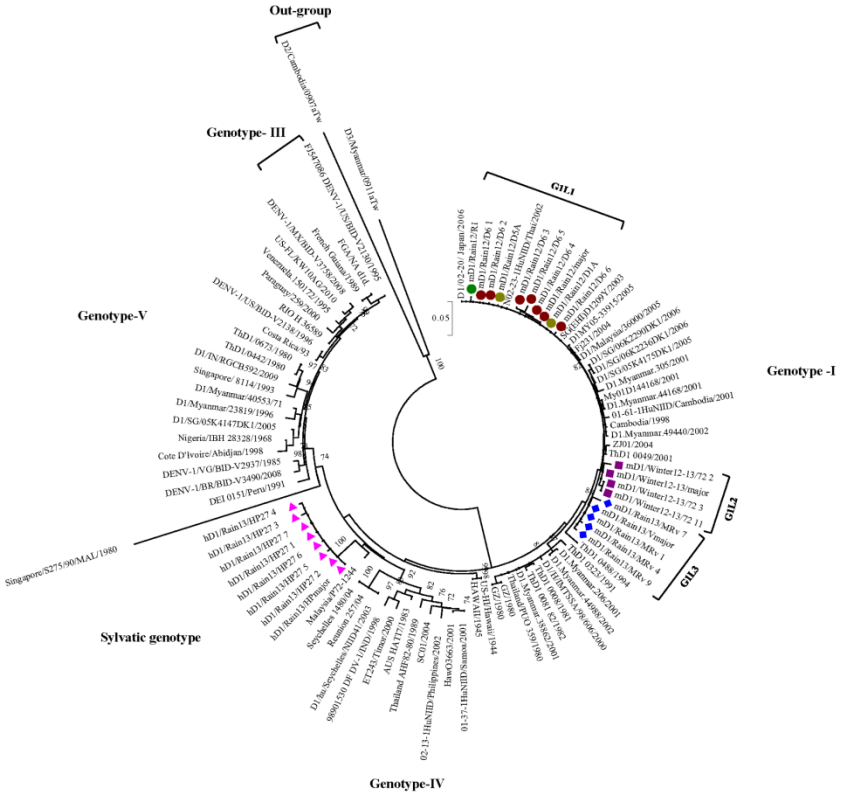


Figure 38 A phylogenetic tree of DENV-1, constructed based on representative E-gene partial sequences. The tree was constructed using 65 sequences of DENV-1; including 38 referent sequences and 27 sequences of DENV-1 from mosquitoes and human volunteers in the study, separated by season during 2012-2013 (mosquitoes: 10 sequences from rainy season represented by green, red and dark-yellow solid circles, 4 sequences from winter season are represented by purple-solid rectangles, 5 sequences from rainy season are represented by blue solid diamond square quadruples, 8 sequences from local human volunteers in the early-rainy season of 2013 are represented by pink-solid upturned-triangles).

The tree of DENV-1 (Fig. 38) was analyzed with 27 sequences from the vector by different season (rainy season in 2012, winter season in 2013, rainy season in 2013) and from local subjects in 2013. DENV-1 strains from the mosquito vector between the rainy season and dry season belonged to genotype-I (Table 9A), which has been proposed that it also originated in Southeast Asian countries (98).

There are three lineages within genotype-I clustered by season. The first lineages (G1L1; rainy season) were classified in the same cluster of the strain from Asian countries (Singapore, Malaysia and Myanmar), Japan (02-20/2006) and Fiji (Fj231/2004), which represented the number of serotype-1 circulating in that area. G1L2 (winter season) and G1L3 (dry season) clustered strains were found from Thailand (ThD1 0049 strain and THD1/0488/1994), where only one strain had been maintained and survived after the last monsoon season.

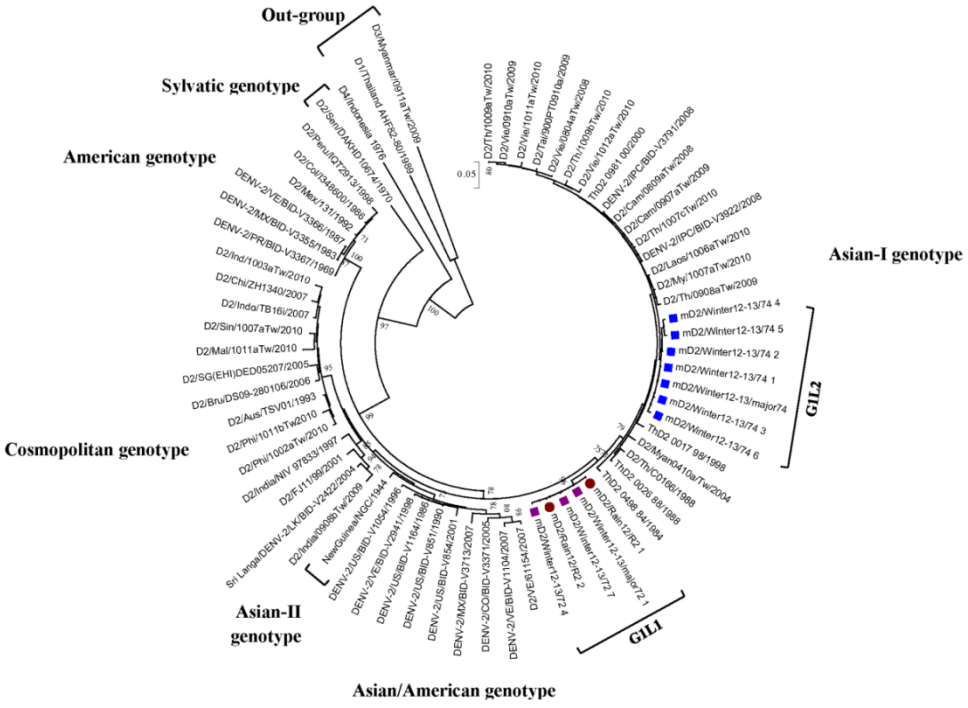


Figure 39 A phylogenetic tree of DENV-2, constructed based on representative E-gene partial sequences. The tree was constructed with 67 sequences of DENV-2 including; 55 sequences of referent sequences and 12 sequences of DENV-2 from mosquitoes in the study, separated by season during 2012-2013 (2 sequences from rainy season of 2012 represented by red solid-circles, 10 sequences from winter season of 2013 represented by blue and purple rectangles).

All virus from the mosquito vector were represented the epidemic strain. Interestingly, the sequences from humans belonged to the sylvatic genotype. The strain was referred to as enzootic cycle which might be transmitted to local humans via the infected-foreign mosquito (67). On the other hand, the genotype might be transmitted to the area via the local mosquito carried the virus from the infected monkey (for turning the coconut) or from infected-foreigners such as Burmese laborer, who has been working in the industry.

When considering to pattern of p-distance, declined values from rainy (p=1.20) in 2012 last to the rainy season (1.36) in 2013 were found (Table 4A and Figure 42). This might be the evidence the virus (from the mosquito) showed the lineage shift by season and also found the sylvatic, attenuate strain. In addition, seasonal-varied patterns of codon preference of the mosquito's virus (Figure 37A) and the host-biased patterns (Figure 36A) could have more confirmed the situation of the transmission dynamic.

In DENV-2, the tree was analyzed with 12 sequences of infected mosquito from two-season (rainy in 2012- and winter season in 2013) (Fig. 39). All strains belonged to Asian-I genotype (Table 9B). There were two lineages found. Both lineages (G1L1) were found in the clustered strains from Thailand (ThD2_0498_84 strain for rainy season and D2/Th/0909atw strain for winter season). Although the virus was classified in only genotype-I, this has also been associated with dengue spread in the region including lineage shifts during the season was found. Although the pattern of p-distance value was showed no different by season (p=0.01 in rainy season 2012 and p=0.02 in winter season in February 2013) as mention in Table 4B and Figure 42, the degree of the codon preference has increased during the same period (Figure 37B). The increase might be the evidence of the lineage shift within the Asian-I genotype.

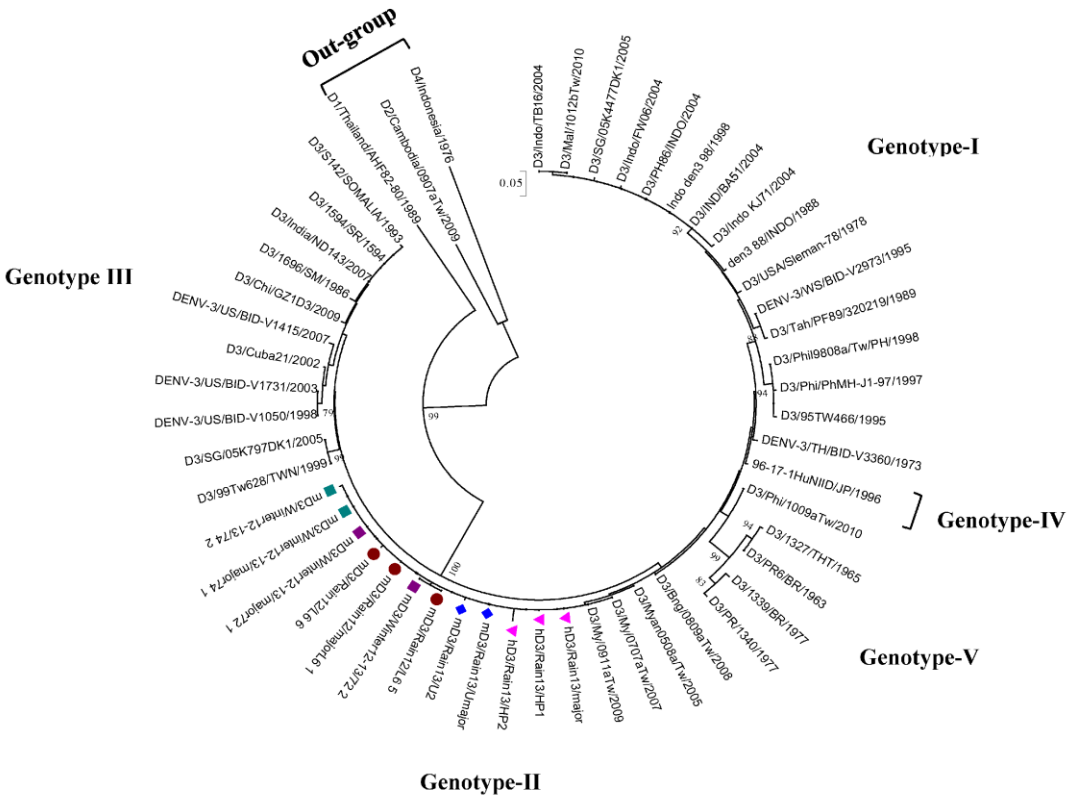


Figure 40 A phylogenetic tree of DENV-3, constructed based on representative E-gene partial sequences. The tree was constructed with 52 sequences of DENV-3 including; 40 sequences of referent sequences and 12 sequences of DENV-3 from mosquitoes and human volunteers from the study, separated by season during 2012-2013 (mosquitoes: 3 sequences from rainy season in 2012 represented by red solid-circles, 4 sequences from winter season in 2013 are represented by green and purple solid-rectangles, 3 sequences from local human volunteer in 2013 are represented by blue solid-diamond inverted-triangles and 2 sequences from rainy season in 2013 are represented by blue diamond-square quadrangles).

DENV-3, 12 sequences from different season and different host type which is from the mosquitoes collected during the rainy season in 2012 and winter season in 2013, and with additional collection from local human volunteers in early-rainy season in 2013 (Fig. 40). Although, they were amplified from both mosquito vector and human hosts, the clustering is similar to that of DENV-2, where all sequences belonged to genotype-II as shown in Table 9C, but only the lineage was shown. The

virus was found in the clustered strained from Myanmar spanning 2005-2008. There is only one-strain (similar to that from Myanmar) been circulated during the time period. The virus might have the low effect on the local residents, remaining preexisting neutralizing antibodies against the previous exposure with the same viral strains. In the case, both patterns of the p-distance values (Table 4C and Figure 42) from both host types and that of codon preference of the virus from the mosquito showed no different by season (Figure 37C). This evidence could support the virus to group in the same cluster within the genotype.

For DENV-4, 23 sequences were partially derived during the rainy season in 2012, winter and dry seasons in 2013, while the rest were from local human volunteers (Fig.41). Two-clusters of the virus were clearly separated by year. The first-cluster (G1L1) is the sequences derived from the rainy season in 2012, which belongs to lineage-1 of genotype-I (Table 9D), found in the cluster from Thailand during 1984 to 2005. On the other hand, the second lineage was derived during the winter and early rainy season in 2013 from human subjects belonging to the genotype-II. It is closely related to the viral strains from the Asian country, Indonesian strain in 1977. Seasonal-different patterns of both p-distances (Table 4D and Figure 42) and codon preference (Figure 36D & figure 37D) during the study period might associate with the situations of both genotype and lineage shifts within genotype. Moreover, the genotype-I have shifted to be the genotype-II during 2012 and 2013 might contribute to viral survival and also continuous transmission thereafter.

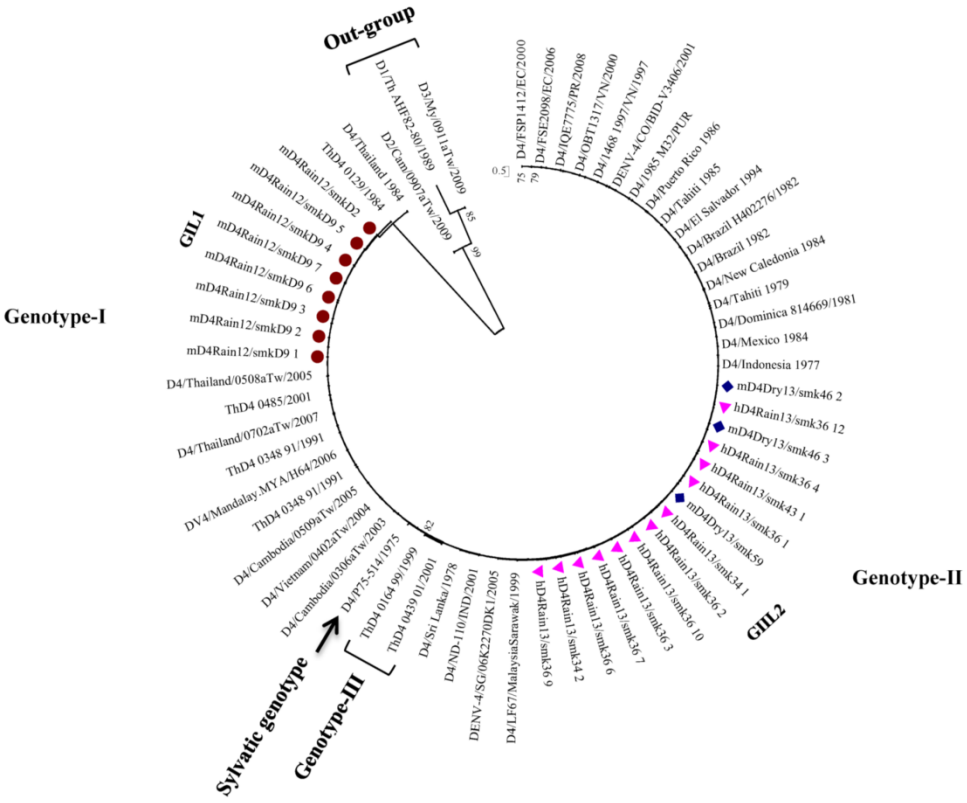


Figure 41 A phylogenetic tree of DENV-4, constructed based on representative E-gene partial sequences. The tree was constructed with 60 sequences of DENV-4 including; 37 sequences of referent sequences and 23 sequences of DENV-1 from mosquitoes and human volunteers from the study, separated by season during 2012-2013 (mosquitoes: 8 sequences from rainy season in 2012 are represented by red solid-circles, 3 sequences from dry season in 2013 are represented by blue solid-diamond square quadrangles, 12 sequences from local human volunteers are represented by pink solid-inverted triangles).

Therefore, the event of genotype and lineage shifts of the circulating DENV serotypes should be the important factor which correlate and associate with the evolutionary dynamic by space and time. This would be used to predict and determine trends of up-coming DENV transmission in the area.

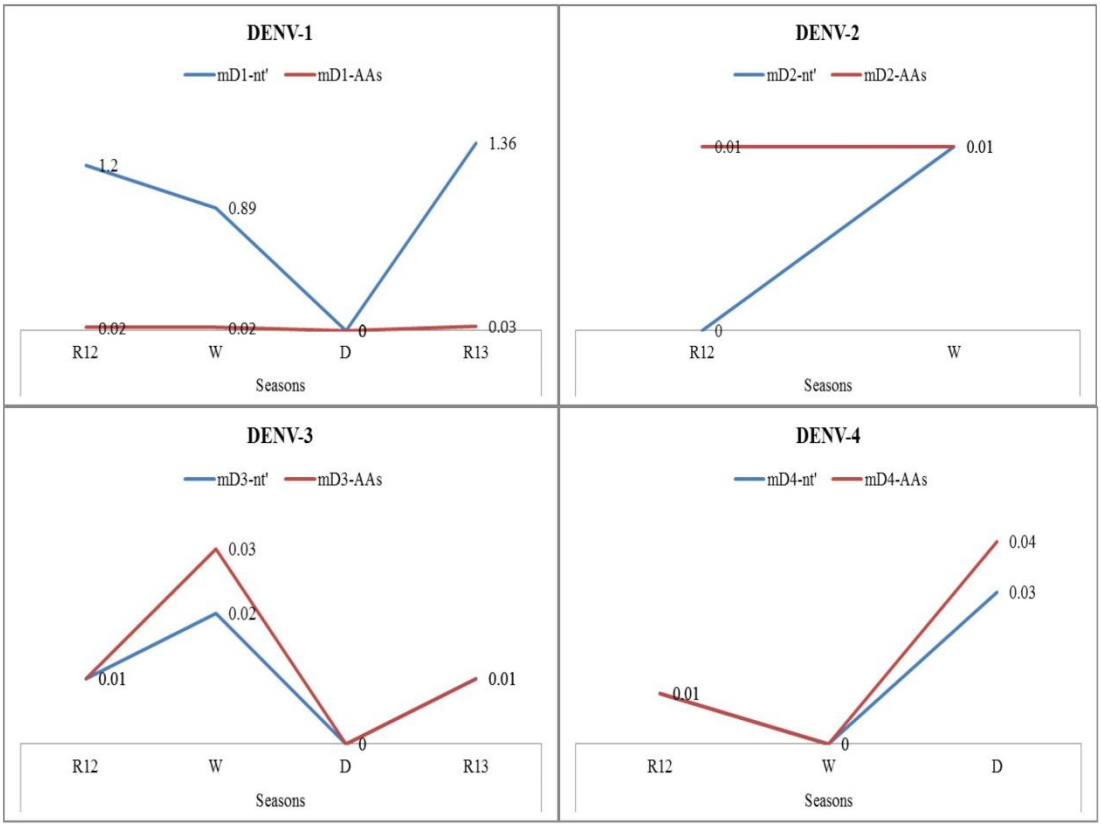


Figure 42 Patterns of pairwise p-distance values each dengue serotype were shown by season.

Table 9 Dengue viruses were indicated to specific genotype within serotype, which analyses by using http://www.denguedb.org/viewGenotypeResult.aspx. DENV1 sequences were deposited to Gen Bank with accession number of KM0039-KM003970, DENV2: KM003971-003982, DENV3: N stand for non-submit to Gen Bank since these sequence lengths are less than 200 bp and DENV4: KM003983-KM004010. A.

DENV-1	Accession no.	Serotype/ Genotypes	Prediction confidence	Seasons	Collected periods	Host types
***m*D1/Rain12/major**	**KM003944**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D6_1**	**KM003945**	**1/1**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypt***
***m*D1/Rain12/D6_2**	**KM003946**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D6_3**	**KM003947**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D6_4**	**KM003948**	**1//I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D6_5**	**KM003949**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D6_6**	**KM003950**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/R1**	**KM003951**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D1A**	**KM003952**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Rain12/D5A**	**KM003953**	**1/I**	**100%**	**Rainy**	**Oct 2012**	***Aedes aegypti***
***m*D1/Winter12-13/major**	**KM003954**	**1/I**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D1/Winter12-13/72_2**	**KM003955**	**1/I**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D1/Winter12-13/72_3**	**KM003956**	**1/I**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D1/Winter12-13/72_11**	**KM003957**	**1/I**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D1/Rain13/Vmajor**	**KM003958**	**1/I**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***m*D1/Rain13/MRv_1**	**KM003959**	**1/I**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***m*D1/Rain13/MRv_4**	**KM003960**	**1/I**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***m*D1/Rain13/MRv_7**	**KM003961**	**1/I**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***m*D1/Rain13/MRv_9**	**KM003962**	**1/I**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***h*D1/Rain13/HPmajor**	**KM003963**	**1/Sylvatic**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D1/Rain13/HP27_1**	**KM003964**	**1/Sylvatic**	**100%**	**Rainy**	**May 2013**	**Subjects**

DENV-1	Accession no.	Serotype/ Genotypes	Prediction confidence	Seasons	Collected periods	Host types
*h*D1/Rain13/HP27_2	KM003965	1/Sylvatic	100%	Rainy	May 2013	Subjects
*h*D1/Rain13/HP27_3	KM003966	1/Sylvatic	100%	Rainy	May 2013	Subjects
*h*D1/Rain13/HP27_4	KM003967	1/Sylvatic	100%	Rainy	May 2013	Subjects
*h*D1/Rain13/HP27_5	KM003968	1/Sylvatic	100%	Rainy	May 2013	Subjects
*h*D1/Rain13/HP27_6	KM003969	1/Sylvatic	100%	Rainy	May 2013	Subjects
*h*D1/Rain13/HP27_7	KM003970	1/Sylvatic	100%	Rainy	May 2013	Subjects

(Table continued)

B.

DENV-2	Accession no.	Serotype/ Genotype	Prediction confidence	Seasons	Collected periods	Host types
*m*D2/Rain12/R2_1	KM003971	2/Asian-II	1.0239%	Rainy	Oct-Nov 2012	*Aedes aegypti*
*m*D2/Rain12/R2_2	KM003972	2/Asian-II	1.0239%	Rainy	Oct-Nov 2012	*Aedes aegypti*
*m*D2/Winter13 /major72	KM003973	2/Asian-II	1.0239%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/72_4	KM003974	2/Asian-II	1.0239%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/72_7	KM003975	2/Asian-II	1.0239%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13 /major74	KM003976	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_1	KM003977	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_2	KM003978	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_3	KM003979	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_4	KM003980	2/Asian-II	3.0303%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_5	KM003981	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*
*m*D2/Winter13/74_6	KM003982	2/Asian-II	2.3891%	Winter	Feb-2013	*Aedes aegypti*

(Table continued) C.

DENV-3	Accession no.	Serotype/ Genotype	Prediction confidence	Seasons	Collected periods	Host types
***m*D3/Rain12/ majorL6**	**N**	**3/II**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D3/Rain12/L6_5**	**N**	**3/II**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D3/Rain12/L6_6**	**N**	**3/II**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D3/Winter 12-13/major72**	**N**	**3/II**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D3/Winter 12-13/72_2**	**N**	**3/II**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D3/Winter 12-13/72_3**	**N**	**3/II**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D3/Winter 12-13/major74**	**N**	**3/II**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D3/Winter 12-13/74_2**	**N**	**3/II**	**100%**	**Winter**	**Feb 2013**	***Aedes aegypti***
***m*D3/Rain13/ U1major**	**N**	**3/II**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***m*D3/Rain13/ U2**	**N**	**3/II**	**100%**	**Rainy**	**Jun 2013**	***Aedes aegypti***
***h*D3/Rain13/major**	**N**	**3/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D3/Rain13/HP36_1**	**N**	**3/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D3/Rain13/HP36_2**	**N**	**3/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D3/Rain13/HP36_3**	**N**	**3/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D3/Rain13/HP36_4**	**N**	**3/II**	**100%**	**Rainy**	**May 2013**	**Subjects**

N: stand for non-submitting to Gen Bank since this sequence length is less than 200 bp.

(Table continued)

DENV-4	Accession no.	Serotype/ Genotypes	Prediction confidence	Seasons	Collected periods	Host types
***m*D4Rain12/smkD9_1**	**KM003983**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_2**	**KM003984**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_3**	**KM003985**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_4**	**KM003986**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_5**	**KM003987**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_6**	**KM003988**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD9_7**	**KM003989**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Rain12/smkD2**	**KM003990**	**4/I**	**100%**	**Rainy**	**Oct-Nov 2012**	***Aedes aegypti***
***m*D4Dry13/smk46_1**	**KM003991**	**4/II**	**100%**	**Dry**	**Apr 2013**	***Aedes aegypti***
***m*D4Dry13/smk46_2**	**KM003992**	**4/II**	**100%**	**Dry**	**Apr 2013**	***Aedes aegypti***
***m*D4Dry13/smk46_3**	**KM003993**	**4/II**	**100%**	**Dry**	**Apr 2013**	***Aedes aegypti***
***m*D4Dry13/smk59**	**KM003994**	**4/II**	**100%**	**Dry**	**Apr 2013**	***Aedes aegypti***
***h*D4Rain13/smk34_1**	**KM003995**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk34_2**	**KM003996**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_1**	**KM003997**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_2**	**KM003998**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_3**	**KM003999**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_4**	**KM004000**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_6**	**KM004001**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_7**	**KM004002**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_8**	**KM004003**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_9**	**KM004004**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_10**	**KM004005**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_12**	**KM004006**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk36_11**	**KM004007**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk43_1**	**KM004008**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk43_2**	**KM004009**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**
***h*D4Rain13/smk43_3**	**KM004010**	**4/II**	**100%**	**Rainy**	**May 2013**	**Subjects**

CHAPTER V
DISCUSSION AND CONCLUSION

Multiple-DENV virus infection was also possibly occurred in endemic countries of many continents such as, Southeast Asia (99-101), southern and central America (86), and the Caribbean (102) and south Pacific (103). Lower rates of infections were reported in Africa but it may be possibly due to insensitive surveillance techniques (3). Speculation of the naturally transmission dynamics of the virus in small scales (regions, provinces, in and out-municipal or district) could be focused. This is because the small area may contribute the origin of the disease spread thereafter. Additionally, it has not been shown yet how the evolutions of co-circulation of multiple dengue serotypes are occurring during each season of the year-round. The seasonal transmission of DENV serotypes in an endemic area was related to either the transmission dynamic and virulent strains, associated to the high pathogenesis and the spread of the virus (104).

Thailand is the one of dengue endemic area located between neighboring countries which has been exposed to all four serotypes but with different dominant strains such as DENV-1 & 3 in Laos during 2007-2012 (105) and DENV-2 in Singapore during 2008-2010 (106). Monitoring of dengue infection in the mosquito vector is the effectively alternative way to predict the upcoming occurrence of DHF situation within endemic areas (107).

In the year 2012, Samut Sakhon province was ranked the 3th for dengue case report in Thailand (138.05 per 100,000). Moreover, death rate (0.12%) had also been reported year after year. This province has annually reported the increase of infection rate, although it is in a well-controlled environment by local government. The incidence of the disease is associated with vector population density (17), biting ability, vector competent and host immune range (90, 108). So, this gap led to observation of seasonal prevalence of the currently circulatory virus in the studied site, however, the dengue transmission in the mosquito vector through local populations based on the dynamics of infection rates was studied by season, during 2012 to 2013.

Dengue prevalence and transmission cycle in the area

The dengue prevalent rates of three Thai season that the rate in the rainy season was significantly highest comparing those of the winter and dry seasons (Fig. 19 and Fig. 21A). Interestingly when considering the transmission rate (in the mosquito) by season, during rainy season (2012) the prevalent rate of dengue serotype viruses were DENV-3 > DENV-1> DENV-2 > DENV-4. DENV-3 was especially highly prevalent since strain was dominantly found. Prevalence of DENV-3 was found twice as much higher than DENV-4. On the other hand the rates of DENV-1 and DENV-2 are similar but still also higher than DENV-4. The pattern of multiple virus circulation mentioned above was accompanied with the study in hospitalized children with dengue disease at the Queen Sirikit National Institute of Child Health, Bangkok, Thailand during 1973-1999 (12), and in Singapore (17). Although, the multiple dengue virus infection in mosquitoes was found in this study which similar to the previous study reported by Thavara in 2006 (14). In this study we performed by using higher sensitivity nested RT-PCR system than that in the previous study. The mosquitoes collected from two of three-houses of the infected volunteers were found to have all dengue strains (8.7%). The sample from the 1st home only DENV-1 was found. On the other hand, the 2nd home was found with all serotypes. Additionally, DENV-3 and DENV-4 were the dominant strains and had the same infection rates (4.35%) which were noticed twice as higher than DENV-1, which had the infection rate of 2.17%. Moreover, dengue incidence seems likely to correlate with the infectious mosquito in the same house cluster (109). Therefore *Ae. aegypti* in the area might be a more competent strain by hosting a different proportion of circulating virus strains (110). In addition, only the selected variant strain could be transmitted to next generation which may be associated with the viral quasispecies (106, 111). This indicates that monitoring and surveillance of DENV is beneficial to help plan the prevention and control of the disease in the near future of this area (54).

Dengue persistence in asymptomatic subjects

Dengue viruses were detected in plasmas of individuals based on the nested RT-PCR using the *E* gene's primer set with high sensitivity of detection ranged 0.1-

1.0 PFU/ ml [10]. There is DENV-1, DENV-3 and DENV-4 infections found from plasma samples with both single and double infections. This is the first report of DENV-1 & 4 and DENV-3 & 4 co-infected within two-asymptomatic volunteers (Figure 19A-B). It is possible for local humans to acquire dengue viruses via multiple bites of many mosquito vectors with single or multiple infections (14). Additionally, the co-infection situation probably occurred depending on the hosts' immune status, supporting the viral replication (59) and then severe form happened especially in foreigner who have travelled to endemic countries (112). In contrast, our subjects who had co-infections did not show any clinical appearances of dengue infection. Previous studies reported that the closely incidence rates of asymptomatic and symptomatic virus infection in the primary school children were found (109, 113). The issue mentioned as above, the important proportion of inapparent dengue infection could be an important role by serving the virus transmission. The longitudinal studied illustrated the prevalence of symptomatic and asymptomatic dengue infections of 18 cases and 56 cases per 1 000 person-years in adult residents living in the endemic area, Bandung, west Java, Indonesia (114). However, studies often reported the co-infection of patients with acute phase during outbreaks (86). Previous studies reported that the virus can be detected in the white blood cell (115), platelet (116), saliva and urine (117) collected from the patients (during acute and convalescent phases). However, no studies have suggested that these viruses are attenuated or virulent strains. This clue should be confirmed in further researches. Moreover, DENV presence in patients with convalescence phase could be observed by RT-PCR because of an irrespective antibody response (86). We ensured that the result from nested RT-PCR, expressed without false positive through pre-analytical and post analytical stages of testing, which have been performed based on the standard criteria of PCR to reduce the false negative (118). The sample which provided the result of multiple-infections was done by triplicate with the re-extracted template. This might be involved with long-term storage or the sample might contain a limited amount of the virus which was under the threshold for detection. Therefore, well-adaption or its way of escaping from the host's immune response could be the main purpose the virus have been living in the host long-term.

It is possible that some locals-have been infected with the homologous/ heterologous viruses via a single or multiple biting of the mosquito vector (14). Demonstration of more than one dengue virus strains were detected in mosquito vectors collected from Thailand (114). Afterwards, the virus might have remained in their host, producing various quantities, which resulted in rapid viral clearance or viremic titers (119). The situation mentioned above might attribute to the persistent infection and development of secondary infection with heterologous infection (42). Due to, long-term dengue infection through co-circulatory of multiple infections and increase of evolutionary distance, this could contribute to the virus, increased virulence. This led to the study of dengue infection, focusing on the adult population of asymptomatic. Therefore, the co-circulation of multiple dengue infection in asymptomatic populations is a significant novel issue. Concentrating on the study of subclinical infections can help provide a better opportunity in comprehending the viral existence and the pathogenesis of the disease in the human host. Moreover, the basic knowledge of co-infection and concurrent transmission of the virus throughout the endemic areas was the key-factors for the epidemiological design of an effective program for controlling the spread of the disease in the future.

Sequence variation and determination of amino acid changes

The numbers of amino acid mutations were found in each serotype due to these derived from the mosquito. The mutation varied by serotypes and seasons within a year. We found new mutation positions in DENV1-3 both in the mosquito and human. Only DENV-4 have ever been reporting as E359D, N362S, T300K, S375N, S385R (F-strand), A329T (BC-loop), A364V (DE-loop) (96, 120, 121). The mean diversity reveal that DENV-1 (0.59% to 0.21%) has shown declined pattern during 2012-2013 while it contrasted to that of DENV-4 (0.2% to 0.80%). There are not different patterns of the same parameter in DENV-2 & 3. Previous researches have been suggested that different sequence variation in the mosquito and human contributes to viral survival, of which derived from human have more variant strains (quasispecies structure) more than that from the vector (55, 56, 59, 115). Moreover, positive values were measured in DENV-1 in rainy season both 2012 and 2013. The virus might be transmitted from human with current symptomatic appearance (122,

123). The positive selection of DENV-4 in human, the virus might be an effect of preexisting neutralizing antibodies-exposure and then adaption of the viral *E* protein could contribute to viral survival (77).

When considering the sequence variation from only human host, there are numbers of AAs mutations in the *E* gene sequences in all three-dengue viruses which is also known as the structure of viral quasispecies (55, 59). Although only one stop codon in DENV-1 was found, this is significant, representing viral defective genome, associated with variant strain during transmission (55). Moreover, eight clones showed the same mutation position (N375S) in DENV-4; this could be the molecular signature of the serotype in adapting and surviving in the environmental condition of the human cells (Table. 10).

Interestingly, the numbers of host-specific amino acid were found in all four serotypes as V324I (B-strand) and A345V (CD-loop) for DENV-1; H343L (CD loop) and K323L (B-strand) for DENV-3; and A329T, (BC-loop), L357F, A364V (DE-loop), M383D (FG-loop) for DENV-4. These represented preference by host of each dengue virus and contribute to highly transmit between hosts (122).

E glycoprotein of RNA virus in flaviviridae family has been reported that was the main target for studying the potent neutralizing mAbs and treatment-drug (22, 124-126). All domains and hinge region between domains of the protein seemed to be the significant epitope against both type specific and cross reactive DENV-mAb (26, 126, 127), WNV (126) and JEV (124). Number of questions to the potent epitope of the E protein still has been unknown. The mutation position whether effects on the epitope of the protein and could be associated with viral infectivity (122), the surface exposed-3D structure of the protein must be done. The result revealed that the surface-located mutations of the *E* protein comprised F337S (Fig. 25); T303A (Fig. 24); and A345V (Fig. 23) for DENV-1. DENV-2 was T352A (Fig. 27). DENV-3 were F333S (Fig. 31); D326N (Fig. 30); and H343L and K323L (Fig. 29). DENV-4 were; R340G (Fig. 35); S385R (Fig. 34); and M383D (Fig. 33). All of positions are new mutation. These mutations might affect by preexisting cross-reactive neutralizing antibodies. Other mutations which were not shown the protein surface, might not affect the structure or the protein activity (22). According to the result of codon usage

bias showed significant pattern by season in the DENV-1 to 4, these might represented dynamic of evolutionary distance.

Table 10 Substitution positions of amino acid sequences corresponding to regions of E genes. The positions of the protein were determined respected to the basis of the sequence of Hawaii strain for DENV-1, H87 strain for DENV-3 and 814609 strain for DENV-4, respectively.

Serotypes	**Amino acid substitution positions of *E* gene**		
	EDI	**Connecting**	**EDIII**
DENV-1	T276I A280V K284*	T303A	A368V P370L P371L G381E
DENV-3	-	-	S303A Q312H K323T D326N E336K H343L‡ R346K
DENV-4	S272P N277R	-	C333G K342E T353A N364S N375S‡

- ; represented for non-analysis. * ; represented for stop-codon on the codon-frame. ǂ and ǂǂ ; indicated that there are two-(DENV-3) and seven-(DENV-4) clones showed the same-substitution positions of the same amino acid on EDIII, respectively.

Additionally, when comparing the degrees of codon biased of the virus by host it reveal that DENV1, 3 & 4 have shown significant values. The implication associated with the viral infectivity (14). Several studies of genetic distribution of the virus have been tried to explain, that its virulence was the result of the mutation of structural (66, 102, 121, 122), and non-structural coding genes (121, 128). *E*-gene is still the most popular gene candidate involved in the study of evolutionary distance of the virus. In the case of DENV-1, we found that sylvatic genotype has been circulating in the local populations and it represented to attenuate strain (18, 38, 63, 94, 101, 129). This represented that there are enzootic and epidemic transmission cycles occurred in the same area (67). In addition, the sylvatic might travelled to the area by infectious laborer, who emigrated from our neighbor-border countries. Moreover, preexisting non-protective neutralizing antibodies in population might mediate the infection with the sylvatic. Although, this evolution scenario was analyzed by short-period studies when compared to other studies, replacement event by a new genotype was found (62, 63, 79, 86, 97, 130). This result, found that the replacement event was done by nature.

Dengue dynamic and weather data

Considering the trend of dengue dynamic during the studied period (2012-2013), the transmission cycle of mosquito infection was comparable (Fig. 20A) with the cycle based the number of case reports (Fig.18). The atmospheric moister reflects the combined influence of temperature and relative humidity which associated with the growth of vector population (9, 11, 76). The massive vector population and the length of the vector survival were the important factor driving the disease spread eventually (94). According to the result mentioned as above, the temporal dengue disease resulted from the vector density and also the risk to spread the disease (131). In this study, increase of RH in L-phase might contribute to grow the vector population lasting to I- and P-phase. Clear magnitude of the humidity seems likely to correlate with both dengue transmission cycle both mosquito and human. However,

the humidity is also affected by temperature. Interestingly, temperature was directly correlated with the transmission dynamic of each phase (75, 76). In Figure 18, the range of temperature around 28-33^{o}C in P-phase is the most suitable for the disease spread more than the lower temperature. This is because the temperature affected on EIP of the viral replication in the mosquito by reducing the length of the period (75, 93, 94). The event contributed to accelerate the viral transmission between the vector and human host which led to increase the morbidity later.

Conclusions

We proposed that seasonal transmission cycles of the DENV in *Ae. aegypti* mosquitoes and that in human population is comparable. In addition, dynamic spread of all four viruses was mainly associated with the viral evolution and competition inhibition based on more genetic variation. The events might be controlled by viral genetic, gene flow (migration by host), and also weather condition, which drove the density of the vector population and high risk of dengue spread later. Hence, the year-round surveillance should be performed, due to it would be beneficial for planning the effective control and the vaccination should be considered the effect on viral-genetic diversification.

REFERENCES

1. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Report of the Scientific Working Group meeting on Dengue, Geneva, 1-5 October 2006. Geneva: World Health Organization; 2007. 160 p. p.
2. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. Nature reviews Microbiology. 2010 Dec;8(12 Suppl):S7-16. PubMed PMID: 21079655.
3. Thomas SJ, Strickman D, Vaughn DW. Dengue epidemiology: virus epidemiology, ecology, and emergence. Advances in virus research. 2003;61:235-89. PubMed PMID: 14714434.
4. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nature reviews Microbiology. 2007 Jul;5(7):518-28. PubMed PMID: 17558424.
5. Balmaseda A, Standish K, Mercado JC, Matute JC, Tellez Y, Saborio S, et al. Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua. The Journal of infectious diseases. 2010 Jan 1;201(1):5-14. PubMed PMID: 19929380. Pubmed Central PMCID: 3724236.
6. Seidahmed OM, Siam HA, Soghaier MA, Abubakr M, Osman HA, Abd Elrhman LS, et al. Dengue vector control and surveillance during a major outbreak in a coastal Red Sea area in Sudan. Eastern Mediterranean health journal = La revue de sante de la Mediterranee orientale = al-Majallah al-sihhiyah li-sharq al-mutawassit. 2012 Dec;18(12):1217-24. PubMed PMID: 23301396.
7. Naish S, Dale P, Mackenzie JS, McBride J, Mengersen K, Tong S. Spatial and temporal patterns of locally-acquired dengue transmission in northern Queensland, Australia, 1993-2012. PloS one. 2014;9(4):e92524. PubMed PMID: 24691549. Pubmed Central PMCID: 3972162.
8. Gubler DJ. Dengue and dengue hemorrhagic fever. Clinical microbiology reviews. 1998 Jul;11(3):480-96. PubMed PMID: 9665979. Pubmed Central PMCID: 88892.
9. Gubler DJ, Reiter P, Ebi KL, Yap W, Nasci R, Patz JA. Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases. Environmental health perspectives. 2001 May;109 Suppl 2:223-33. PubMed PMID: 11359689. Pubmed Central PMCID: 1240669.
10. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. The American journal of tropical medicine and hygiene. 1988 Jan;38(1):172-80. PubMed PMID: 3341519.
11. Cazelles B, Chavez M, McMichael AJ, Hales S. Nonstationary influence of El Nino on the synchronous dengue epidemics in Thailand. PLoS medicine. 2005 Apr;2(4):e106. PubMed PMID: 15839751. Pubmed Central PMCID: 1087219.
12. Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, Scott RM, et al. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. The American journal of tropical medicine and hygiene. 2003 Feb;68(2):191-202. PubMed PMID: 12641411.
13. McBride WJ, Bielefeldt-Ohmann H. Dengue viral infections; pathogenesis and epidemiology. Microbes and infection / Institut Pasteur. 2000 Jul;2(9):1041-50. PubMed PMID: 10967284.

14. Thavara U, Siriyasatien P, Tawatsin A, Asavadachanukorn P, Anantapreecha S, Wongwanich R, 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. The Southeast Asian journal of tropical medicine and public health. 2006 May;37(3):468-76. PubMed PMID: 17120966.
15. World Health Organization. Dengue guidelines for diagnosis, treatment, prevention and control : new edition. Geneva: World Health Organization; 2009. x, 147 p. p.
16. Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, et al. Unifying the epidemiological and evolutionary dynamics of pathogens. Science. 2004 Jan 16;303(5656):327-32. PubMed PMID: 14726583.
17. Schreiber MJ, Holmes EC, Ong SH, Soh HS, Liu W, Tanner L, et al. Genomic epidemiology of a dengue virus epidemic in urban Singapore. Journal of virology. 2009 May;83(9):4163-73. PubMed PMID: 19211734. Pubmed Central PMCID: 2668455.
18. Raghwani J, Rambaut A, Holmes EC, Hang VT, Hien TT, Farrar J, et al. Endemic dengue associated with the co-circulation of multiple viral lineages and localized density-dependent transmission. PLoS pathogens. 2011 Jun;7(6):e1002064. PubMed PMID: 21655108. Pubmed Central PMCID: 3107208.
19. Rabaa MA, Klungthong C, Yoon IK, Holmes EC, Chinnawirotpisan P, Thaisomboonsuk B, et al. Frequent in-migration and highly focal transmission of dengue viruses among children in Kamphaeng Phet, Thailand. PLoS neglected tropical diseases. 2013;7(1):e1990. PubMed PMID: 23350000. Pubmed Central PMCID: 3547850.
20. Nybakken GE, Nelson CA, Chen BR, Diamond MS, Fremont DH. Crystal structure of the West Nile virus envelope glycoprotein. Journal of virology. 2006 Dec;80(23):11467-74. PubMed PMID: 16987985. Pubmed Central PMCID: 1642602.
21. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nature reviews Microbiology. 2005 Jan;3(1):13-22. PubMed PMID: 15608696.
22. Lin HE, Tsai WY, Liu IJ, Li PC, Liao MY, Tsai JJ, et al. Analysis of epitopes on dengue virus envelope protein recognized by monoclonal antibodies and polyclonal human sera by a high throughput assay. PLoS neglected tropical diseases. 2012 Jan;6(1):e1447. PubMed PMID: 22235356. Pubmed Central PMCID: 3250511.
23. Clyde K, Harris E. RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. Journal of virology. 2006 Mar;80(5):2170-82. PubMed PMID: 16474125. Pubmed Central PMCID: 1395379.
24. Pryor KV, Young JE, Rumsey FJ, Edwards KJ, Bruford MW, Rogers HJ. Diversity, genetic structure and evidence of outcrossing in British populations of the rock fern Adiantum capillus-veneris using microsatellites. Molecular ecology. 2001 Aug;10(8):1881-94. PubMed PMID: 11555233.
25. Zidane N, Dussart P, Bremand L, Villani ME, Bedouelle H. Thermodynamic stability of domain III from the envelope protein of flaviviruses and its improvement by molecular design. Protein engineering, design & selection : PEDS. 2013 Jun;26(6):389-99. PubMed PMID: 23479674.

26. Brien JD, Austin SK, Sukupolvi-Petty S, O'Brien KM, Johnson S, Fremont DH, et al. Genotype-specific neutralization and protection by antibodies against dengue virus type 3. Journal of virology. 2010 Oct;84(20):10630-43. PubMed PMID: 20702644. Pubmed Central PMCID: 2950583.
27. Beltramello M, Williams KL, Simmons CP, Macagno A, Simonelli L, Quyen NT, et al. The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell host & microbe. 2010 Sep 16;8(3):271-83. PubMed PMID: 20833378. Pubmed Central PMCID: 3884547.
28. Chavez JH, Silva JR, Amarilla AA, Moraes Figueiredo LT. Domain III peptides from flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization. Biologicals : journal of the International Association of Biological Standardization. 2010 Nov;38(6):613-8. PubMed PMID: 20817489.
29. Batra G, Nemani SK, Tyagi P, Swaminathan S, Khanna N. Evaluation of envelope domain III-based single chimeric tetravalent antigen and monovalent antigen mixtures for the detection of anti-dengue antibodies in human sera. BMC infectious diseases. 2011;11:64. PubMed PMID: 21401963. Pubmed Central PMCID: 3068959.
30. Brandler S, Ruffie C, Najburg V, Frenkiel MP, Bedouelle H, Despres P, et al. Pediatric measles vaccine expressing a dengue tetravalent antigen elicits neutralizing antibodies against all four dengue viruses. Vaccine. 2010 Sep 24;28(41):6730-9. PubMed PMID: 20688034.
31. Zhao H, Jiang T, Zhou XZ, Deng YQ, Li XF, Chen SP, et al. Induction of neutralizing antibodies against four serotypes of dengue viruses by MixBiEDIII, a tetravalent dengue vaccine. PloS one. 2014;9(1):e86573. PubMed PMID: 24466156. Pubmed Central PMCID: 3897746.
32. Halstead SB, Scanlon JE, Umpaivit P, Udomsakdi S. Dengue and chikungunya virus infection in man in Thailand, 1962-1964. IV. Epidemiologic studies in the Bangkok metropolitan area. The American journal of tropical medicine and hygiene. 1969 Nov;18(6):997-1021. PubMed PMID: 4390977.
33. de Souza LJ, Nogueira RM, Soares LC, Soares CE, Ribas BF, Alves FP, et al. The impact of dengue on liver function as evaluated by aminotransferase levels. The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases. 2007 Aug;11(4):407-10. PubMed PMID: 17873994.
34. Nguyen TL, Nguyen TH, Tieu NT. The impact of dengue haemorrhagic fever on liver function. Research in virology. 1997 Jul-Aug;148(4):273-7. PubMed PMID: 9272578.
35. Salcuni P, Rizzo C. [West Nile disease: review of clinical features and risk factors associated with severe disease]. Le infezioni in medicina : rivista periodica di eziologia, epidemiologia, diagnostica, clinica e terapia delle patologie infettive. 2011 Mar;19(1):5-15. PubMed PMID: 21471741. Malattia da virus West Nile: caratteristiche cliniche e associazione tra fattori di rischio e sviluppo di forme cliniche gravi. Una revisione dei dati di letteratura.
36. Wichmann O, Hongsiriwon S, Bowonwatanuwong C, Chotivanich K, Sukthana Y, Pukrittayakamee S. Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi,

Thailand. Tropical medicine & international health : TM & IH. 2004 Sep;9(9):1022-9. PubMed PMID: 15361117.
37. Sabin AB, Schlesinger RW. Production of Immunity to Dengue with Virus Modified by Propagation in Mice. Science. 1945 Jun 22;101(2634):640-2. PubMed PMID: 17844088.
38. Rico-Hesse R. Microevolution and virulence of dengue viruses. Advances in virus research. 2003;59:315-41. PubMed PMID: 14696333. Pubmed Central PMCID: 3045824.
39. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science. 1988 Jan 29;239(4839):476-81. PubMed PMID: 3277268.
40. World Health Organization. Dept. of Immunization Vaccines and Biologicals., UNICEF., World Health Organization. Expanded Programme on Immunization. WHO-UNICEF guidelines for developing a comprehensive multi-year plan (cMYP). Geneva: World Health Organization; 2005. vii, 60 p. p.
41. World Health Organization. Regional Office for South-East Asia. Comprehensive school health education : recommendations and guidelines for implementing and strengthening comprehensive school health education in the South-East Asia Region. New Delhi: WHO Regional Office for Sourh-East Asia; 1992. 63 p. p.
42. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013 Apr 25;496(7446):504-7. PubMed PMID: 23563266. Pubmed Central PMCID: 3651993.
43. World Health Organization. Global strategy for dengue prevention and control 2012-2020 (in IRIS). Geneva: World Health Organization; 2012. v, 35 p. p.
44. Alves MJ, Fernandes PL, Amaro F, Osorio H, Luz T, Parreira P, et al. Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(6). PubMed PMID: 23410256.
45. Huhtamo E, Korhonen E, Vapalahti O. Imported dengue virus serotype 1 from Madeira to Finland 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2013;18(8). PubMed PMID: 23449230.
46. Kumar A, Gittens-St Hilaire M, Nielsen AL. Epidemiological trends and clinical manifestations of Dengue among children in one of the English-speaking Caribbean countries. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2013 Apr;107(4):254-60. PubMed PMID: 23423432.
47. Torres-Aponte JM, Luce RR, Hunsperger E, Munoz-Jordan JL, Beltran M, Vergne E, et al. Enhanced West Nile virus surveillance in a dengue-endemic area--Puerto Rico, 2007. The American journal of tropical medicine and hygiene. 2013 May;88(5):997-1002. PubMed PMID: 23478583. Pubmed Central PMCID: 3752770.
48. Rodriguez-Roche R, Villegas E, Cook S, Poh Kim PA, Hinojosa Y, Rosario D, et al. Population structure of the dengue viruses, Aragua, Venezuela, 2006-2007. Insights into dengue evolution under hyperendemic transmission. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2012 Mar;12(2):332-44. PubMed PMID: 22197765. Pubmed Central PMCID: 3919160.

49. De La Cruz Hernandez SI, Ortiz-Navarrete V, Ludert JE, Del Angel RM. Dengue specific IgM seropositivity correlates with severe clinical outcome. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2013 Dec;58(4):751. PubMed PMID: 24144672.
50. Guo X, Zhao Q, Wu C, Zuo S, Zhang X, Jia N, et al. First isolation of dengue virus from Lao PDR in a Chinese traveler. Virology journal. 2013;10:70. PubMed PMID: 23497045. Pubmed Central PMCID: 3606315.
51. Wilson MR. Emerging viral infections. Current opinion in neurology. 2013 Jun;26(3):301-6. PubMed PMID: 23591683.
52. Alzahrani AG, Al Mazroa MA, Alrabeah AM, Ibrahim AM, Mokdad AH, Memish ZA. Geographical distribution and spatio-temporal patterns of dengue cases in Jeddah Governorate from 2006-2008. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2013 Jan;107(1):23-9. PubMed PMID: 23222946.
53. Cook AR, Carrasco LR, Lee VJ, Ooi EE, Chen MI, Lye DC, et al. Higher risk of infection with dengue at the weekend among male Singaporeans. The American journal of tropical medicine and hygiene. 2012 Dec;87(6):1116-8. PubMed PMID: 23128291. Pubmed Central PMCID: 3516085.
54. Chakravarti A, Chauhan MS, Kumar S, Ashraf A. Genotypic characterization of dengue virus strains circulating during 2007-2009 in New Delhi. Archives of virology. 2013 Mar;158(3):571-81. PubMed PMID: 23129129.
55. Lin SR, Hsieh SC, Yueh YY, Lin TH, Chao DY, Chen WJ, et al. Study of sequence variation of dengue type 3 virus in naturally infected mosquitoes and human hosts: implications for transmission and evolution. Journal of virology. 2004 Nov;78(22):12717-21. PubMed PMID: 15507664. Pubmed Central PMCID: 525091.
56. Wang WK, Lin SR, Lee CM, King CC, Chang SC. Dengue type 3 virus in plasma is a population of closely related genomes: quasispecies. Journal of virology. 2002 May;76(9):4662-5. PubMed PMID: 11932434. Pubmed Central PMCID: 155095.
57. Weaver SC, Brault AC, Kang W, Holland JJ. Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. Journal of virology. 1999 May;73(5):4316-26. PubMed PMID: 10196330. Pubmed Central PMCID: 104213.
58. Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, et al. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. Journal of virology. 1992 May;66(5):3225-9. PubMed PMID: 1313927. Pubmed Central PMCID: 241092.
59. Steinhauer DA, Holland JJ. Rapid evolution of RNA viruses. Annual review of microbiology. 1987;41:409-33. PubMed PMID: 3318675.
60. Sanchez IJ, Ruiz BH. A single nucleotide change in the E protein gene of dengue virus 2 Mexican strain affects neurovirulence in mice. The Journal of general virology. 1996 Oct;77 (Pt 10):2541-5. PubMed PMID: 8887488.
61. Goncalvez AP, Escalante AA, Pujol FH, Ludert JE, Tovar D, Salas RA, et al. Diversity and evolution of the envelope gene of dengue virus type 1. Virology. 2002 Nov 10;303(1):110-9. PubMed PMID: 12482662.
62. Anez G. [Molecular evolution of dengue virus: a necessary field of research]. Investigacion clinica. 2007 Sep;48(3):273-6. PubMed PMID: 17853786. Evolucion molecular del virus dengue: un area de investigacion prioritaria.

63. Kukreti H, Dash PK, Parida M, Chaudhary A, Saxena P, Rautela RS, et al. Phylogenetic studies reveal existence of multiple lineages of a single genotype of DENV-1 (genotype III) in India during 1956-2007. Virology journal. 2009;6:1. PubMed PMID: 19126194. Pubmed Central PMCID: 2667167.
64. Anoop M, Mathew AJ, Jayakumar B, Issac A, Nair S, Abraham R, et al. Complete genome sequencing and evolutionary analysis of dengue virus serotype 1 isolates from an outbreak in Kerala, South India. Virus genes. 2012 Aug;45(1):1-13. PubMed PMID: 22729802.
65. Huang JH, Su CL, Yang CF, Liao TL, Hsu TC, Chang SF, et al. Molecular characterization and phylogenetic analysis of dengue viruses imported into Taiwan during 2008-2010. The American journal of tropical medicine and hygiene. 2012 Aug;87(2):349-58. PubMed PMID: 22855770. Pubmed Central PMCID: 3414576.
66. Ke R, Aaskov J, Holmes EC, Lloyd-Smith JO. Phylodynamic analysis of the emergence and epidemiological impact of transmissible defective dengue viruses. PLoS pathogens. 2013 Feb;9(2):e1003193. PubMed PMID: 23468631. Pubmed Central PMCID: 3585136.
67. Wang E, Ni H, Xu R, Barrett AD, Watowich SJ, Gubler DJ, et al. Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. Journal of virology. 2000 Apr;74(7):3227-34. PubMed PMID: 10708439. Pubmed Central PMCID: 111823.
68. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. Journal of clinical microbiology. 1992 Mar;30(3):545-51. PubMed PMID: 1372617. Pubmed Central PMCID: 265106.
69. Libraty DH, Endy TP, Houng HS, Green S, Kalayanarooj S, Suntayakorn S, et al. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. The Journal of infectious diseases. 2002 May 1;185(9):1213-21. PubMed PMID: 12001037.
70. Jarman RG, Holmes EC, Rodpradit P, Klungthong C, Gibbons RV, Nisalak A, et al. Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. Journal of virology. 2008 Jun;82(11):5494-500. PubMed PMID: 18367520. Pubmed Central PMCID: 2395201.
71. Slifer EH, Sekhon SS. The fine structure of the sense organs on the antennal flagellum of the yellow fever mosquito Aedes aegypti (Linnaeus). Journal of morphology. 1962 Jul;111:49-67. PubMed PMID: 13913860.
72. Adams CF. Aedes Aegypti Linnaeus, the Yellow Fever Mosquito, in Central Missouri. Science. 1942 Jan 2;95(2453):19. PubMed PMID: 17752151.
73. Malavige GN, Ranatunga PK, Jayaratne SD, Wijesiriwardana B, Seneviratne SL, Karunatilaka DH. Dengue viral infections as a cause of encephalopathy. Indian journal of medical microbiology. 2007 Apr;25(2):143-5. PubMed PMID: 17582186.
74. Malavige GN, Fernando S, Fernando DJ, Seneviratne SL. Dengue viral infections. Postgraduate medical journal. 2004 Oct;80(948):588-601. PubMed PMID: 15466994. Pubmed Central PMCID: 1743110.
75. Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A. Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. The American journal of tropical medicine and hygiene. 1987 Jan;36(1):143-52. PubMed PMID: 3812879.

76. Chan M, Johansson MA. The incubation periods of Dengue viruses. PloS one. 2012;7(11):e50972. PubMed PMID: 23226436. Pubmed Central PMCID: 3511440.
77. Endy TP, Nisalak A, Chunsuttitwat S, Vaughn DW, Green S, Ennis FA, et al. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. The Journal of infectious diseases. 2004 Mar 15;189(6):990-1000. PubMed PMID: 14999601.
78. Ander M, Meiswinkel R, Chirico J. Seasonal dynamics of biting midges (Diptera: Ceratopogonidae: Culicoides), the potential vectors of bluetongue virus, in Sweden. Veterinary parasitology. 2012 Feb 28;184(1):59-67. PubMed PMID: 21944873.
79. Wongkoon S, Jaroensutasinee M, Jaroensutasinee K. Assessing the temporal modelling for prediction of dengue infection in northern and north-eastern, Thailand. Tropical biomedicine. 2012 Sep;29(3):339-48. PubMed PMID: 23018496.
80. Aguiar M, Ballesteros S, Kooi BW, Stollenwerk N. The role of seasonality and import in a minimalistic multi-strain dengue model capturing differences between primary and secondary infections: complex dynamics and its implications for data analysis. Journal of theoretical biology. 2011 Nov 21;289:181-96. PubMed PMID: 21907213.
81. Kovats RS, Campbell-Lendrum DH, McMichael AJ, Woodward A, Cox JS. Early effects of climate change: do they include changes in vector-borne disease? Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2001 Jul 29;356(1411):1057-68. PubMed PMID: 11516383. Pubmed Central PMCID: 1088500.
82. World Health Organization. Dengue haemorrhagic fever : diagnosis, treatment, prevention and control. 2nd ed. Geneva: World Health Organization; 1997.
83. Yenchitsomanus PT, Sricharoen P, Jaruthasana I, Pattanakitsakul SN, Nitayaphan S, Mongkolsapaya J, et al. Rapid detection and identification of dengue viruses by polymerase chain reaction (PCR). The Southeast Asian journal of tropical medicine and public health. 1996 Jun;27(2):228-36. PubMed PMID: 9279982.
84. Zhou MY, Clark SE, Gomez-Sanchez CE. Universal cloning method by TA strategy. BioTechniques. 1995 Jul;19(1):34-5. PubMed PMID: 7669292.
85. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution. 2013 Dec;30(12):2725-9. PubMed PMID: 24132122. Pubmed Central PMCID: 3840312.
86. Figueiredo RM, Naveca FG, Oliveira CM, Bastos Mde S, Mourao MP, Viana Sde S, et al. Co-infection of Dengue virus by serotypes 3 and 4 in patients from Amazonas, Brazil. Revista do Instituto de Medicina Tropical de Sao Paulo. 2011 Nov-Dec;53(6):321-3. PubMed PMID: 22183455.
87. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics. 2006 Jan 15;22(2):195-201. PubMed PMID: 16301204.
88. Pond SL, Frost SD. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics. 2005 May 15;21(10):2531-3. PubMed PMID: 15713735.
89. Campbell KM, Lin CD, Iamsirithaworn S, Scott TW. The complex relationship between weather and dengue virus transmission in Thailand. The

American journal of tropical medicine and hygiene. 2013 Dec;89(6):1066-80. PubMed PMID: 23958906. Pubmed Central PMCID: 3854883.
90. Pongsiri P, Themboonlers A, Poovorawan Y. Changing pattern of dengue virus serotypes in Thailand between 2004 and 2010. Journal of health, population, and nutrition. 2012 Sep;30(3):366-70. PubMed PMID: 23082638. Pubmed Central PMCID: 3489952.
91. Carrington LB, Seifert SN, Armijos MV, Lambrechts L, Scott TW. Reduction of Aedes aegypti vector competence for dengue virus under large temperature fluctuations. The American journal of tropical medicine and hygiene. 2013 Apr;88(4):689-97. PubMed PMID: 23438766. Pubmed Central PMCID: 3617853.
92. Carrington LB, Seifert SN, Willits NH, Lambrechts L, Scott TW. Large diurnal temperature fluctuations negatively influence Aedes aegypti (Diptera: Culicidae) life-history traits. Journal of medical entomology. 2013 Jan;50(1):43-51. PubMed PMID: 23427651.
93. Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD, Thomas MB, et al. Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. Proceedings of the National Academy of Sciences of the United States of America. 2011 May 3;108(18):7460-5. PubMed PMID: 21502510. Pubmed Central PMCID: 3088608.
94. Kramer LD, Ebel GD. Dynamics of flavivirus infection in mosquitoes. Advances in virus research. 2003;60:187-232. PubMed PMID: 14689695.
95. Farrar J, Focks D, Gubler D, Barrera R, Guzman MG, Simmons C, et al. Towards a global dengue research agenda. Tropical medicine & international health : TM & IH. 2007 Jun;12(6):695-9. PubMed PMID: 17550466.
96. Chu PY, Ke GM, Chen PC, Liu LT, Tsai YC, Tsai JJ. Spatiotemporal dynamics and epistatic interaction sites in dengue virus type 1: a comprehensive sequence-based analysis. PloS one. 2013;8(9):e74165. PubMed PMID: 24040199. Pubmed Central PMCID: 3767619.
97. Behura SK, Severson DW. Nucleotide substitutions in dengue virus serotypes from Asian and American countries: insights into intracodon recombination and purifying selection. BMC microbiology. 2013;13:37. PubMed PMID: 23410119. Pubmed Central PMCID: 3598932.
98. Volz EM, Koelle K, Bedford T. Viral phylodynamics. PLoS computational biology. 2013;9(3):e1002947. PubMed PMID: 23555203. Pubmed Central PMCID: 3605911.
99. Lee VJ, Chen MI, Yap J, Ong J, Lim WY, Lin RT, et al. Comparability of different methods for estimating influenza infection rates over a single epidemic wave. American journal of epidemiology. 2011 Aug 15;174(4):468-78. PubMed PMID: 21719743. Pubmed Central PMCID: 3148265.
100. Jing QL, Yang ZC, Luo L, Xiao XC, Di B, He P, et al. Emergence of dengue virus 4 genotype II in Guangzhou, China, 2010: survey and molecular epidemiology of one community outbreak. BMC infectious diseases. 2012;12:87. PubMed PMID: 22497881. Pubmed Central PMCID: 3375192.
101. Tripathi SK, Gupta P, Khare V, Chatterjee A, Kumar R, Khan MY, et al. Emergence of new lineage of Dengue virus 3 (genotype III) in Lucknow, India. Iranian journal of microbiology. 2013 Mar;5(1):68-75. PubMed PMID: 23466682. Pubmed Central PMCID: 3577557.

102. Perez-Ramirez G, Diaz-Badillo A, Camacho-Nuez M, Cisneros A, Munoz Mde L. Multiple recombinants in two dengue virus, serotype-2 isolates from patients from Oaxaca, Mexico. BMC microbiology. 2009;9:260. PubMed PMID: 20003526. Pubmed Central PMCID: 2804599.
103. A AN, Berlioz-Arthaud A, Chow V, Endy T, Lowry K, Mai le Q, et al. Sustained transmission of dengue virus type 1 in the Pacific due to repeated introductions of different Asian strains. Virology. 2004 Nov 24;329(2):505-12. PubMed PMID: 15518827.
104. Barbazan P, Yoksan S, Gonzalez JP. Dengue hemorrhagic fever epidemiology in Thailand: description and forecasting of epidemics. Microbes and infection / Institut Pasteur. 2002 Jun;4(7):699-705. PubMed PMID: 12067829.
105. Khampapongpane B, Lewis HC, Ketmayoon P, Phonekeo D, Somoulay V, Khamsing A, et al. National dengue surveillance in the Lao People's Democratic Republic, 2006-2012: epidemiological and laboratory findings. Western Pacific surveillance and response journal : WPSAR. 2014 Jan-Mar;5(1):7-13. PubMed PMID: 24734212. Pubmed Central PMCID: 3984965.
106. Lee KS, Lo S, Tan SS, Chua R, Tan LK, Xu H, et al. Dengue virus surveillance in Singapore reveals high viral diversity through multiple introductions and in situ evolution. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2012 Jan;12(1):77-85. PubMed PMID: 22036707.
107. Chen CF, Shu PY, Teng HJ, Su CL, Wu JW, Wang JH, 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 and zoonotic diseases. 2010 Dec;10(10):1017-25. PubMed PMID: 21128850.
108. Gibbons RV, Kalanarooj S, Jarman RG, Nisalak A, Vaughn DW, Endy TP, et al. Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions and dengue hemorrhagic fever, and serotype sequences. The American journal of tropical medicine and hygiene. 2007 Nov;77(5):910-3. PubMed PMID: 17984352.
109. Yoon IK, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, Koenraadt CJ, et al. Fine scale spatiotemporal clustering of dengue virus transmission in children and Aedes aegypti in rural Thai villages. PLoS neglected tropical diseases. 2012;6(7):e1730. PubMed PMID: 22816001. Pubmed Central PMCID: 3398976.
110. Sakudo A, Onodera T, Shintani H, Ikuta K. Dengue virus presence and surveillance in Okinawa (Review). Experimental and therapeutic medicine. 2012 Jan;3(1):15-7. PubMed PMID: 22969837. Pubmed Central PMCID: 3438643.
111. Khoo CC, Doty JB, Held NL, Olson KE, Franz AW. Isolation of midgut escape mutants of two American genotype dengue 2 viruses from Aedes aegypti. Virology journal. 2013;10:257. PubMed PMID: 23937713. Pubmed Central PMCID: 3751248.
112. Cnops L, Domingo C, Van den Bossche D, Vekens E, Brigou E, Van Esbroeck M. First dengue co-infection in a Belgian traveler returning from Thailand, July 2013. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2014 Oct 22;61(4):597-9. PubMed PMID: 25453334.

113. Endy TP, Anderson KB, Nisalak A, Yoon IK, Green S, Rothman AL, et al. Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS neglected tropical diseases. 2011;5(3):e975. PubMed PMID: 21390158. Pubmed Central PMCID: 3046956.
114. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, Rudiman PI, et al. Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. The American journal of tropical medicine and hygiene. 2005 Jan;72(1):60-6. PubMed PMID: 15728868.
115. Wang HL, Lin KH, Yueh YY, Chow L, Wu YC, Chen HY, et al. Efficient diagnosis of dengue infections using patients' peripheral blood leukocytes and serum/plasma. Intervirology. 2000;43(2):107-11. PubMed PMID: 10971129.
116. Noisakran S, Gibbons RV, Songprakhon P, Jairungsri A, Ajariyakhajorn C, Nisalak A, et al. Detection of dengue virus in platelets isolated from dengue patients. The Southeast Asian journal of tropical medicine and public health. 2009 Mar;40(2):253-62. PubMed PMID: 19323010.
117. Ma X, Zhen W, Yang P, Sun X, Nie W, Zhang L, et al. First confirmation of imported dengue virus serotype 2 complete genome in urine from a Chinese traveler returning from India. Virology journal. 2014;11:56. PubMed PMID: 24666930. Pubmed Central PMCID: 3986945.
118. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature. 1989 May 18;339(6221):237-8. PubMed PMID: 2716852.
119. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. The Journal of infectious diseases. 2000 Jan;181(1):2-9. PubMed PMID: 10608744.
120. Bedi SK, Prasad A, Mathur K, Bhatnagar S. Positive selection and evolution of dengue type-3 virus in the Indian subcontinent. Journal of vector borne diseases. 2013 Sep;50(3):188-96. PubMed PMID: 24220077.
121. Chen WJ, Wu HR, Chiou SS. E/NS1 modifications of dengue 2 virus after serial passages in mammalian and/or mosquito cells. Intervirology. 2003;46(5):289-95. PubMed PMID: 14555849.
122. Erb SM, Butrapet S, Moss KJ, Luy BE, Childers T, Calvert AE, et al. Domain-III FG loop of the dengue virus type 2 envelope protein is important for infection of mammalian cells and Aedes aegypti mosquitoes. Virology. 2010 Oct 25;406(2):328-35. PubMed PMID: 20708768.
123. Rodpothong P, Auewarakul P. Positive selection sites in the surface genes of dengue virus: phylogenetic analysis of the interserotypic branches of the four serotypes. Virus genes. 2012 Jun;44(3):408-14. PubMed PMID: 22222691.
124. Goncalvez AP, Chien CH, Tubthong K, Gorshkova I, Roll C, Donau O, et al. Humanized monoclonal antibodies derived from chimpanzee Fabs protect against Japanese encephalitis virus in vitro and in vivo. Journal of virology. 2008 Jul;82(14):7009-21. PubMed PMID: 18480437. Pubmed Central PMCID: 2446956.
125. Zhang S, Vogt MR, Oliphant T, Engle M, Bovshik EI, Diamond MS, et al. Development of resistance to passive therapy with a potently neutralizing humanized monoclonal antibody against West Nile virus. The Journal of infectious diseases.

2009 Jul 15;200(2):202-5. PubMed PMID: 19527169. Pubmed Central PMCID: 2752978.
126. Vogt MR, Moesker B, Goudsmit J, Jongeneelen M, Austin SK, Oliphant T, et al. Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step. Journal of virology. 2009 Jul;83(13):6494-507. PubMed PMID: 19386704. Pubmed Central PMCID: 2698525.
127. Crill WD, Hughes HR, Delorey MJ, Chang GJ. Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens. PloS one. 2009;4(4):e4991. PubMed PMID: 19337372. Pubmed Central PMCID: 2659788.
128. Hanley KA, Manlucu LR, Gilmore LE, Blaney JE, Jr., Hanson CT, Murphy BR, et al. A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. Virology. 2003 Jul 20;312(1):222-32. PubMed PMID: 12890635.
129. Manakkadan A, Joseph I, Prasanna RR, Kunju RI, Kailas L, Sreekumar E. Lineage shift in Indian strains of Dengue virus serotype-3 (Genotype III), evidenced by detection of lineage IV strains in clinical cases from Kerala. Virology journal. 2013;10:37. PubMed PMID: 23360780. Pubmed Central PMCID: 3598737.
130. Mammen MP, Pimgate C, Koenraadt CJ, Rothman AL, Aldstadt J, Nisalak A, et al. Spatial and temporal clustering of dengue virus transmission in Thai villages. PLoS medicine. 2008 Nov 4;5(11):e205. PubMed PMID: 18986209. Pubmed Central PMCID: 2577695.
131. Thu HM, Aye KM, Thein S. The effect of temperature and humidity on dengue virus propagation in Aedes aegypti mosquitos. The Southeast Asian journal of tropical medicine and public health. 1998 Jun;29(2):280-4. PubMed PMID: 9886113.

APPENDICES

APPENDIX A
RESEARCH INSTRUMENTS

1) Automatic adjustable micropipette (Eppendorf, Germany)

2) Combs (Bio-Rad, USA)

3) DNA thermal cycler (Eppendorf, Germany)

4) Electrophoresis chamber set (Bio-Rad, USA)

5) Heat block (Bockel Scientific)

6) Para film (American Naation Can, USA)

7) Pipet tip (Eppendorf, Germany)

8) Micro centrifuge (Eppendorf, Germany)

9) Votex mixture (Scientific industry, USA)

10) CFX96 (Bio-Rad, USA)

11) Incubator chaker

APPENDIX B
CHEMICAL AND RAGENT COMPONENTS

1) 10X TAE buffer

- 11.4 mL of glacial acetic acid (17.4 M)
- 3.7 g of EDTA, disodium salt
- deionized water
- Prepare the 10X TAE Electrophoresis Buffer

Dissolve the Tris, glacial acetic acid and EDTA in 800 ml of deionized water. Dilute the buffer to 1 L. You do not need to sterilize the solution. Store the bottle of 10X buffer solution at room temperature.

2) 10X PBS buffer

- 137 mM (80g) Sodium chloride
- 2.7 mM (2.0g) Potassium chloride
- 10 mM (14.4g) Disodium hydrogen phosphate
- 1.8 mM (2.4g) Potassium dihydrogen phosphate

Dissolve the following in 800ml distilled H_2O and then adjust pH to 7.4. Adjust volume to 1L with additional distilled H2O. Sterilize by autoclaving and store at room temperature.

3) 0.5 M EDTA (pH8.0)

- 0.5M, 1L: 148 g EDTA
- 30-40 g NaOH to adjust pH

pH adjusted by NaOH is essential for solubility and the reagent would be autoclaved.

4) 1 M Tris-HCL 1L

- 121.14 g Tris
- Absolute HCL
- Deionized water

Dissolve Tris in 800 ml dH_2O and djust pH of the solution to 7.0 with the absolute HCl solution. Add final volume up to 1 liter with dH2O. Autoclave and store at room temperature.

5) 1X SOC outgrowth Medium

- 2% Vegetable Peptone
- 0.5% Yeast Extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl2
- 10 mM MgSO4
- 20 mM Glucose

Dissolve all components in 100 ml dH2O. Autoclave and store at 4^oC.

6) 1X SOB outgrowth medium 1L

- 20 g Bacto tryptone
- 5 g Bacto-Yeast extract
- 0.5 g NaCl
- 10 ml 250 mM KCl

Dissolve all components in 800 ml dH_2O and adjust pH of the solution to 7.0. Add final volume up to 1 liter with dH_2O. Autoclave and store at 4^oC. Add add 5 ml of 2mM $MgCl_2$ before use.

7) 1000x Antibiotics

- 100 mg/ml Ampicillin in H_2O

8) 5X DNA loading sample buffer, 100 ml

- 40% sucrose
- 0.01-0.02% bromophenol blue

Add 40 g sucrose to 80 ml dH_2O, add 2 ml 1% BPB solution and adjust total volume up to 100 ml by dH_2O.

9) LB medium (Luria-Bertani Medium)

- 10 g Bacto Tryptone
- 5 g Bacto-Yeast extract
- 10 g NaCl

Dissolve all components in 1000 mL dH_2O, adjust pH to 7.0 and autoclave 1 h. Before store at 4^{o}C, add 1ml of 100 mg/ml Ampicillin.

10) 1 M IPTG

- 1 g IPTG resolved in 4.2 ml of dH_2O and then filters through 0.22 µm filters. Aliquot 1 ml in Eppendorf tube before store at -20^{o}C.

11) 20mg/ml X-gal

- DMSO solution
- 100 mg X-gal

Add 5 ml DMSO solution with 100 mg X-gal bottom and then aliquot 1 ml in Eppendorf tube before store at -20^{o}C.

APPENDIX C

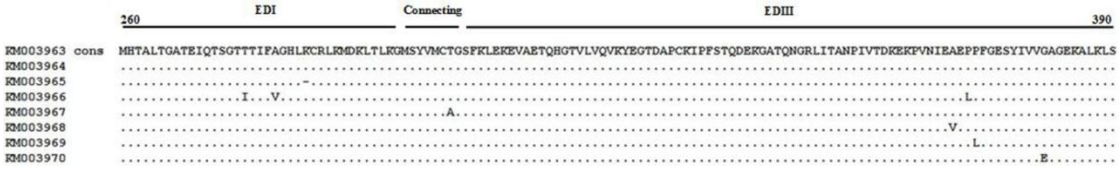
DETECTION OF DENGUE INFECTION AND MULTIPLE ALIGNMENTS

Table 11 Results of amplification of E genes based on RT-PCR and Nested RT-PCR which were prepared from plasma samples. The sample was collected from local asymptomatic populations without the historical data of dengue infection during the studied period.

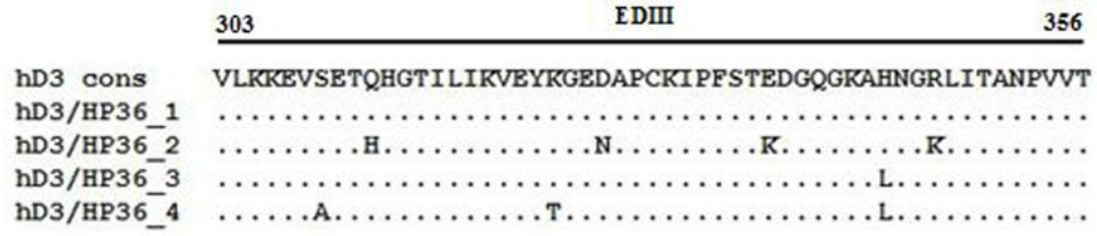
No. (Samples)	**Sex**	**Age**	***E* gene (Plasma)**	***E* gene (Typing)**
1 (S27)	F	59	+	DENV-1 &DENV-4
2 (S34)	F	59	+	DENV-4
3 (S36)	F	60	+	DENV-3 &DENV-4
4 (S43)	F	45	+	DENV-4
5 (S51)	F	27	+	DENV-4

F: female, M: male, +: positive result, -: negative result

A.



B.



C.

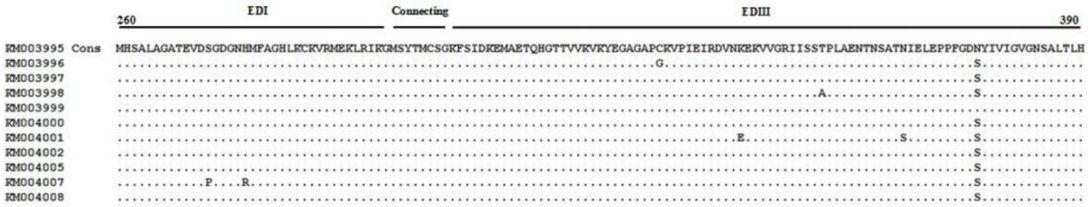


Figure 43 Amino acid sequences alignment of E-proteins of multiple clones from local asymptomatic populations. The positions of amino acid sequences and the equivalent subunits within the mentioned protein as determined based on Hawaii strain of DENV-1 (A), H87 strain of DENV-3 (B) and 814609 strain of DENV-4 (C), respectively. A consensus sequence (Cons) was constructed for each serotype. Dashes (.); represented for sequence identity and an underline (-); represented for an in-frame stop codon. Accession numbers (DENV-1 & 4) and names (DENV-3) of individuals are shown at the left-side. For uncomplicatedness, only one-pattern of samples with identical sequences is displayed.

APPENDIX D

THE REFERENCE SEQUENCE FOR CONSTRUCTING THE PHYLOGENETIC TREES

Table 12 Previously published sequences of DENV-1 and outgroup for constructing the phylogenetic tree of DENV-1.

Strains	Serotypes	Acc. no. (GenBank)
D1.02-20. Japan	1	AB178040
SG(EHI)D1209Y03	1	FJ469907
N02-23-1HuNIID/Thai/02	1	AB111079
D1MY05-33915	1	FN429890
D1/Malaysia/36000/05	1	FR666924
Fj231/04	1	DQ193572
D1/SG/06K2290DK1/2006	1	EU081281
D1/SG/06K2236DK1/2006	1	EU081280
D1/SG/05K4175DK1/2005	1	EU081264
D1.Myanmar.305/01	1	AY713476
My01D144168	1	AY618211
01-61-1HuNIID/Cambodia/01	1	AB111071
Cambodia/1998	1	AF309641
D1.Myanmar.49440/02	1	AY726553
ZJ01/2004	1	AY835999
ThD1 0049 01	1	AY732401
ThD1 0488 94	1	AY732427
D1.Myanmar.206/01	1	AY713475
ThD1 0323 91	1	AY732478
D1.Myanmar.44988/02	1	AY726552
D1/H/IMTSSA/98/606	1	AF298808
ThD1 0008 81	1	AY732385
ThD1 0081 82/1982	1	AY732397
D1.Myanmar.38862/01	1	AY726550
Thailand/PUO 359/80	1	AF425630
01-37-1HuNIID/Samoa/2001	1	AB111068
HawO3663/2001	1	DQ672564
02-13-1HuNIID/Philippines/2002	1	AB111074
SC01	1	AY858983
Thailand AHF82-80:98	1	D00502
AUS HATI7/83	1	AF425612
ET243/Timor/2000	1	EF440432
98901530 DF DV-1/IND/1998	1	AB189121
D1/hu/Seychelles/NIID41/2003	1	AB195673
Reunion 257/04	1	DQ285560
Malaysia/P72-1244	1	AF425622
Singapore S275/90/MAL/1980	1	M87512

Strains	Serotypes	Acc. no. (GenBank)
DEI 0151	1	AF425626
DENV-1/VG/BID-V2937/1985	1	GQ868601
DENV-1/MX/BID-V3758/2008	1	GQ868537
Cote D-Ivoire:Abidjan/98	1	AF298807
Nigeria/IBH 28328/68	1	AF425625
D1/SG/05K4147DK1/2005	1	EU081258
D1.Myanmar.23819/96	1	AY722802
D1.Myanmar.40553/71	1	AY713473
Singapore 8114/93	1	AY762084
D1/IN/RGCB592/2009	1	JN903581
Costa Rica/93	1	AY153755
DENV-1/US/BID-V2138/1996	1	FJ478457
RIO H 36589	1	AF425610
Paraguay.259/00	1	AF514883
Venezuela.150172/95	1	AF425633
US-FL/KW10AG/2010	1	JF519855
DENV-1/MX/BID-V3758/2008	1	GQ868537
French Guiana/89	1	AF226687
FGA/NA d1d	1	AF226686
DENV-1/US/BID-V2130/1995	1	FJ547086
ThD1 0442 80	1	AY732476
ThD1 0673 80	1	AY732474
D2/Cambodia/0907aTw	2	JF967989 (outgroup)
D3/Myanmar/0911aTw	3	JF968088 (outgroup)

Table 13 Previously published sequences of DENV-2 and outgroup for constructing the phylogenetic tree of DENV-2.

Strains	Serotypes	Acc. no. (GenBank)
D2/Th/1009aTw/2010	2	JF968041
D2/Vietnam/0910aTw/2009	2	JF967999
D2/Taiwan/900PT0910a/2009	2	JQ403523
D2/Vietnam/1011aTw/2010	2	JF968048
D2/Vietnam/0804aTw/2008	2	JF967961
D2/Thailand/1009bTw/2010	2	JF968042
D2/Vietnam/1012aTw/2010	2	JF968052
ThD2_0981_00/2000	2	DQ181872
DENV-2/IPC/BID-V3791/2008	2	GQ868631
D2/Cambodia/0809aTw/2008	2	JF967966
D2/Cambodia/0907aTw/2009	2	JF967989
D2/Thailand/1007cTw/2010	2	JF968030
DENV-2/IPC/BID-V3922/2008	2	GU131924
D2/Laos/1006aTw/2010	2	JF968020
D2/Myanmar/1007aTw/2010	2	JF968026
D2/Thailand/0908aTw/2009	2	JF967990
ThD2_0017_98/1998	2	DQ181799
Th/C0166/1988	2	AF100463
Myan0410a/Tw/2004	2	DQ518653
ThD2_0026_88/1988	2	DQ181802
ThD2_0498_84/1984	2	DQ181804
VE/61154/2007	2	HQ332189
DENV-2/VE/BID-V1104/2007	2	EU482605
DENV-2/CO/BID-V3371/2005	2	GQ868555
DENV-2/MX/BID-V3713/2007	2	GQ868515
DENV-2/US/BID-V854/2001	2	EU482593
DENV-2/US/BID-V851/1990	2	EU482590
DENV-2/US/BID-V1164/1986	2	EU482568
DENV-2/VE/BID-V2941/1998	2	FJ898465
DENV-2/US/BID-V1054/1996	2	EU482561
New Guinea/NGC/1944	2	M29095
D2/India/0908bTw/2009	2	JF967992
DENV-2/LK/BID-V2422/2004	2	GQ252677
FJ11/99/2001	2	AF359579
India/NIV_97833/1997	2	FJ538913
D2/Philippines/1002aTw/2010	2	JF968004
D2/Philippines/1011bTw2010	2	JF968047
Australia/TSV01/1993	2	AY037116
Brunei/DS09-280106/2006	2	EU179859
SG(EHI)DED05207/2005	2	GQ357790
D2/Malaysia/1011aTw/2010	2	JF968049
D2/Singapore/1007aTw/2010	2	JF968025
Indo/TB16i/2007	2	AY858036

Strains	Serotypes	Acc. no. (GenBank)
Chi/ZH1340/2007	2	EU359009
D2/Indonesia/1003aTw/2010	2	JF968008
DENV-2/PR/BID-V3367/1969	2	GQ868600
DENV-2/MX/BID-V3355/1983	2	GQ868589
DENV-2/VE/BID-V3366/1987	2	GQ868599
Mexico/131/1992	2	AF100469
Colombia/I348600/1986	2	AY702040
Peru/IQT2913/1998	2	AF100468
Senegal/DAKHD10674/1970	2	AF231720
Indonesia 1976	4	U18429 (outgroup)
D1/Thailand AHF82-80/1989	1	D00502 (outgroup)
D3/Myanmar/0911aTw/2009	3	JF968088 (outgroup)

Table 14 Previously published sequences of DENV-3 and outgroup for constructing the phylogenetic tree of DENV-3.

Strains	Serotypes	Acc. no. (GenBank)
D3/Indo/TB16/2004	3	AY858047
D3/Mal/1012bTw/2010	3	JF968112
D3/SG/05K4477DK1/2005	3	EU081223
D3/Indo/FW06/2004	3	AY858041
D3/PH86/INDO/2004	3	AY858045
Indo den3_98/1998	3	AY858039
D3/IND/BA51/2004	3	AY858037
D3/Indo KJ71/2004	3	AY858044
den3_88/INDO/1988	3	AY858038
D3/USA/Sleman-78/1978	3	AY656170
DENV-3/WS/BID-V2973/1995	3	FJ898456
D3/Tah/PF89/320219/1989	3	AY744678
D3/Phil9808a/Tw/PH/1998	3	DQ518671
D3/Phi/PhMH-J1-97/1997	3	AY496879
D3/95TW466/1995	3	DQ675519
DENV-3/TH/BID-V3360/1973	3	GQ868593
96-17-1HuNIID/JP/1996	3	AB111084
D3/Phi/1009aTw/2010	3	JF968103
D3/1327/THT/1965	3	L11439
D3/PR6/BR/1963	3	L11433
D3/1339/BR/1977	3	AY146761
D3/PR/1340/1977	3	L11434
D3/Bng/0809aTw/2008	3	JF968064
D3/Myan0508a/Tw/2005	3	DQ518666
D3/My/0707aTw/2007	3	EU448443
D3/My/0911aTw/2009	3	JF968088
D3/99Tw628/TWN/1999	3	DQ675533
D3/SG/05K797DK1/2005	3	EU081183
DENV-3/US/BID-V1050/1998	3	EU482559
DENV-3/US/BID-V1731/2003	3	FJ205870
D3/Cuba21/02	3	AY702031
DENV-3/US/BID-V1415/2007	3	EU596492
D3/Chi/GZ1D3/2009	3	GU363549
D3/1696/SM/1986	3	L11435
D3/India/ND143/2007	3	FJ644564
D3/1594/SR/1594	3	L11436
D3/S142/SOMALIA/1993	3	DQ341208
D1/Thailand/AHF82-80/1989	1	D00502 (outgroup)
D2/Cambodia/0907aTw/2009	2	JF967989 (outgroup)
D4/Indonesia/1976	4	U18429 (outgroup)

Table 15 Previously published sequences of DENV-4 and outgroup for constructing the phylogenetic tree of DENV-4.

Strains	**Serotypes**	**Acc. no. (GenBank)**
FSP1412/EC/2000	4	GQ139557
FSE2098/EC/2006	4	GQ139572
IQE7775/PR/2008	4	GQ139553
OBT1317/VN/2000	4	GQ139583
1468_1997/VN/1997	4	GQ139578
DENV-4/CO/BID-V3406/2001	4	GQ868579
1985_M32/PUR	4	AY152856
Puerto Rico 1986	4	U18436
Tahiti 1985	4	U18439
El Salvador 1994	4	U18427
Brazil H402276	4	JN559740
Brazil 1982	4	U18425
New Caledonia 1984	4	U18432
Tahiti 1985	4	U18439
Dominica 814669	4	AF326573
Mexico 1984	4	U18431
Indonesia 1977	4	U18430
LF67/MalaysiaSarawak99	4	FM986668
DENV-4/SG/06K2270DK1/2005	4	GQ398256
ND-110/IND/01	4	HM237349
Sri Lanka 1978	4	U18437
ThD4_0439_01/2001	4	AY618940
ThD4_0164_99/1999	4	AY618986
Malaysia/P75-514/1975	4	AF231723
D4/Cambodia/0306aTw	4	EU448453
D4/Vietnam/0402aTw	4	EU448452
D4/Cambodia/0509aTw	4	EU448455
ThD4_0348_91	4	AY618965
DV4/Mandalay.MYA/H64/2006	4	EU478408
D4/Thailand/0702aTw/2007	4	EU448454
ThD4_0485_01	4	AY618992
D4/Thailand/0508aTw/2005	4	EU448456
ThD4_0129_84	4	AY618958
Thailand 1984	4	U18442
D2/Cambodia/0907aTw	2	JF967989 (outgroup)
Thailand AHF82-80	1	D00502 (ooutgroup)
D3/Myanmar/0911aTw	3	JF968088 (outgroup)

VITA

NAME: Veerayuth Kittichai

SEX: Male

DATE OF BIRTH: 3 March 1983

PLACE OF BIRTH: Kalasin, Thailand

E-MAIL ADDRESS: K.veerayuth@gmail.com

EDUCATION:

2002-2006: Bachelor of Sciences in Medical Technology, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand and passed Medical Technology License Examination

2007-2011 Master of Science in Medical Sciences, Program Graduate Division, Faculty of Medicine, Chulalongkorn University, Thailand

RESEARCH GRANTS:

Ratchadapiseksompotch Research Fund, Faculty of Medicine, Chulalongkorn University and the Chulalongkorn University's Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumiphol Adulyadej for Ph.D. Student