ผลของการติดเชื้อไวรัสเดงกี่และเชื้อไวรัสไข้ปวดข้อยุงลายร่วมกันในยุงลายและเซลล์เพาะเลี้ยงจากยุงลายสวน *Aedes albopictus* (C6/36)

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THE EFFECT OF DENGUE AND CHIKUNGUNYA VIRUS MIXED INFECTION IN AEDES ALBOPICTUS (C6/36) CELL LINE AND AEDES SPP. MOSQUITOES

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รัชรินทร์ โพธิวัฒน์: ผลของการติดเชื้อไวรัสเดงกี่และเชื้อไวรัสไข้ปวดข้อยุงลายร่วมกันในยุงลายและเซลล์เพาะเลี้ยงจากยุงลายสวน *Aedes albopictus* (C6/36). (The Effect of dengue and chikungunya virus mixed infection in *Aedes albopictus* (C6/36) cells line and *Aedes spp.* mosquitoes) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. นพ. เผด็จ สิริยะเสถียร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นฤมล โกมลมิศร์ 150 หน้า.

ยุงลายบ้านและยุงลายสวนเป็นยุงพาหะที่มีสำคัญต่อการถ่ายทอดเชื้อไวรัสไข้เลือดออกและเชื้อไวรัสไข้ปวดข้อยุงลายในทวีปเอเชียและเอเชียตะวันออกเฉียงใต้ ปัจจุบันพบว่ายุงลายบ้านมีแนวโน้มที่จะมาแทนที่แหล่งเพาะพันธุ์ยุงลายสวนในหลายประเทศ เช่น แอฟริกา ยุโรปและอเมริกา อีกทั้งยุงลายสวนยังถ่ายทอดเชื้อไวรัสไข้ปวดข้อยุงลายชนิดกลายพันธุ์ตรงตำแหน่ง A226V ได้ดีกว่ายุงลายบ้าน ทุกๆปีจะมีรายงานการระบาดของเชื้อไวรัสเดงกี่ทั้ง 4 สายพันธุ์ในประเทศไทยซึ่งแสดงให้เห็นถึงการแพร่กระจายของไวรัสทั้ง 4 ชนิดนี้ในแหล่งเดียวกันและที่สำคัญในปี 2552 ประเทศไทยยังมีการระบาดครั้งใหญ่ของโรคไข้ปวดข้อยุงลาย โดยครั้งนี้มีผู้ป่วยจากไวรัสไข้ปวดข้อยุงลายกว่า 24,029 ราย ถึงแม้จะมีรายงานการติดเชื้อไวรัสทั้งสองชนิดพร้อมกันในซีรั่มผู้ป่วยมาก่อน แต่การติดเชื้อพร้อมกันของเชื้อไวรัสเดงกี่และไวรัสไข้ปวดข้อยุงลายโดยมียุงพาหะยังมีรายงานน้อยมาก เพื่อประเมินว่าไวรัสทั้งสองชนิดนี้สามารถถ่ายทอดสู่คนโดยยุงพาหะเพียงตัวเดียวได้หรือไม่ เราจึงใช้เซลล์เพาะเลี้ยงจากยุงลายสวน (C6/36) และยุงลายเพศเมียเพื่อเป็นแบบจำลองการติดเชื้อไวรัสในหลอดทดลองและในสัตว์ทดลองตามลำดับ จากผลการศึกษาพบการติดเชื้อไวรัสทั้งสองชนิดนี้พร้อมกันในเซลล์เพาะเลี้ยงและปริมาณไวรัสทั้งสองชนิดนี้ยังมีปริมาณเพิ่มขึ้นเมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ยังพบการควบคุมการติดเชื้อแบบแข่งขันเกิดขึ้นในยุงลายบ้านและยุงลายสวนในกลุ่มการทดลองที่ใช้อนุภาคไวรัสในปริมาณมากกว่าหรือเท่ากันแต่ตรวจพบไวรัสเพียงสายพันธุ์เดียวที่สามารถเพิ่มปริมาณได้ในยุงพาหะและเป็นที่น่าประหลาดใจว่าการติดเชื้อพร้อมกันของไวรัสทั้งสองชนิดนี้เกิดขึ้นในยุงลายบ้านเท่านั้นไม่พบการติดเชื้อพร้อมกันในยุงลายสวน ผลการศึกษานี้นำมาซึ่งความรู้ใหม่เพื่อให้เราเข้าใจความสัมพันธ์ระหว่างพาหะก่อโรคกับเชื้อไวรัสรวมถึงกระบวนการการถ่ายทอดเชื้อไวรัส ซึ่งอาจนำไปประยุกต์ใช้เพื่อการทำนายและการควบคุมการระบาดของโรคในอนาคต

สาขาวิชาชีวเวชศาสตร์.....ลายมือชื่อนิสิต .....

ปีการศึกษา...2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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RUTCHARIN POTIWAT: THE EFFECT OF DENGUE AND CHIKUNGUNYA VIRUS MIXED INFECTION IN AEDES ALBOPICTUS (C6/36) CELL LINE AND AEDES SPP. MOSQUITOES. THESIS ADVISOR: ASSOCIATE PROFESSOR PADET SIRIYASATIEN, THESIS CO-ADVISOR: ASSOCIATE PROFESSOR NARUMON KOMALAMISRA, 150 pp.

Aedes aegypti and *Aedes albopictus* are an important mosquito's vector disease that supported dengue and chikungunya virus transmission in Asia and South East Asia countries. In recently, *Ae. albopictus* has a trend to displace to *Ae. aegypti* habitat in Africa, Europe and America, and *Ae. albopictus* is a great mainly vectors which contribute to mutant chikungunya virus transmission than *Ae. aegypti*, A226V substitute on *E1* gene. All 4 serotype of dengue viruses are widely spread in Thailand annually which presents cocirculation in the same region. Additionally, reemergence of chikungunya outbreaks are reported in southern parts of Thailand in 2009, more than 24,029 cases of chikungunya fever have been reported. Although dual infections of two viruses in sera's patient was reported but mixed infection in mosquito vector have been rarely studied. To evaluate whether both virus can be transmitted to human host by passing through single mosquito. We used *Aedes albopictus* C6/36 cell line and female *Aedes spp.* mosquitoes as a model of viral transmission *in vitro* and *in vivo*, respectively. The results show mixed-infection in C6/36 cell line and viral production are increase. Competitive suppression was found in two mosquitoes with using high viral concentration or equal titer and surprisingly, *Ae. aegypti* are supposed to carrying two viruses particle in single mosquitoes whereas *Ae. albopictus* presents only one strain of viral mixed-infection. The results provided the new knowledge in order to understand the effect of vector-virus interactions and virus transmission which might be applies to predict the outbreak and control the diseases in future.

Field of Study : Biomedical Sciences Student's Signature…….
Academic Year : 2010 Advisor's Signature……
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LIST OF ABBREVIATIONS

Abbreviations		Term
Ab	=	Antibody/antibodies
ATCC	=	American Type Culture Collection
bp	=	base pair
°C	=	Degree Celsius
cm	=	centimeter
CPE	=	Cytopathic effects
ΔCt	=	cycle threshold
DAB	=	3, 3’ diaminobenzidine tetrahydrochloride
DENV-1, DENV-2, DENV-3, DENV-4	=	dengue virus serotype 1, 2, 3, 4
DEPC	=	Diethypyrocarbonate
DF	=	dengue fever
DHF	=	dengue hemorrhagic fever
DMEM	=	Dulbecco’s Modified Eagle’s medium
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
DSS	=	dengue shock syndrome
DTT	=	dithiothreitol
E protein	=	envelope protein
EDTA	=	Ethylenediaminetetraacetic acid
et. al.	=	and others
FBS	=	Fetal bovine serum
h	=	Hour (s)
HRP	=	Horseradish peroxidase hydrochloric acid
kDa	=	kilodaltons
l	=	liter
M	=	Molar

LIST OF ABBREVIATIONS (Continued)

Abbreviations		Term
MEM	=	Minimum Essential Medium Eagle
$MgCl_2$	=	Magnesium chloride
min	=	minute (s)
ml	=	milliliter
mM	=	millimolar
MOI	=	Multiplicity of infection
mRNA	=	Messenger ribonucleic acid
NaCl	=	sodium chloride
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
prM E	=	Pre-Membrane Envelope
RNA	=	Ribonucleic acid
rpm	=	revolutions per minute
rRNA	=	Ribosomal ribonucleic acid
sec	=	second(s)
Tris-HCl	=	Tris-(hydroxymethyl)-aminomethane
x g	=	Gravity centrifugal force
µl	=	microliter
µM	=	micromolar
WHO	=	World Health Organization
FITC	=	Fluorescein isothiocyanate

CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Dengue viruses (DENV) are the majority infectious disease that belonging to family *Flaviviridae*, genus *Flavivirus*. DENV are composed of 4 serotypes such as DENV-1, DENV-2, DENV-3 and DENV-4. All four serotype are caused public health problem in many parts of the world [1-3].

According to The Ministry of Public Health (Thailand) reported dengue fevers and dengue hemorrhagic fever cases are often represent in Thailand annually. In 2009, Annual Epidemiological Surveillance Report (AESR 2009), Thailand was present 56,651 cases of DF, DHF and DSS that higher than last year 2.23 fold, with 50 cases of deaths. These case are consist of DF (44.48%), DHF (53.8%), DSS (1.72%) and 50 cases of deaths. From 555 cases of dengue sample were revealed that 47.6% infectious with DENV-1, 28.2% with DENV-2, 20.2% with DENV-3, and 3.4% with DENV-4. Co-circulation between dengue four serotype were reported every year and immunological test presents the incidence was higher during rainy seasons. All four dengue virus (DENV) serotypes were permanently in circulation, though the predominant serotype has changed year by year. In Cambodia also reported DENV-2 co-circulation with DENV-3 during 1980-2008 and the predominant serotype has alternated between DENV-3 and DENV-2 since 2000. These data were supported dengue virus co-circulation in Thai people and the diseases are constantly presented in endemic area such as Thailand and South East Asia countries. In 2006 co-circulation with DENV-1 and DENV-3 in Thai's sera has been reported while DENV-1 is shown primary infectious and DENV-3 is shown secondary infection by ELISA test respectively [4].

Moreover, DENV-2 was found triple co-infection persistency with other virus in *Aedes albopictus* (C6/36) cell line (Kanthong et. al., 2010). The triple infections are located by JEV mixed infection with DENV-2 and following with densovirus (DNV) infection. The phenomenon provides an opportunity for genetic exchange between

diverse viruses and it may have important medical and veterinary implications for arboviruses.

Double infection of heteroserotypes of dengue viruses in field population of *Ae. aegypti* and *Ae. albopictus* mosquitoes were reported in southern Thailand. There are three dengue serotype (DENV-2, DENV-3 and DENV-4) were detected in *Ae. aegypti* male and female, and two serotype of DENV-2 and DENV-3 were detected in *Ae. aegypti* female and *Ae. albopictus* female. DENV-2 and DENV-1 were the most prevalent serotypes that found in the serum of the patients in this area, followed by DENV-4 and DENV-3 circulation. The incidences of multiple serotypes of dengue virus were found in *Ae. aegypti* and *Ae. albopictus* in the same area point toward a high risk for an endemic of DHF.

Both of DENV and Chikungunya virus (CHIKV) are arbovirus that transmitted by *Aedes spp.* mosquitoes. *Ae. aegypti* and *Ae. albopictus* are important mosquitoes which distribution in Thailand and subtropical countries. CHIKV are caused chikungunya fever, is a member of family *Togaviridae*, genus *Alphavirus* that was originally isolated from the serum of febrile illness patients from the Mankonde Plateau in Tanzenia in 1952 [5]. In recently, reemergence of chikungunya fever was occurred in southern parts of Thailand, 24,029 cases of chikungunya fever have been reported in 36 provinces, no cases of deaths, and *Ae. albopictus* is expected as a major vector because it was found as a pre-dominant in epidemic area[6-7] . *Ae. aegypti* and *Ae. albopictus* are several feeding in light day time and after World War II, *Ae. aegypti* was eliminated by vector control program in many parts of the world as a results of *Ae. albopictus* are become a silent vector; in addition, *Ae. albopictus,* are spread through central of Africa and EU, not only transmitted of chikungunya virus but it is also a minor vector which supported DENV transmission.

Additionally, the current reported from India, Malaysia and Germany were show dengue and chikungunya virus mixed infection in the sera patient and chikungunya fever is always misdiagnosis because it was a similarity symptom with dengue fever such as high fever, headache, arthralgia and myalgia [8-11]. The

specifically clinical feather and laboratory diagnostic are mainly importance for identify both of viruses infection.

In human, CHIKV produce disease about 48 h after mosquito bit. Patients have a high viraemia during the first 2 day of illness. Viraemia declines around 3 or 4 days, unusually disappearing by day 5. Haemaggutination inhibition (HI) and neutralizing antibodies can usually be detected after day 5 with fading viraemia. Neuro-invasive cases and haemorrhagic manifestation related to CHIKV infection have been conclusively documented. The “Silent” of CHIK infections are occur, but how does commonly mechanism represent are still not known. CHIKV infection (Whether clinical or silent) is thought to confer lifelong immunity. During the acute diseases, most patients will have headache, but it is not usually severe. Prolong joint pain, arthralgia, myalgia and rash were used to initially identify chikungunya fever from dengue fever [12].

Although, dengue and chikungunya virus was found concurrent infection but they have never been record to support the dengue and chikungunya virus can be transmitted to human by one mosquito vectors; thus, our research are focus on dual infection between dengue and chikungunya virus *in vitro* and *in vivo*. To evaluate the hypothesis we used *Aedes albopictus* mosquito (C6/36) cell line as a model to reveal the replication of two viral infections in once cell line and design the experiment as single infection, mixed infection (co-infection) and super-infection (after infected primary viral 1 hr followed with secondary infection) and determine the viral production in the supernatant by D-RT-PCR, real time RT-PCR and plaque assay.

For evaluate the viral infection on these cell lines, we used the immunocytochemistry (ICC) and immunofluorecence staining (IFA) to visualize the specifically binding between DENV-3 integrated into C6/36 cell line and penetrated to head parts of *Ae. aegypti* mosquitoes, respectively [13-14]. The benefit of this study will give the new knowledge of viral transmission and vectors relationship that due to vector control and specific viral treatment in further.

Hypothesis

Dengue and chikungunya virus are infected in the same culture and both of virus can be replicated and transmitted together by one mosquito.

Research question

1. Are dengue and chikungunya viruses multiply in the same culture?
2. Are there any relation between this virus and vectors?

Objectives

1. To produce dengue or chikungunya virus strain in cell culture by using larvae stage of mosquito cell line, *Aedes albopictus* (C6/36), and mammalian cell line (Vero cell).
2. To estimate both of virus titration by standard plaque assay.
3. To determine the viral replication *in vitro* by using *Aedes albopictus* (C6/36) cell line as a model of viral infection in order to single infection, mixed infection (co-infection) and super-infection by Duplex-Reverse Transcriptase Polymerase chain reaction (D- RT-PCR).
4. To evaluate the position of viral assembly in *Aedes albopictus* (C6/36) cell line by Immunocytochemistry (ICC) staining.
5. To evaluate the amount of virus concentration that can be multiplied into mosquito body by intrathoracic injection method.
6. To establish the virus infection by using *Ae. albopictus* and *Ae. aegypti* mosquito as a model of viral replication, Oral feeding technique, *in vivo* transmission.
7. To identify DENV-3 and CHIKV replication in *Ae. albopictus* and *Ae. aegypti* mosquito in order to single infection, mixed infection (co-infection) and super-infection by D-RT-PCR method.
8. To determine the virus transmission on a head parts of mosquito by using Indirect Immunofluorescence Assay (IFA).

Experiment I; To propagate dengue and chikungunya virus in C6/36 cell line and identified the viral titer by plaque assay.

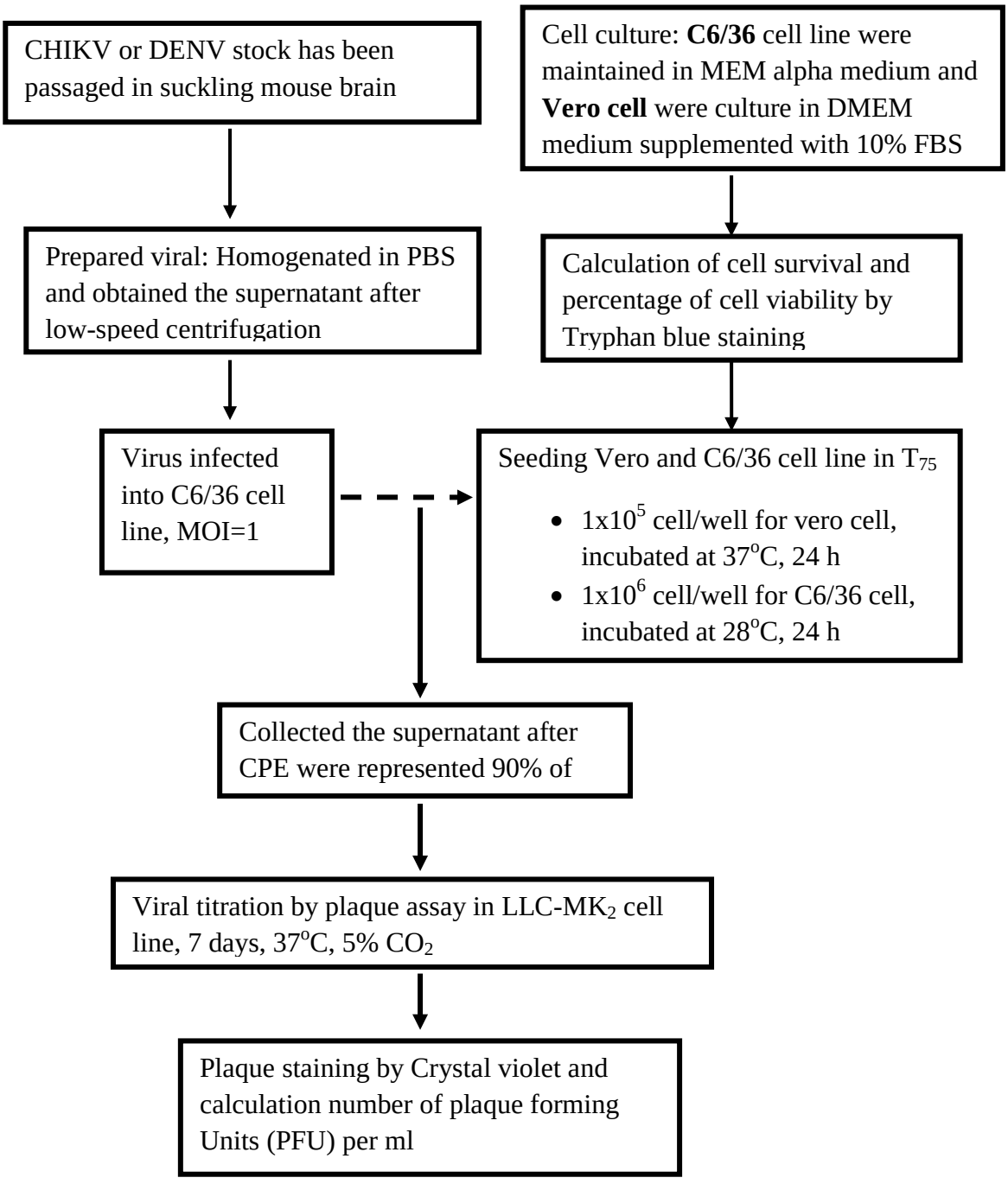


Figure 1. Conceptual flame work (I)

Experiment II; To compare the viral replication in single infection, mixed infection (co-infection) and super-infection by real time RT-PCR and evaluate the position of viral infection in *Aedes albopictus* (C6/36) cell line by ICC

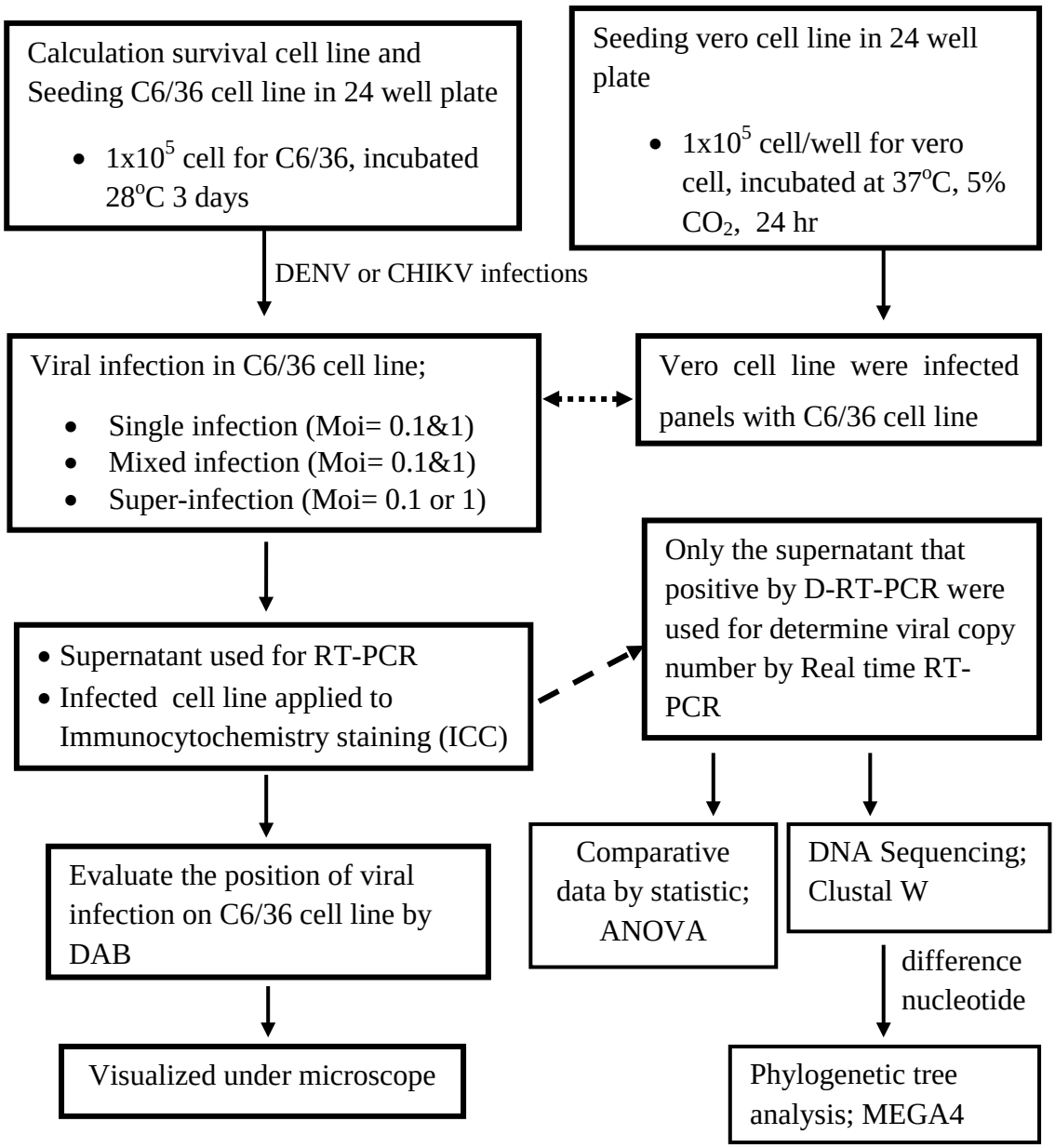


Figure 2. Conceptual flame work (II)

Experiment III; To evaluate DENV and CHIKV replication in *Ae. albopictus* and *Ae. aegypti* mosquito in order to single infection, mixed infection (co-infection) and super-infection by using Oral feeding technique and determine the positive sampling by D-RT-PCR

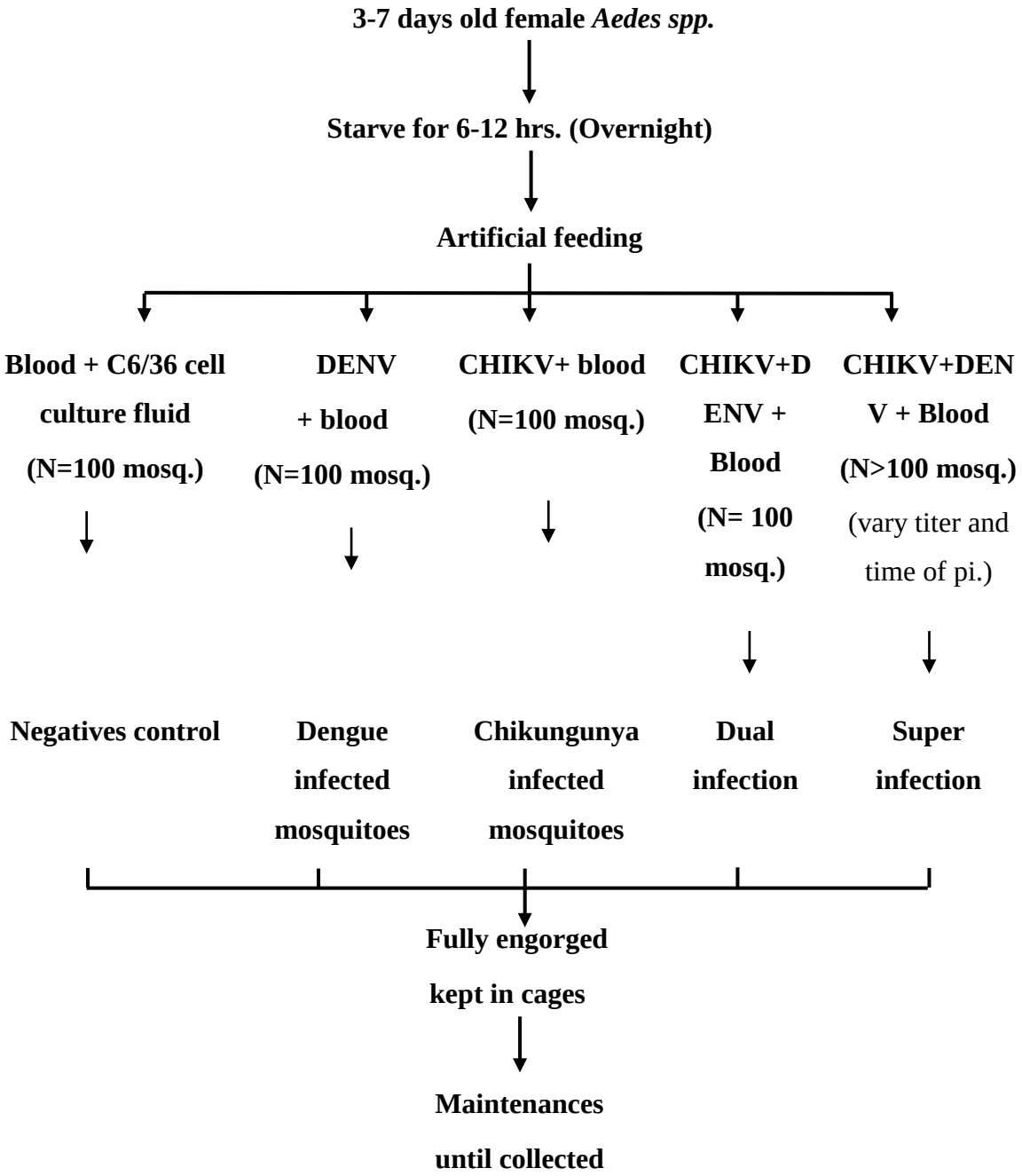


Figure 3. Conceptual flame work (III)

CHAPTER II
LITERATURE REVIEW

2.1 Dengue and Chikungunya virus genome and structure

Dengue and chikungunya virus are arbovirus (Arthropod borne viruses diseases) that transmitted by *Aedes* mosquito vector. Arboviruses could be discriminated by serological tests. Using hemagglutination inhibition and neutralization assays, it was suggested that Western equine encephalitis virus (WEEV), Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and Semliki Forest virus (SFV) are related but distinct. They were clustered in a group called A, whereas a second interrelated group B included dengue virus (DENV), Japanese encephalitis virus (JEV), Ntaya virus (NTAV), West Nile virus (WNV), and yellow fever virus (YFV) [15].

Subsequently, electron microscopy, biochemical and genetic characterization of these viruses led to placement of both group A and group B in a single virus family named *Togaviridae.* More recently, the classification was revisited based upon gene sequences, replication strategies, and structure of these viruses which resulted in the final assignment of the different arbovirus groups into *Flavivirus* or *Alphavirus* genus [16-17]. The *Flavivirus* genus includes more than 70 single-stranded RNA viruses divided into 8 serogroups [18]. The *Alphavirus* genus consists of 29 species of arboviruses that have been classified into 7 antigenic complexes: Barmah Forest (BF), Eastern equine encephalitis (EEE), Middelburg (MID), Ndumu (NDU), Semliki Forest (SF), Venezuelan equine encephalitis (VEE), and Western equine encephalitis (WEE). *Alphaviruses* have a wide geographic distribution [19].

CHIKV is part of the SF group of Old World *Alphaviruses* that includes SFV, ONNV, and RRV. Most of the Old World *Alphaviruses* cause fever, rash and arthralgia while many of the New World *Alphaviruses* cause encephalitis. The seven antigenic complexes are indeed reflecting clades of viruses that share medically important characteristics. Evolutionary studies have also been reported for the SF group in Old World *Alphaviruses* that revealed some interesting genetic features

relevant to evolution; an ancestor of the SFV, RRV, ONNV, and CHIKV groups which originated from the New World was introduced in the Old World with complicity of the migratory animal reservoir, likely birds [20], and next is expected to have spread in the Old World. It has been postulated that CHIKV evolved in Africa where it is maintained in a sylvatic cycle involving wild primates and *Aedes spp.* mosquitoes, and was subsequently introduced into Asia, where it was typically associated with *Ae. aegypti* mosquitoes [21]. This virus is currently transmitted by *Ae. albopictus* in urban areas [22]. More information is described as below.

2.1.1 Dengue virus genome and structure

Dengue, a mosquito-borne viral disease, is becoming an increasing public health problem in developing countries, is caused by any of four antigenically and genetically distinct viruses of the family *Flaviviridae*, genus *Flavivirus* named DENV-1, DENV-2, DENV-3 and DENV-4 [18].

Dengue virus (DENV) contains a single-stranded, positive-sense RNA genome. The DENV genome comprises approximately 10,600 nucleotides and is capped by a 5′-type 1 structure (m7GpppA), but it does not contain a polyadenylated 3′-tail sequence. Both the 5′- and 3′-untranslated regions (UTRs) have key roles in the regulation of translation and genomic RNA synthesis. Translation of viral RNA yields a single polypeptide that is co-translationally processed by viral and cellular proteases, generating three structural proteins and at least seven non-structural (NS) proteins (see the figure, part a). The DENV virion is a spherical, enveloped virus that has a diameter of approximately 50 nm (see the Figure 4b). The virion contains three structural proteins (capsid (C), membrane (M) and envelope (E)) and the RNA genome. The membrane precursor, prM, is believed to aid in the folding of the E glycoprotein and both are integrated in the lipid bilayer of the virion by two transmembrane regions that surround a nucleocapsid of unknown structure (Figure 4a). At a late stage of virion assembly in the trans-Golgi network, prM is cleaved by furin, which results in a rearrangement of the M and E proteins on the virion surface yielding mature infectious virions.

The surface of the mature DENV virion is smooth with the envelope proteins aligned in pairs parallel to the virion surface. The E glycoprotein mediates cell attachment and fusion and is also the major target of protective antibodies.

The E glycoprotein can be divided into three structural or functional domains: the central domain; the dimerization domain which presents a fusion peptide; and the receptor-binding domain. Virions enter cells by receptor-mediated endocytosis, which is followed by fusion of the viral and cellular membranes mediated by the E protein under acidic conditions within the endosome. Following fusion, the RNA genome dissociates from the viral nucleocapsid and enters the cytoplasm where it functions as mRNA and is translated.

Translation of the RNA generates a polyprotein that is co-translationally and post-translationally processed by the virus-encoded serine protease, NS2B/NS3, and by host-encoded proteases, including signalase and furin, to produce the 3 structural proteins and 7 nonstructural proteins in the order C-prM-E -NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 [23]. NS3 (70 kDa) and NS5 (104 kDa) are the best characterized nonstructural proteins, with multiple enzyme activities that are required for viral replication. NS3 has three distinct activities: serine protease together with the cofactor NS2B, required for polyprotein processing; helicase/NTPase activity, required for unwinding the double-stranded replicative form of RNA; RNA triphosphatase, required for capping nascent viral RNA [24-28]. Mutations that affect each activity impair viral replication [29-30].

NS5 is the largest and most highly conserved flaviviral protein, with greater than 75% sequence identity across all DEN serotypes. It contains two distinct enzymatic activities, separated by an interdomain region: an S-adenosyl methyltransferase (SAM) [31-32] and an RNA-dependent RNA polymerase (RdRp) [33-37]. NS1 (46 kDa) is required for flavivirus replication and presumably involved in negative-strand synthesis by an unknown mechanism. NS2A (22 kDa) is a small hydrophobic transmembrane protein that is involved in generation of virus-induced membranes during virus assembly [38]. NS4A (16 kDa) is an integral membrane

protein which may induce membrane rearrangements to form the viral replication complex [39-40].

NS4B (27 kDa) inhibits the type I interferon response of host cells [41], and may modulate viral replication via its interaction with NS3[42]. Virion assembly occurs in the endoplasmic reticulum and viruses exit the cell through the Golgi network (figure 5).

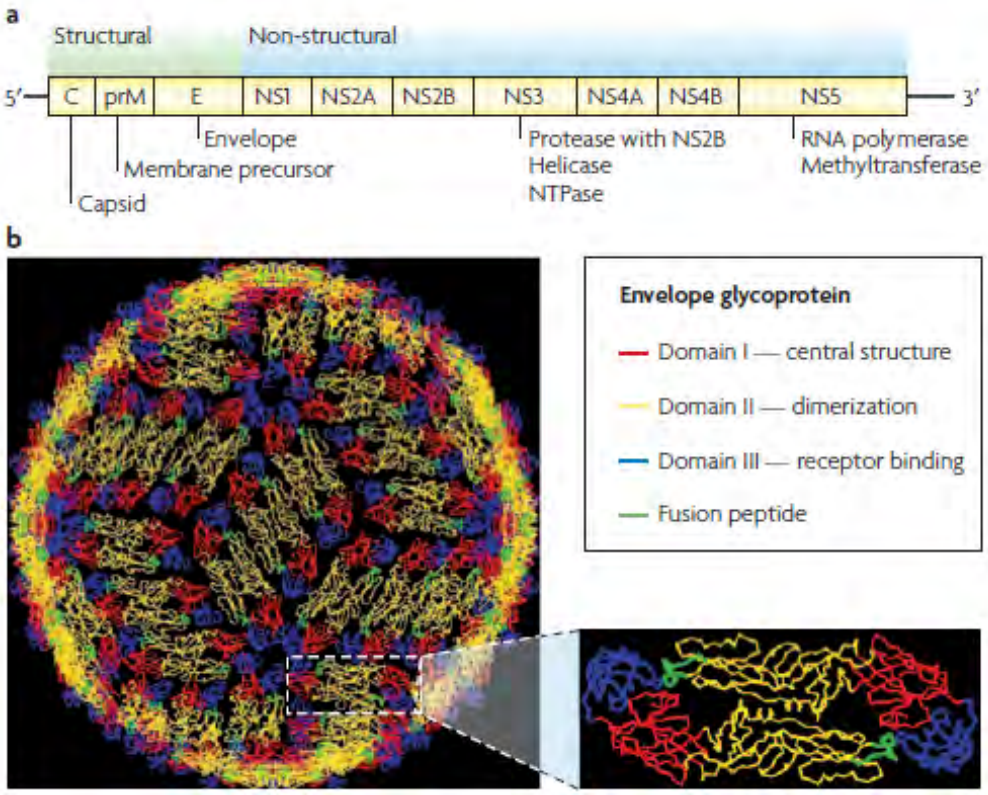


Figure 4. The dengue virus genome and virus particle

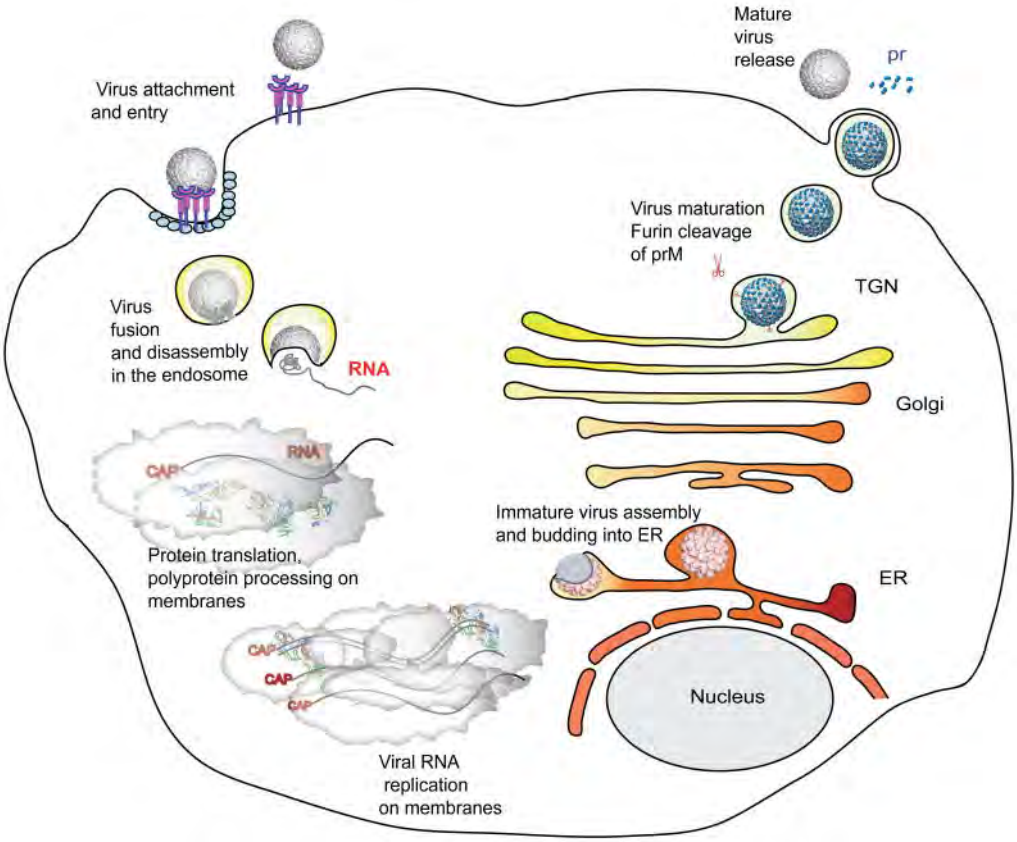


Figure 5. The dengue virus replication cycle. Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis. In the low pH of the endosome, viral glycoproteins mediate fusion of the viral and cellular membranes, allowing disassembly of the virion and release of its RNA into the cytoplasm. The viral RNA is translated into a polyprotein that is processed by viral and cellular proteases. Viral non-structural proteins then replicate the genomic RNA. Virion assembly occurs at the ER membrane. Capsid protein and viral RNA are enveloped by the membrane and its embedded glycoproteins to form immature virus particles, which are then transported through the secretory pathway. In the low pH of the trans-Golgi network (TGN), prM is cleaved by furin. Mature virions are then released into the cytoplasm.

2.1.2 Chikungunya virus genome and structure

As a member of the *Alphavirus genus*, CHIKV is a small (about 60–70 nm-diameter), spherical, enveloped, positive-strand RNA virus [19, 43-45]. Alphaviruses are among the simplest membrane enveloped viruses. Until recently, only two complete nucleotide sequences of CHIKV isolated from humans infected during the 1952 Tanzania outbreak were available corresponding to the strain Ross and the S27 CHIKV African prototype, respectively [46].

The genome of CHIKV is organized as follows: 5′ cap -nsP1-nsP2- nsP3-nsP4-(junction region)-C-E3-E2-6k-E1-poly(A)-3′ and is 11,805 nucleotides long, excluding the 5′ cap nucleotide, an I -poly(A) tract and the 3′poly(A) tail (CHIKV S27 strain) (Fig. 6). The CHIKV genome resembles eukaryotic mRNAs in that it possesses 5′ cap structures and 3′ poly (A) tail. Although it has not been specifically investigated for CHIKV, it was documented with related Alphaviruses that the 5′end capped with a 7-methylguanosine. For CHIKV (e.g., S27 strain), ′the 5 - non-translatable regions (NTR) of CHIKV are composed of 76 nucleotides.

CHIKV replication (Figure 7) is likely to start with the attachment of the viral envelope to host receptors (step 1). Receptor(s) for CHIKV have yet to be identified, but a role has been proposed for the laminin receptor, glycosaminoglycans and DC-SIGN molecules in vertebrate cell infection by alphaviruses. The virus enters the target cell by endocytosis of clathrin-coated vesicles (step 2). During transfer to endosomes, the low pH environment leads to conformational reorganization of the E1–E2 viral envelope complex, exposure of the E1 fusion peptide and subsequent fusion with the endosomal membrane enabling delivery of the nucleocapsid into the cytoplasm (step 3). Then CHIKV replication proceeds along parallel pathways (steps 4, 5 and 6).

Early in infection, the viral genome serves as an mRNA for the translation of the P1234 precursor and matures nonstructural proteins (step 4). RNA replication then occurs through the synthesis of a full-length minus-strand RNA intermediate (step 5) used as a template for synthesis of the genome-length RNA and for transcription of

the 26S subgenomic plus-strand RNA from an internal promoter (step 6). Transcription of plus-strand RNA (produced at a constant rate throughout the replicative cycle) and minus-strand RNA (no longer detected at late infection stages) is temporally regulated by proteolytic processing of the P1234 nonstructural precursor. Early in infection, P1234 is cleaved into nsP4, which in association with nsP123 polyprotein and host partners, probably behaves as a minus strand RNA replicase. During replication, when the cell concentration of nsP123 is raised sufficiently, the precursor is further processed into mature proteins (step 8), which both regulate plus-strand RNA synthesis and amplify the subgenomic 26S positive-strand mRNA using the negative-strand RNA as a template (step 6). This step is regulated by helicase and proteinase functions of nsP2. A possible role was proposed for the migration of nsP2 into the nucleus of vertebrate cells, because de novo viral production is decreased in enucleated vertebrate cells. In step 7, the 26S subgenomic RNA serves as mRNA for the translation of the C–pE2–6k–E1 polyprotein precursor and production of structural proteins.

At late replicative steps the C–pE2–6k–E1 polyprotein precursor is processed cotranslationally and post-translationally into mature structural proteins. Autoproteolytic serine proteinase activity releases the capsid (C) from the N-terminus of nascent polypeptide (step 9). The envelope polyprotein precursor that inserts into the endoplasmic reticulum bilayer through an N-terminal signal sequence is processed into pE2 and E1. Step 10 then takes place in the Golgi complex, where E1 and pE2 associate and are exported to the plasma membrane.

During transport, and probably just before arrival at the cell surface, pE2 is expected to be cleaved by furin or a related host proteinase into E2 and E3. Meanwhile, the C-terminus transmembrane insertion in endocytoplasmic reticulum bilayer is disrupted, thus enabling the release and maturation of the E1–E2 heterodimer in the viral envelope. This probably induces the reorientation of the E2 C-terminus from the luminal to the cytoplasmic side, required for the correct interaction between nucleocapsid and envelope glycoproteins. In parallel, mature nucleocapsids diffuse freely in cytoplasm toward the plasma membrane (step 11).

Viral assembly is directed through electrostatic binding of the nucleocapsid to RNA and the recruitment of the plasma membrane-associated glycoproteins. The assembled alphavirus particle, which consists of an icosahedral nucleocapsid, finally buds through the cell membrane and becomes an enveloped virion (step 12).

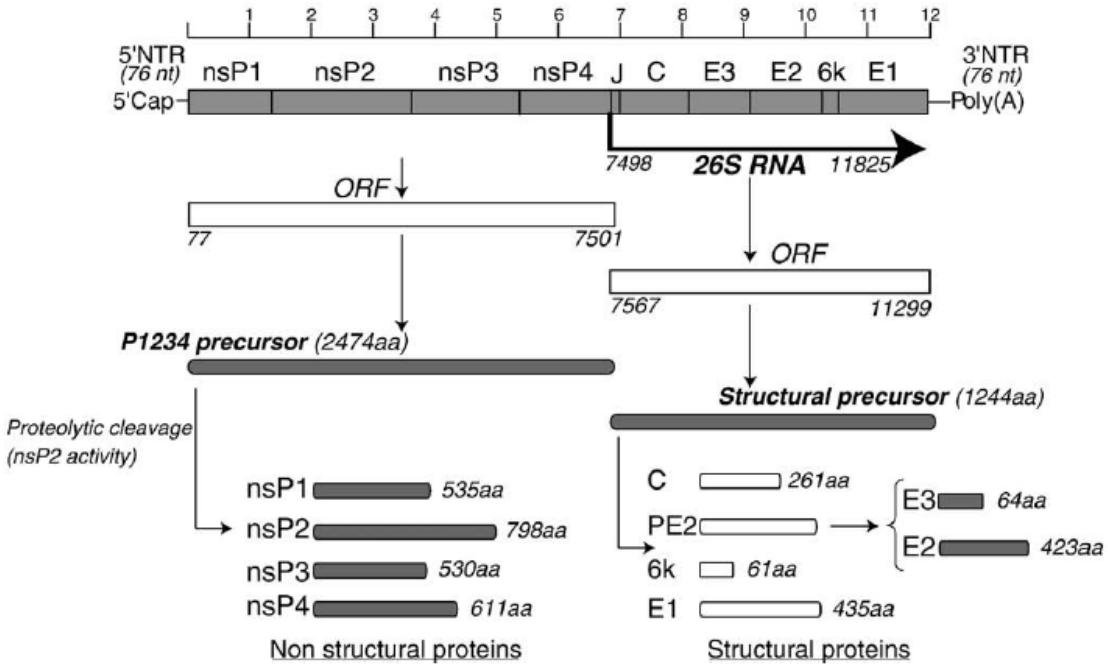


Figure 6. Organization of the CHIKV genome and gene products. The CHIKV genome resembles eukaryotic mRNAs in that it possesses 5′ cap structures and 3′ poly (A) tail. The 5 and 3′ proximal sequences of CHIKV genome carry non -translatable regions (NTR). The junction region (J) is also non-coding. A subgenomic positive-strand mRNA referred to as 26S RNA, is transcribed from a negative-stranded-RNA intermediate and serves as the mRNA for the synthesis of the viral structural proteins. The different non-structural proteins (nsP1–nsP4) and structural proteins (C, Capsid; E1, E2, E3, envelope; 6K) are generated after proteolytic cleavage of polyprotein precursors.

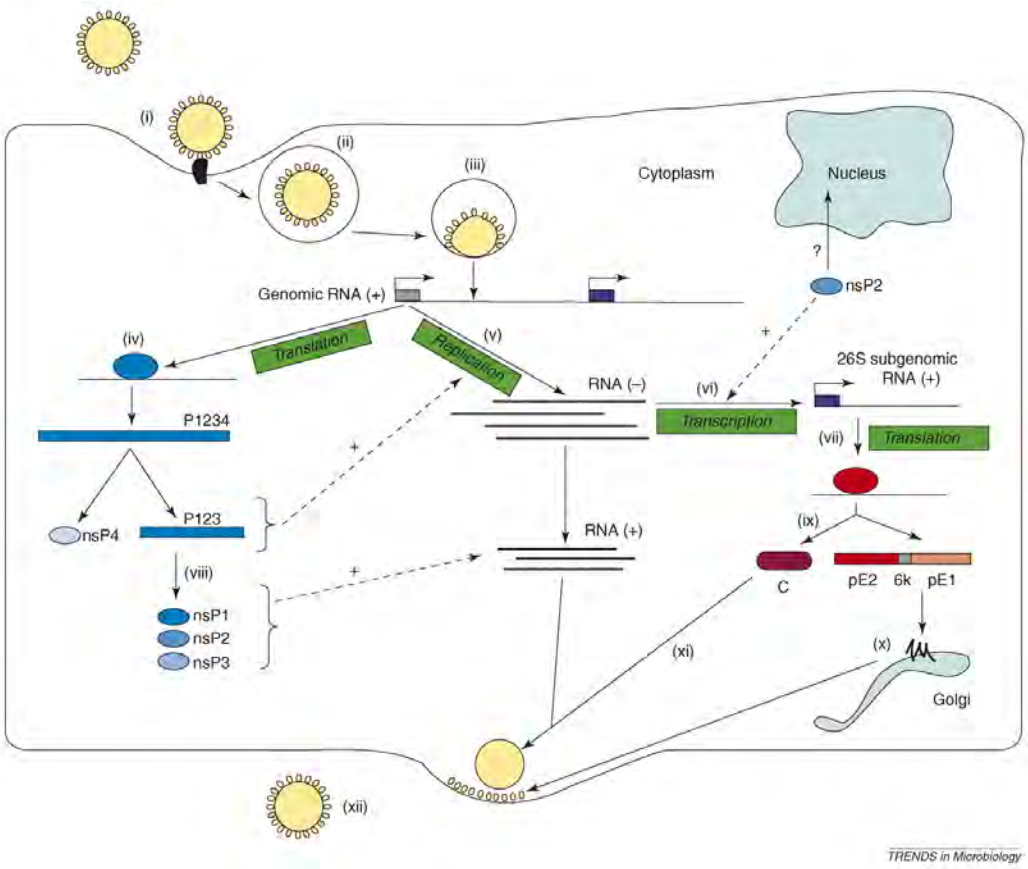


Figure 7. Summary of CHIKV replication cycle in vertebrate cells.

2.2 The clinical feature and tratment

2.2.1 The clinical feature of dengue fever (DF), Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)

Dengue is the most widespread vector-borne virus disease in the world, and DHF/DSS are rapidly increasing in incidence in many tropical areas [47-49]. This dramatic increase is a result of changes in human lifestyle, increased international travel, and urban crowding. Dengue has been known for at least 200 years, but DHF/DSS has only been routinely recognized since the 1950s, although there is evidence to suggest that DHF occurred as early as 1897 in north-eastern Australia [50].

The case definitions of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are indicated as provided by the WHO. For a diagnosis of DHF Grade I, each of the four criteria listed in part b of the figure 8 must be met. There is a contention from some clinicians that this requirement results in an under-reporting of severe dengue disease as a patient with only two or three severe conditions would be classified as having DF (figure 8a). A generalized time course of the events associated with DF, DHF and DSS is indicated in part d of the figure. The incubation period before the development of signs of infection generally ranges from 4 to 7 days. Hypovolemic shock can develop during the late stage of disease and usually lasts 1 to 2 days.

Dengue fever is characterized by an abrupt onset of fever, headache, myalgia, loss of appetite, and varying gastrointestinal symptoms, often accompanied by rash and bone or joint pains [51-53]. Symptoms persist for 3–7 days, and while convalescence may be prolonged for several weeks, mortality is rare. Homologous immunity is lifelong, but cross-protection to other dengue viruses is not elicited and, indeed, there is evidence to suggest that heterologous antibodies may form infectious immune complexes which may increase the severity of subsequent infections[48, 54-56].

Dengue haemorrhagic fever (DHF) (Figure 8b) and dengue shock syndrome (DSS) (Figure 8c) are more severe manifestations of dengue infection, and are most often associated with infection of people with pre-existing antibodies to dengue, either actively or passively acquired [57-58]

The clinical course follows that of classic dengue fever initially; however, as the febrile phase subsides, the patient's condition rapidly deteriorates with signs of circulatory failure, neurological manifestations, and hypovolemic shock with potential fatal outcome if prompt, proper, clinical management is not implemented (Figure 8d). Haemorrhage may be evident by petechiae, easy bruising, bleeding at injection sites, and occasionally gastrointestinal bleeding, especially where gastric ulcers already exist [59].

There is no specific treatment for dengue fever. Although convalescence may last several weeks following dengue infection, especially among adults, disabling sequelae are not thought to follow infection. Dengue haemorrhagic fever and DSS require symptomatic treatment, coupled with careful assessment of fluid and electrolyte balance for management of shock (WHO: 1986)

Areas where clinicians are not familiar with the proper treatment of DHF/DSS may have case fatality rates substantially higher than those where physicians are experienced in the proper treatment of the disease. Diagnosis is made on clinical grounds and confirmed by laboratory demonstration of specific immune response, virus isolation, or identification of dengue virus nucleic acid sequences in clinical material [60-61].

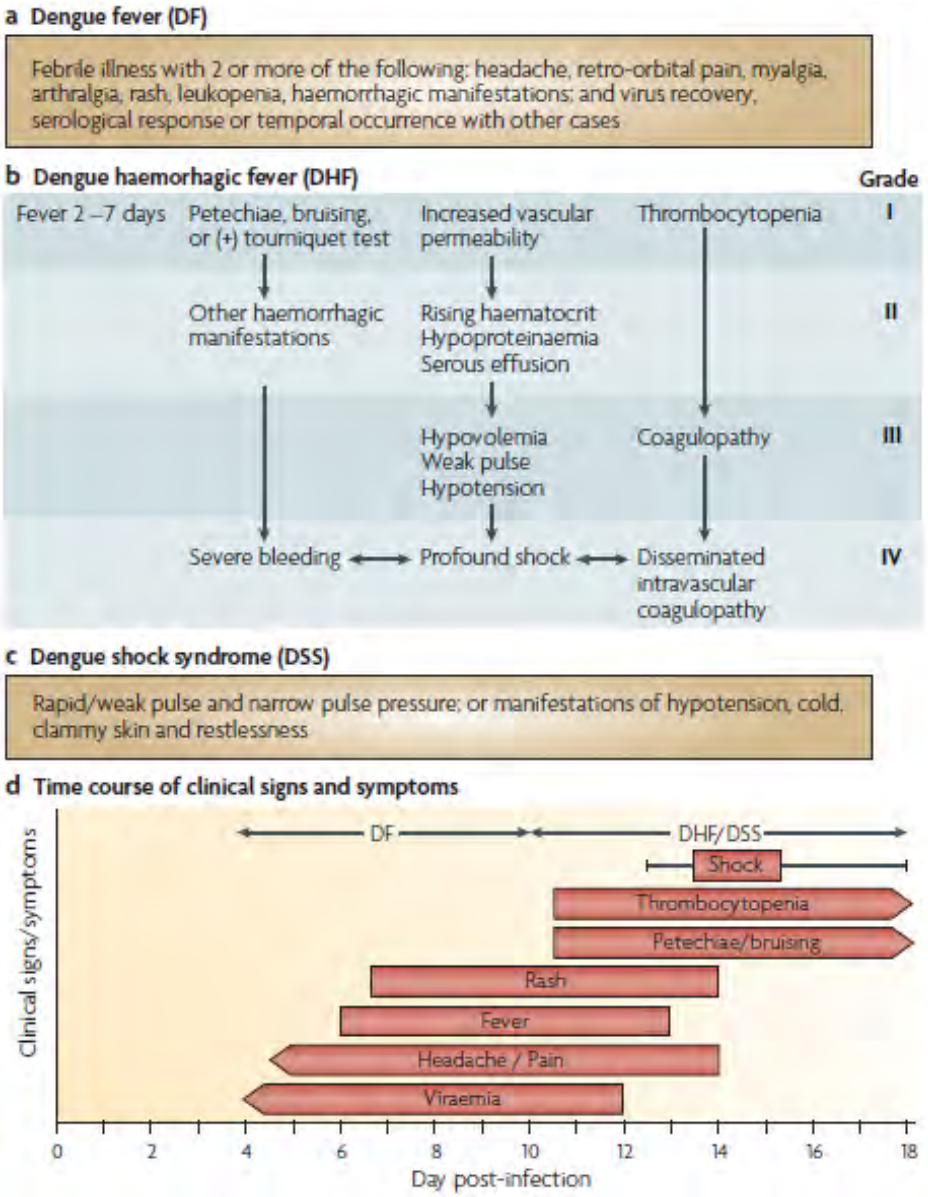


Figure 8. The Clinical sign symptoms of dengue disease in patient; a) dengue fever (DF), b) dengue haemorhagic fever (DHF), c) dengue shock syndrome (DSS), d) Time course of clinical signs and symptoms

2.2.2 The clinical feature of chikungunya fever

The first case series of patients infected with CHIKV, published in 1955, described 115 hospitalized patients in Tanzania with acute onset of high fever, severe joint pain, and rash. The illness was initially diagnosed as a “dengue-like” disease until laboratory evaluation confirmed CHIKV as the source of illness.

Since then, many CHIKV outbreaks have occurred that have helped to further characterize chikungunya fever. The incubation period for chikungunya fever is typically between 3–7 days (range, 2–12 days). Not all individuals infected with the virus develop symptoms. Serological surveys indicate that 3%–25% of persons with antibodies to CHIKV have asymptomatic infections.

Symptoms of CHIKV infection start abruptly with fever (temperature, usually >38.9 ^{o}C). The fevers typically last from several days up to 2 weeks and can be biphasic in nature. Shortly after the onset of fever, the majority of infected persons develop severe, often debilitating polyarthralgias. The joint pains are usually symmetric and occur most commonly in wrists, elbows, fingers, knees, and ankles but can also affect more-proximal joints. Arthritis with joint swelling can also occur. The lower extremity arthralgias can be severely disabling, resulting in a slow, broad-based, halting gait, which can persist for months. The clinical features of chikungunya fever are mimic misdiagnosis with dengue fever whereas dengue fever is endemic area (Table 1).

Table 1. Comparison of the Clinical Features of Chikungunya Fever and Dengue Fever

Clinical feature	CHIKV infection	DENV infection
Fever (temperature, > 38.9 ^{o}C)	+++	++
Myalgias	+	++
Althalgias	+++	+/-
Headache	++	++ [a]
Rash	++	+
Bleeding dyscrasias	+/-	++
Shock	-	+/-
Leukopenia	++	+++
Neutropenia	+	+++
Lymphopenia	+++	++
Thrombocytopenia	+	+++

NOTE. The mean frequencies of symptoms were determined from studies where the 2 diseases were directly compared among patients seeking care. Symbols indicate the percentage of patients exhibiting each feature: +++, 70%–100% of patients; ++, 40%–69%; +, 10%–39%; +/, !10% -, 0%

[a] Headache was often retro-orbital.

2.3 Dengue and chikungunya cases reported

2.3.1 Dengue cases reported

DHF has been reported in Thailand since the late 1950s [52, 62-63]. Since the first DHF epidemic outbreak in 1958 [64], epidemics have been reported from almost all parts of the country. During December 1976 there was an epidemic in a rural area of Indonesia. They observed the patients' age distribution which was similar to the highly endemic area in Jakarta [65]. DEN-3 was found predominantly in severe patients in 1983 in Thailand (WHO, 1997). In 1986, Puerto Rico had the first severe hemorrhagic dengue fever outbreak which was the eleventh outbreak in this century in this country.

The most predominant serotype was DEN-4 with DEN-1 and DEN-2 circulating in the environment. Infants less than one year were at the most risk to develop to DHF and DSS [66]. The Bureau of Epidemiology, has reported highest number of cases in 1987. The incidence rate was as high as 325 cases per 100,000 population based on the number of cases reported. The latest epidemic was in 1998 when the incidence rate was as high as 211 cases per 100,000 populations. This was the second highest incidence rate in the 40 years' history of DHF outbreaks (Source: Bureau of Epidemiology, 2002).

In Thailand, the incidence has increased 20 fold in the last four decades from 9/100,000 in 1958 to 211/100,000 in 1998 [67] whereas it has risen 30 fold worldwide [68]. The circulation of all four serotypes was found in Thailand with one serotype emerging at each epidemic. Serotypes 1, 2, 3 were related to moderately severe dengue epidemic years whereas only DENV-3 correlated with severe outbreak dengue year.

DENV-4 was found mostly in secondary infection and not correlated with serious outbreak. DENV-2 and DEN-3 was associated with DHF, the severe form of dengue fever. The frequency of primary infection was arranged as DENV-3 > DENV-1 > DENV-2 > DENV-4; for secondary infection as DENV-2 > DENV-3 > DENV-1

> DENV-4; for DF as DENV-3 > DENV-1 > DENV-2 > DENV-4; and for DHF as DENV-2 > DENV-3 > DENV-1 > DENV-4. Infants were the most susceptible population for severe dengue disease, whose case fatality was three times higher than other children. Secondary infections were correlated with severe DHF while primary infection would cause milder symptoms. DHF/DSS of hospital cases occurs about 85-90% as a secondary infection, in Thailand [47]. All these data was derived from the study at the Queen Sirikit National Institute of Child Health in Bangkok, Thailand from 1973 to 1990 [56, 67]. Dengue disease has become an international public health especially Thailand.

In 2002, the DHF case incidence in southern Thailand was especially high risk 64 cases of deaths from total 33,617 cases, with a fatality rate of 0.77 per 100,000 populations. During 2000–2004, the Southern Epidemiology Department reported 113,591 cases of DHF in southern Thailand, with 251 deaths. In 2005, DHF cases in southern Thailand were as high as 2,991 cases with 5 deaths. This indicated that DHF was a major health risk in southern parts of Thailand.

The total number of reported cases and deaths of DF/DHF from nine countries of the SEA Region for the period 1985-2006 is reported. Total cases ranged from 46,458 (1986) to 218,821 (1998) and 188,684 (2006). Since 2003 the trend of reported dengue cases is rising although the case fatality rate is maintained below 1%. Since 2004 Indonesia reports the highest number of cases from the region. In 2006 57 % of the cases were reported from Indonesia alone.

During January – June 2005 (18 June), DHF cases in southern Thailand were show a high cases report at 2,991 cases with 5 cases of deaths. This indicated that DHF was a major health risk in southern Thailand. The endemic area that risk to dengue transmission was show in Figure 9.

During January-September 2010, Weekly Epidemiological Surveillance Report, Thailand was presented 86,407 cases of DF, DHF and DSS that higher than last year 2.23 fold, with 100 case of deaths. The highest cases were found in student group round about 38,548 cases. (Source: Bureau of Epidemiology, 2010).

In Cambodia, 1980-2008, since virological testing started in 2000, all four dengue virus serotypes have been observed to be in circulation each year, with DENV-2 and DENV-3 being predominant. The predominant circulating serotype changed from DENV-3 to DENV-2 in 2002 and then switched back to DENV-3 and 4 years later. Between 2000 and 2008, both the incidence of dengue and the proportion of cases with DHF were highest in 2006 and 2007, when the predominant serotype was DENV-3. In Cambodia, 1980-2008 was reported the incidence of DF/DHF is higher during rainy seasons. All four dengue virus (DENV) serotypes were permanently in circulation, though the predominant serotype has alternated between DENV-3 and DENV-2 since 2000 (Figure 10). Although larvicide has been distributed in 94 districts since 2002, logistic regression analysis showed no association between the intervention and dengue incidence.

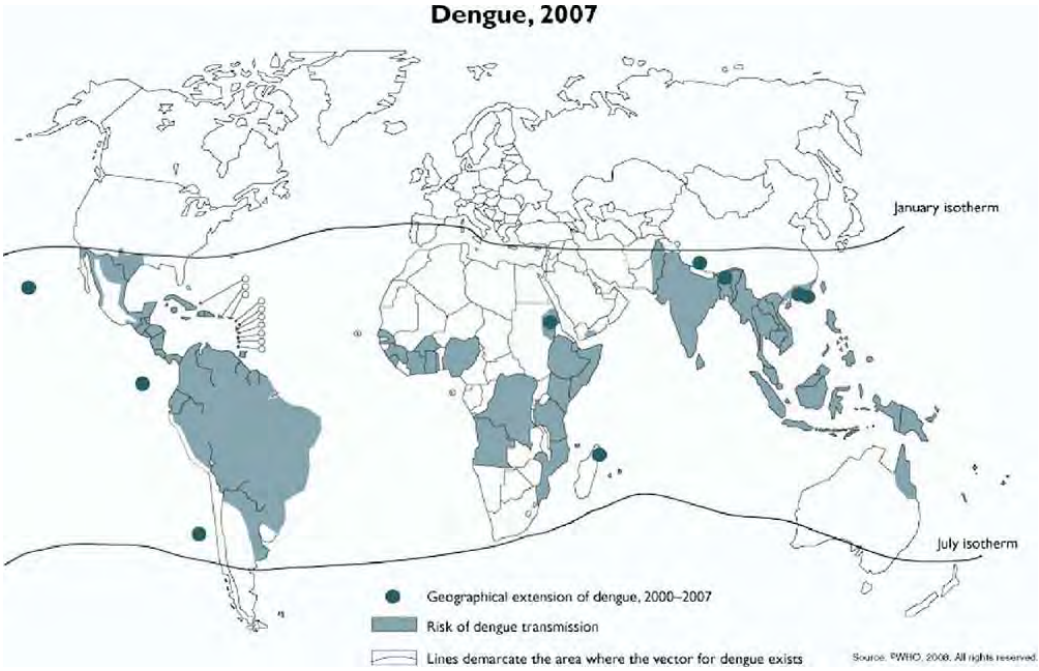


Figure 9. Areas endemic for dengue. (Reproduced with permission of the WHO.)

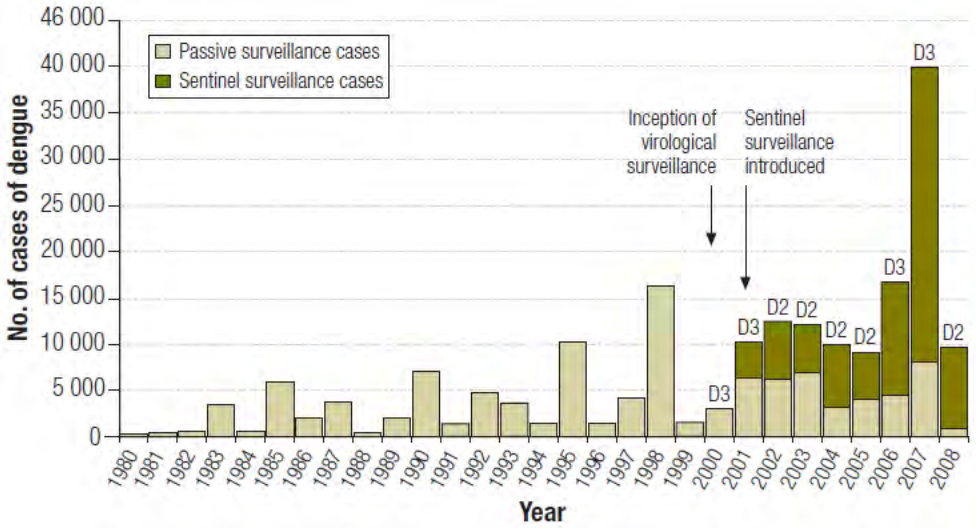


Figure 10. Number of cases of dengue fever reported nationally in Cambodia, 1980–2008. Dengue virus type-2 was predominant in the year; dengue virus type-3 (DENV-3) was predominant in Cambodia in 2007

2.3.2 Chikungunya cases reported

Since the first reported case of chikungunya fever isolated from fibril illness patient in Tanzania in 1952 [69]. The fevers are wildly spreads through many parts of the world. The first significant urban chikungunya fever outbreaks were documented in the early 1960s in Bangkok, Thailand and between 1963 and 1973 in Calcutta and Barsi, India. Only minor outbreaks occurred over the next 30 years until Kenyan outbreaks in 2004 caused by the Central/East African genotype heralded a geographically dispersed series of large outbreaks that have continued through 2009 [8, 70].

In recent years, countries in the South-East Asia Region have been severely affected by the outbreaks of chikungunya fever. During 2004-2005, a substantial chikungunya epidemic with origins in Kenya (2004) and the Comoros (2005) has been underway in the Indian Ocean, India, and South and Southeast Asia [71]. Several million cases have been seen, including thousands in returned travelers to Europe and the Americas, and local transmission of chikungunya, likely imported by an infected

traveler, was reported in Italy in 2007. The current chikungunya epidemic was particularly severe on the Indian Ocean island of La Reunion [72]. Of a population of 770,000, there were 265,000 clinical cases (34% incidence) in 2005 and 2006; overall seroprevalence was 35%, indicating very few asymptomatic infections. As well, relatively high mortality (1:1000 cases), which is traditionally rare with chikungunya, was seen on La Reunion, including in infants and young children. The geographical of chikungunya fever are illustrated in Figure 11.

India was hit in 2006 after a gap of 32 years. Indonesia, Maldives, and Sri Lanka have also been affected by this emerging infection. Various factors that are responsible for the resurgence of chikungunya include mutation of the virus, absence of herd immunity, lack of efficient vector control activities, globalization and emergence of *Ae. albopictus*, in addition to *Ae.aegypti* as an efficient vector for chikungunya virus.

India In 2006, almost 1.5 million cases of chikungunya were reported. During 2007, more than 55,000 suspected cases of chikungunya have been reported from 14 states and Union Territories (UTs) in India. The state of Kerala alone accounts for 43% of the cases. No death has been attributed to chikungunya in India in 2007. The National Vector-Borne Diseases Control Programme undertakes surveillance of suspected chikungunya cases through a network of 40 hospital based sentinel surveillance centres supported by 13 apex laboratories.

Indonesia: Chikungunya occurred sporadically until 1985 after which there were no reports until a series of outbreaks between 2001 and 2007. Between January 2001 and April 2007, more than 15,000 cases were reported from 7 provinces, with a peak in 2003. There have been over 1,200 suspected cases reported from 23 sub-districts in 2007. Most of the cases were reported from the province of Java.

Maldives: The chikungunya started in December 2006 and lasted for three months. Almost 11,000 (4.5% of the total population) suspected cases were reported. Suspected cases were also reported in 2007. Chikungunya has established endemicity in several parts of the South-East Asia Region. The socioeconomic factors and public

health inadequacies that facilitated the spread of this infection continue to exist. There is an urgent need to strengthen national surveillance and response capacity through a multisectoral approach and active participation of the communities to prevent and contain this emerging infectious disease [72].

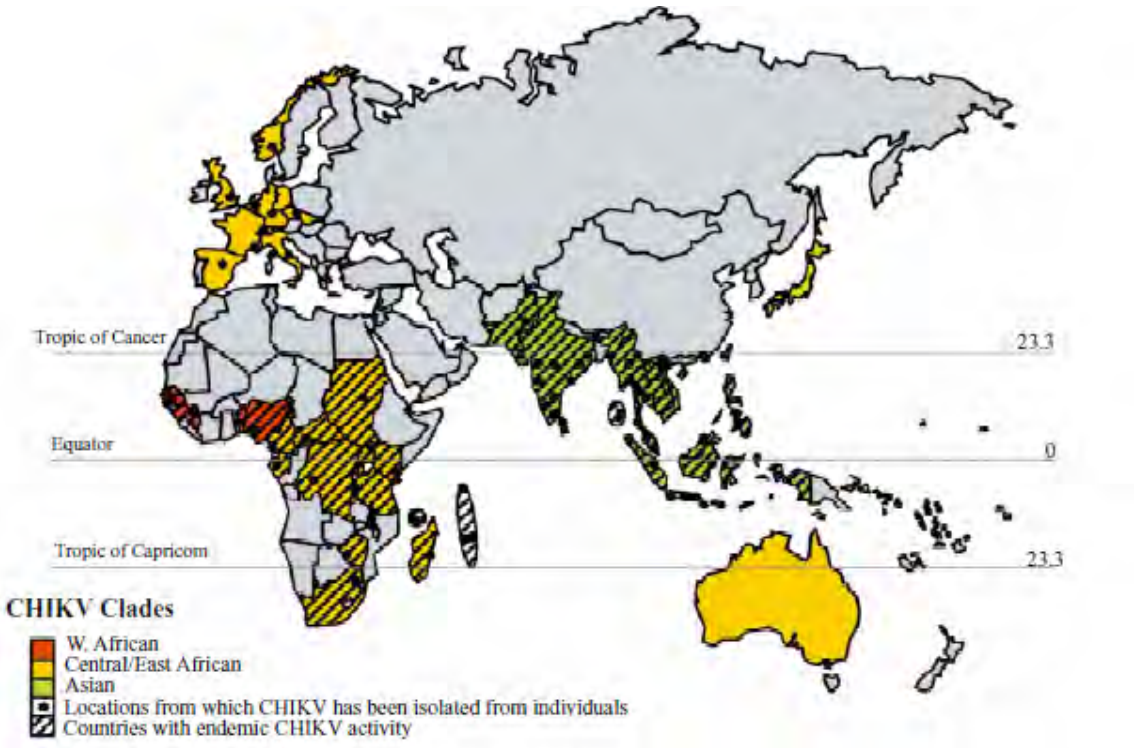


Figure 11. Worldwide distribution of CHIKV. Shading of countries indicates the predominant (or only) genotype reported to have been identified in a given country. India is shaded in green (Asian genotype) as outbreaks from 1963 to 1965 and 1973 were confirmed to have been caused by members of the Asian clade; however, reports from India during 2005–2007 indicate this outbreak was caused by the same CHIKV strains detected during the Indian Ocean outbreaks (Central/East African genotype). Asterisk indicates a location from which CHIKV was isolated [73].

In Asia, re-emergence of chikungunya fever were occurred in south East of Asia; India and Thailand, especially in Narathiwat and Phuket, southern provinces of Thailand, in 2008. Southern provinces of Thailand including tourist destinations are mainly affected areas (Figure 12). In Phuket, 669 Thais and 25 foreigners had been admitted to hospital for chikungunya fever. No death was reported. The spread of the disease in Phuket is slowing down. There are 24,029 cases of chikungunya fever have been reported in 36 provinces with no death reported in Thailand in 2009. (Source: ProMED-mail 10 & 11 June 2009). The infection rate ratio of female is higher than male at 1.5: 1.0 (Source: Bureau of Epidemiology, 2010).

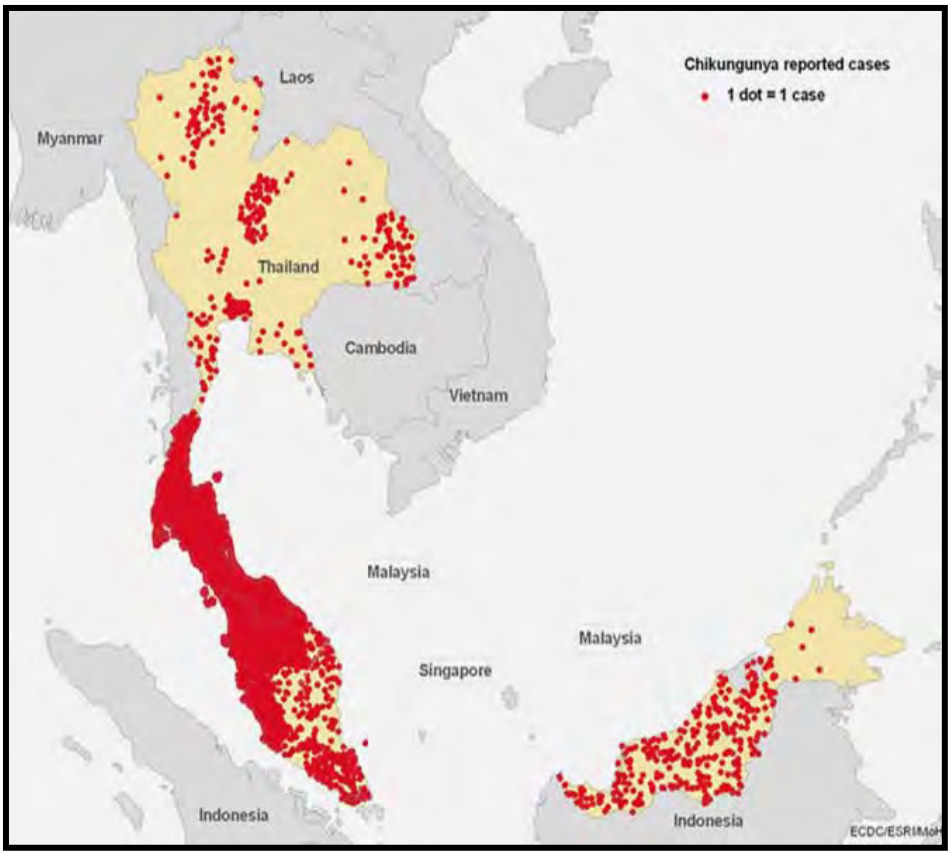


Figure 12. Distribution of Chikungunya cases reported in Singapore, Thailand and Malaysia (Data source: National Ministries of Health)

2.4 Laboratory diagnostic of chikungunya and dengue virus

Chikungunya and dengue virus are diagnosed by blood test. Since the clinical appearances of both are similar, laboratory confirmation is important, especially in areas where dengue is present [74]. In recent years, several new diagnostic techniques have been developed and have proven very useful in dengue diagnosis, such as: nucleic acid and hybridization, Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR).

Currently, dengue diagnosis is based on serology, viral isolation and RNA detection. Enzyme-linked immunosorbant assays (ELISA) are still the most widely used technique for serological diagnosis, but they do not identify the dengue virus serotype responsible for the current infection, so molecular techniques may soon assume a very important role in dengue diagnosis. RT-PCR is definitely the most satisfactory test that can be used on these infections, since it has been shown to be able to detect dengue viruses up to the 10^{th} day after the onset of the symptoms[75]. Furthermore, Pfeffer and they college developed a reverse transcription–polymerase chain reaction (RT-PCR) and Nested PCR assay to detect chikungunya virus [76]. The method can be completed in less than two working days and may serve as a sensitive alternative in chikungunya virus diagnosis.

In the mosquitoes, laboratory diagnosis based on virus isolation [77], immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) [78-80], conventional reverse transcription-polymerase chain reaction (RT-PCR) assays, and real-time RT-PCR assays [70] is used commonly to detect a single pathogen (e.g., dengue virus (DENV), Japanese encephalitis virus (JEV), or West Nile virus) in field-caught mosquitoes [81-82]. Among these methods, real-time RT-PCR has the advantages of rapidity, higher sensitivity, and capability of detecting viral nucleic acid.

2.5 The mosquito vector and viral transmission

2.5.1 Vector of dengue and chikungunya virus

Aedes species are typical small mosquitoes. They usually have black and white stripe markings on their body and legs (Figure 12B).

2.5.1.1 *Ae. aegypti* mosquito: The *Ae. aegypti* mosquito belongs to the sub-genus Stegomygia of the genus *Aedes*. The adults are marked with silvery-white or yellowish-white bands and striped on a nearly black background. It has a “lyrelike” pattern dorsally on its thorax, the legs are conspicuously banded and the last segment of the hind leg is white. It has been considered as being the major urban vector of arboviral diseases such as yellow fever, dengue and dengue haemorrhagic fever (DHF).

Ae. aegypti mainly breeds in the domestic environment, its preferred habitats are water storage tanks, jar inside and outside houses, roof gutters, leaf axils and temporary containers such as used care tyres, garbage tin cans bottles and plant pots. All these habitats typically contain relatively clean water. The eggs are laid singly on damp surfaces just above or near the water line in temporary pools and other habitats where water level rise and fall. The larvae will hatch whenever the eggs are submerged in water. Larvae develop through 4 stars and this stage may be complete in 6-8 days depending on temperature (Figure 13A).

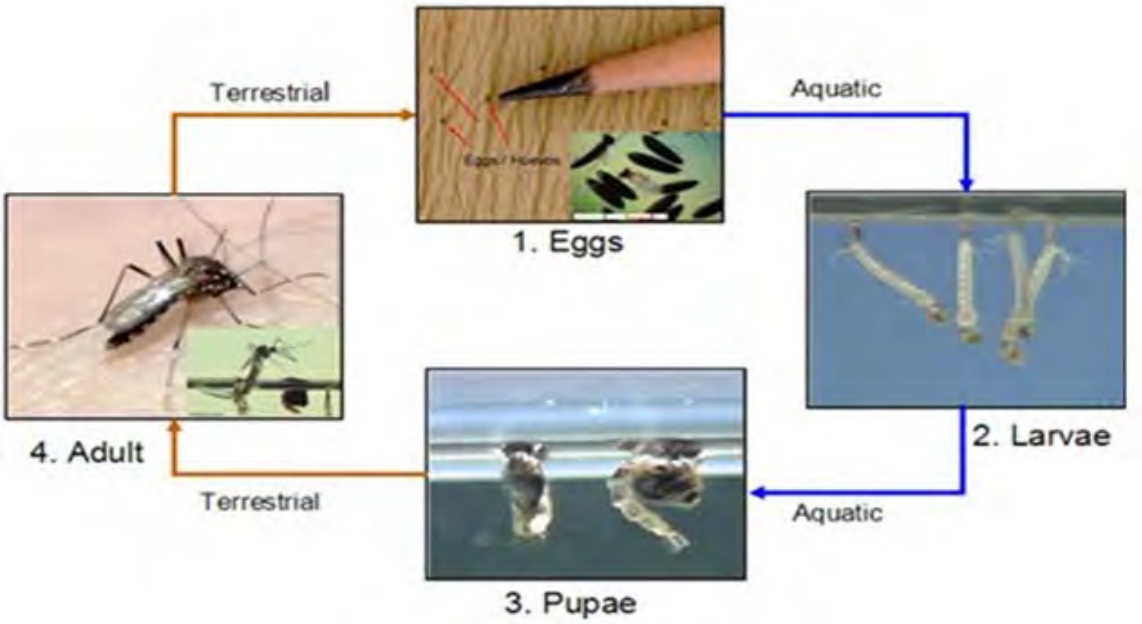
The adult mosquitoes emerge after a pupa stage of 1 to 5 days duration. Initially, post-emergence, the adults require only a sugar meal such as plant juices but 2-3 days post-emergence female mosquitoes will take a blood meal and lay their eggs. *Ae. aegypti* mosquitoes bite mainly in the morning and evening. They breed, feed and rest in and around houses. Only one copulation is necessary for the fertilization of all eggs as the female stores sperm in spermatheca.

2.5.1.2 *Ae. albopictus* mosquito: Adult *Ae. albopictus* are easily recognized by the bold black shiny scales and distinct silver white scales on the palpus and tarsi [83]. The scutum (back) is black with a distinguishing white stripe down the center beginning at the dorsal surface of the head and continuing along the thorax. Differences in morphology between male and female include the antennae of the male are plumous and mouthparts are modified for nectar feeding. The abdominal are covered in dark scales. Legs are black with white basal scales on each tarsal segment. The abdomen narrows into a point characteristic of the genus Aedes. Field identification is very easy because of these distinct features.

Ae. albopictus has a wide geographical distribution (Figure 14), is particularly resilient, and can survive in both rural and urban environments. The mosquito's eggs are highly resistant and can remain viable throughout the dry season, giving rise to larvae and adults the following rainy season. Originating from Asia, and initially sylvatic, *Ae. albopictus* has shown a remarkable capacity to adapt to human beings and to urbanisation, allowing it to supersede *Ae. aegypti* in many places (including China, the Seychelles, and Hawaii), and to become a secondary but important vector of dengue and other arboviruses. It seems that most new introductions of *Ae. albopictus* have been caused by vegetative eggs contained in timber and tyres exported from Asia throughout the world.

Ae. albopictus is a typically vector in both rural and urban, zoophilic and anthropophilic, thriving both in the natural environment, and in habitations and their immediate periphery. *Ae. albopictus* is relatively long-lived (4–8 weeks) and has a flight radius of 400–600 m. It is aggressive, silent, and diurnal, meaning that bednets are ineffective. The adult female appears to transmit the virus vertically to her eggs, although this remains to be documented in the Indian Ocean outbreak.

(A)



(B)

Figure 13. The *Aedes* mosquito life cycle and adults female characteristic of *Ae. aegypti* and *Ae. albopictus* mosquitoes

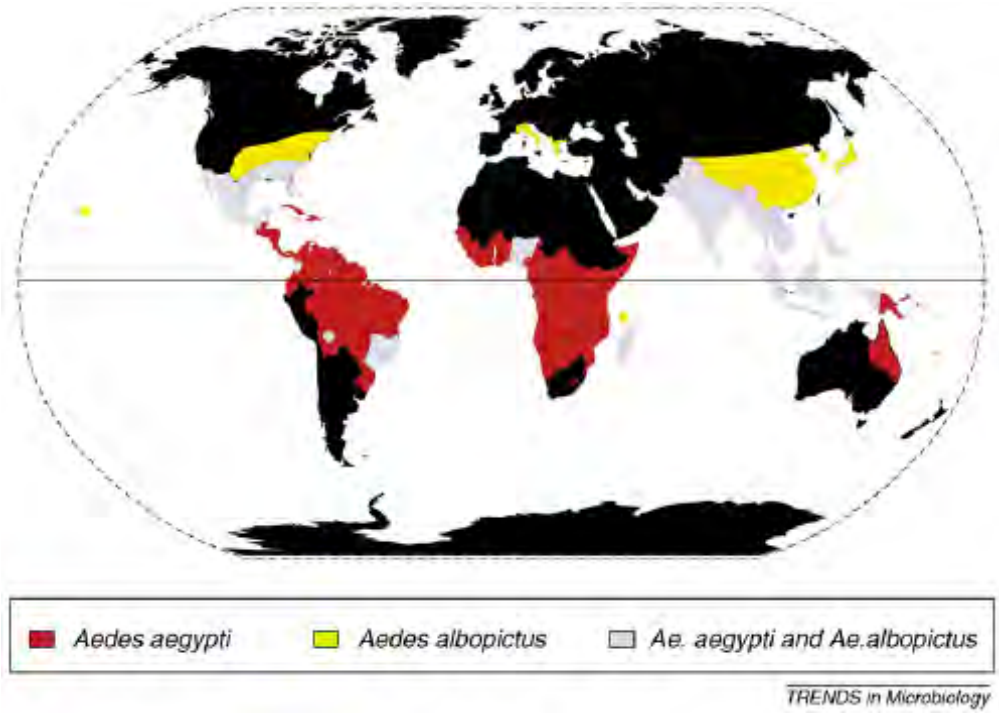


Figure 14. Current distribution of *Ae. aegypti* and *Ae. albopictus*. The *Ae. aegypti* genotypes that invaded the tropical belt belong to the anthropophilic subtype *Ae. aegypti,* and most probably originated from African Atlantic populations. Whereas the origins of the invasions of the Asian *Ae. albopictus* vector remain to be elucidated, the current active invasion by this vector of nontropical urban ecosystems in the New World and Mediterranean area should be noted.

2.5.2 Viral-vector transmission

Ae. aegypti and *Ae. albopictus* are an importance vectors of arboviruses. Both mosquitoes have been incriminated as the vector responsible for dengue virus transmission [84]. *Ae. aegypti* could be transmitted dengue virus pass through transovarial route. The possibility of transovarian transmission was investigated in Malaysia by [84-85]. In contrast, Mourya and they collage has been reported that no transovarian transmission of chikungunya virus in *Ae. aegypti* mosquitoes [86]. *Aedes sp*. are biting several times because they need some blood for development their egg and viral transmission was occurred between the female mosquito uptakes a blood meal.

There are two cycles of DENV infection (Figure 15), one occurring when infected-mosquito is biting human host, the virus is pass through human body and infected to lymphatic tissue and other organ. At this time, human were infected with DENV and present dengue fever symptom. On the other hand, when uninfected mosquito get a blood from the patient, the viral was infected to mosquito cell and propagated into the mosquito body pass through proboscis, midgut and penetrate to heamoceal before infected to salivary gland. Between mosquitoes uptake a blood, the viral progeny in saliva was injected to uninfected host.

Infected people are infective for feeding mosquitoes during the febrile phase of their illness, and mosquitoes may then transmit the virus to others at subsequent blood-meals after an incubation period of approximately one week, depending upon ambient temperatures[87].

Infected female mosquitoes may also pass the virus on to progeny by transovarial transmission[88]. Mosquitoes infected transovarially may transmit dengue virus to susceptible humans at their first blood-meal. Once infected, mosquitoes remain infected for life and may transmit dengue virus either during probing, interrupted blood feeding, or when feeding to repletion. Thus, a single infected mosquito may transmit the virus to several susceptible humans over its lifetime.

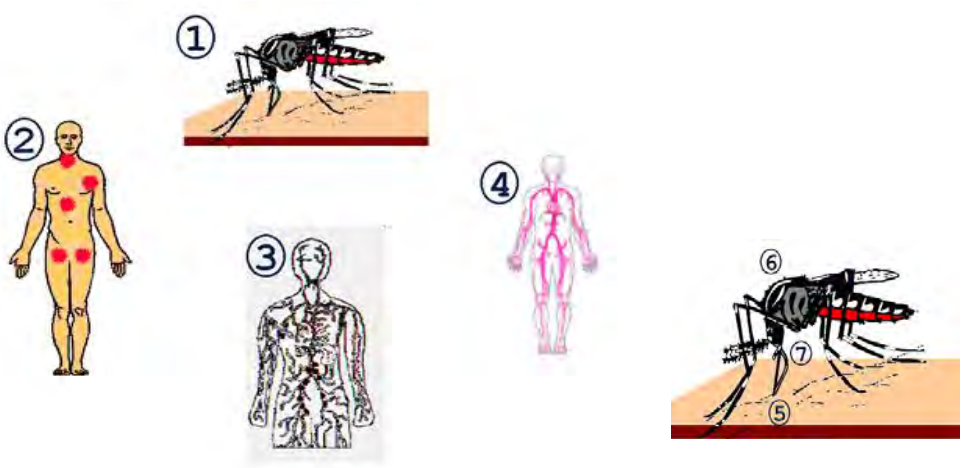


Figure 15. Dengue virus transmission: 1) DENV transmitted to human pass through infected-mosquito saliva, 2) The viral replicates in target organs, 3) Infected white blood cells and lymphatic tissues, 4) Released vireon and circulates in blood, 5) Uninfected mosquito ingests virus with blood, 6) The viral infected to midgut and mosquito tissue, 7) Virus replicates in salivary glands and transmitted to new human host by injection saliva with viremia between uptake a blood meal (www. CDC.gov)

2.5.3 Role of viral-mosquito transmission

2.5.3.1 DENV transmission

The mechanisms of viral transmission are still do not know but many article was support that dengue virus are involve to zoonotic cycle (Figure 16). They reported that humans are the primary vertebrate hosts of dengue viruses, although there is evidence of a silent zoonotic cycle involving mosquitoes and monkeys in parts of Asia and Africa [89-90].

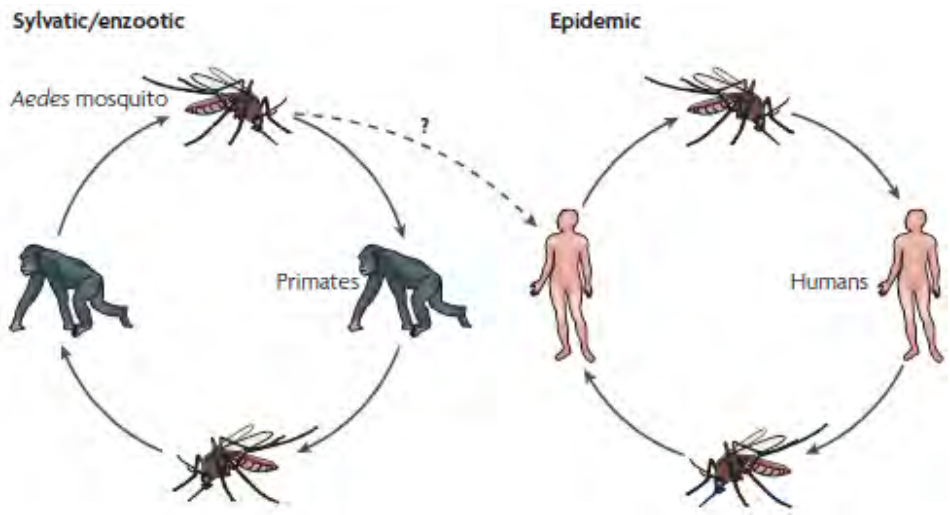


Figure 16. Transmission of dengue viruses. Because of the high level of viraemia resulting from dengue virus (DENV) infection of humans, the viruses are efficiently transmitted between mosquitoes and humans without the need for an enzootic amplification host. DENV is spread principally by the *Ae. aegypti* mosquito, which breeds in domestic and peridomestic water containers, increasing the frequency of contact between mosquitoes and humans. In addition, a sylvatic cycle for dengue transmission has been documented in western Africa and southeast Asia. However, unlike the impact that widespread sylvatic transmission of yellow fever virus has on human disease, the contribution of the observed sylvatic cycle of dengue transmission to human infection is unknown, but appears to be minimal.

2.5.3.1 CHIKV transmission

In Asia and the Indian Ocean region the main chikungunya virus vectors are *Ae. aegypti* and *Ae. albopictus*. A larger range of Aedes species (*A furcifer*, *Ae. vittatus, Ae. fulgens, Ae. luteocephalus, Ae. dalzieli, Ae. vigilax, Ae.camptorhynchites*) transmit the virus in Africa, and *Culex annulirostris*, *Mansonia uniformis*, and anopheles mosquitoes have also occasionally been incriminated. These viruses are maintained in nature in enzootic cycles involving mosquitoes and vertebrates, generally birds and mammals [91]. Like many of the other infections described here, human infections are incidental and occur after receipt of a bite from an infected mosquito. CHIKV produces high-titer viremia in humans sufficient to infect mosquitoes, which permits an urban transmission cycle between humans and *Ae. aegypti* mosquitoes (Figure 17).

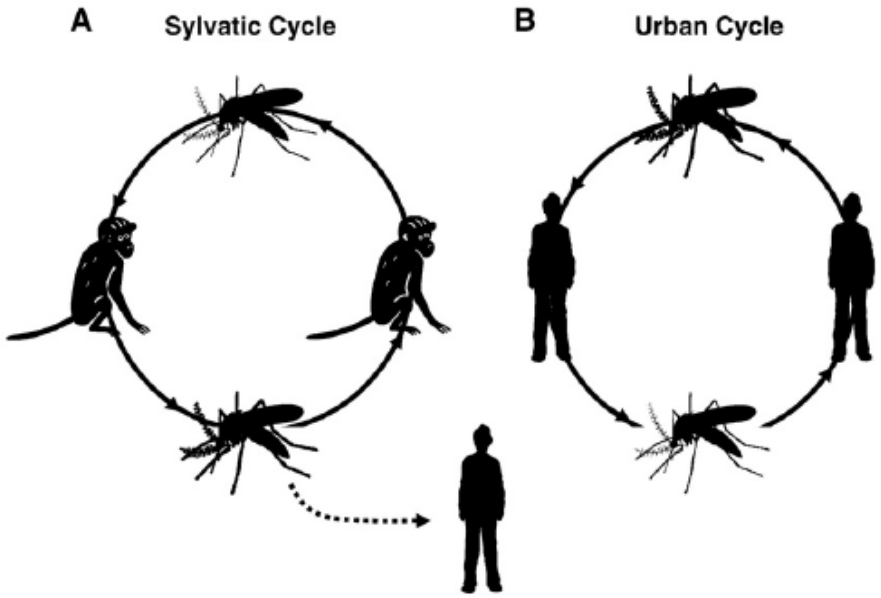


Figure 17. (A) CHIKV in Africa is maintained in a sylvatic cycle involving forest dwelling Aedes spp. mosquitoes and nonhuman primates. When sylvatic mosquito densities increase, often during periods of heavy rainfall, small human epidemics or sporadic human cases may occur. (B) In urban settings, CHIKV circulates in a man-mosquito-man cycle vectored principally by the anthropophilic *Ae. aegypti* mosquito. Although *Ae. albopictus* has been considered an accessory vector, some recent urban outbreaks have been present as a primarily vectors by this mosquito [91].

2.5.4 Vector competence

The vector competence to DENV or CHIKV depends on the susceptibility of a mosquito to become infected and subsequently transmit the virus through the bit. In recently, *Ae. albopictus* have a trend to displace to *Ae. aegypti* in many parts of the world [72]. Reviews of many vector competence studies involving *Ae. albopictus* provide information for 23 arboviruses and for Nodamura virus (probably not an arbovirus). In addition, *Ae. albopictus* has been recently found to be a competent experimental vector of Sindbis virus. A list of viruses included in vector competence experiments involving *Ae. albopictus* is shown in Table 2.

Moreover, *Ae. aegypti* as well as *Ae. albopictus* ensured a high replication of the virus which underwent an efficient dissemination as detectable in the salivary glands at day 2 post-infection (pi) [92]. After 2 day of Oral infection with CHIKV [92], infectious CHIKV particles were delivered and staining with immunocytochemistry. This result was show *Ae. albopictus* is slightly more efficient than *Ae. aegypti* to transmit the variant E1-226V of CHIKV after day 2 pi and the viral titer is show maximum rate at day 6 pi for *Ae. albopictus* ($10^{3.3}$ PFU) and day 7 pi for *Ae. aegypti* ($10^{2.5}$ PFU).

Table 2. The ability of *Ae. albopictus* susceptible to arbovirus by oral infection and ability to transmit by bite.*

Viruses	*Ae. albopictus* strains			
	Hawii and areas outside W. Hemisphere		North and South America	
	Infect.	Trans.	Infect.	Trans
Chikungunya	+	+	+	+
Dengue 1, 2, 3, 4	+	+	+	+
Eastern equine encephalitis	+	+	+	+
Jamestown Canyon			+	+
Japanese encephalitis	+	+		
Keystone			+	-
La Crosse			+	+
Mayaro			+	+
Nodamura	+	?		
Oropouche			+	_
Orungo	+	+		
Potosi			+	+
Rift Valley fever			+	+
Ross River	+	+	+	+
San Angelo	+	+		
Sindbis			+	+
St. Louis encephalitis	+	+		
Trivittatus			+	_
West Nile	+	+		
Western equine encephalitis	+	+	+	+
Venezuelan equine encephalitis			+	+
Yellow fever	+	+	+	+

* Modified from Mitchell (1991)

2.5.5 Host shifts as an ecological rule of arbovirus emergence

The increase in transcontinental travel has facilitated the invasion of urban ecosystems not only by DENV, but also by their peridomestic mosquito vectors, *Ae. aegypti* and *Ae. albopictus*. This led to a ‘snowball effect’ resulting in dengue pandemics; that is, the increase in the number of areas colonized by at least one vector species and one DENV strain directly multiplied their probability of invading new areas (Figure 18). One can debate that CHIKV epidemiology fits either the general zoonotic or the DENV pattern depending on the geographical area considered.

In Senegal, CHIKV epidemics in humans depend more or less directly on the spillover of zoonotic viruses [92]. This might also be the case in other African regions. Long-range human migrations have so far connected the CHIKV epidemics observed among non-African urban ecosystems, raising the question of a risk for future CHIKV pandemics as previously observed for DENV [86]. However, the low sustainability of CHIKV urban transmission at a local scale supported by long interepidemic periods has to be considered. Balancing the arguments for and against the likelihood of CHIKV pandemics requires addressing whether CHIKV and DENV share the same ability to complete their adaptation to new transmission cycles, that is, ultimately to sustain urban epidemics in the absence of any reimmigration of zoonotic-derived variants [93].

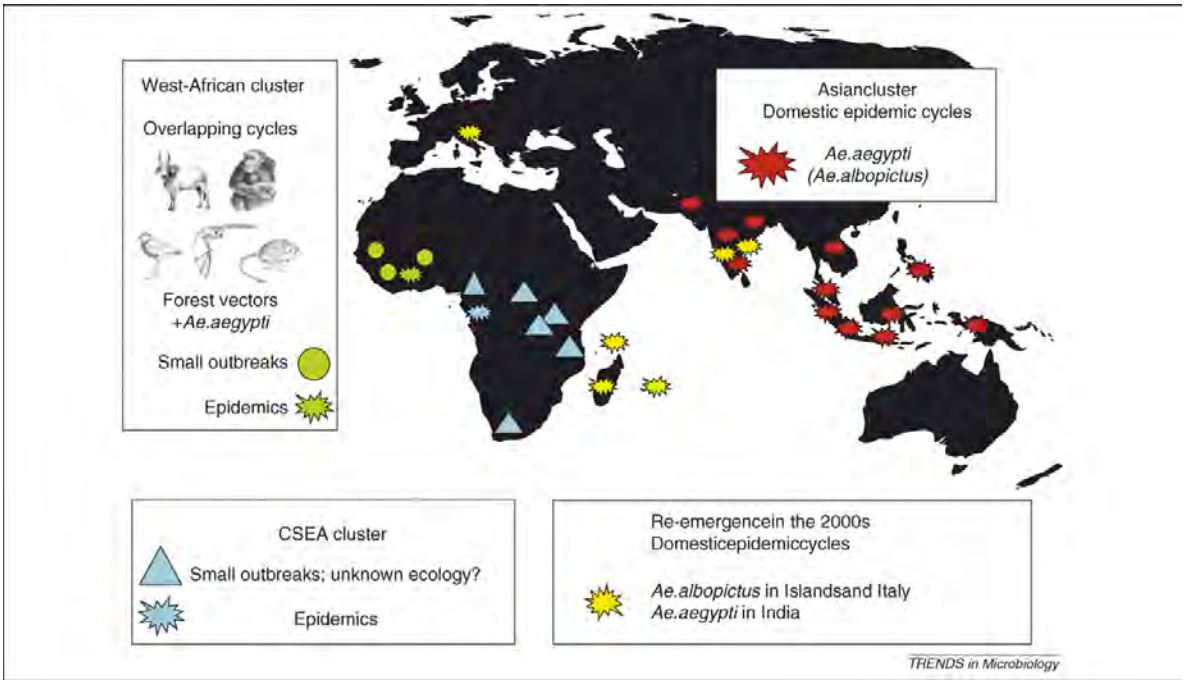


Figure 18. illustrates the historical and geographical variations in CHIKV ecology and associated risks for human health. Viral phylogenetic groups of genotypes are color coded: the ancestral West African group is in green; the second African cluster (or CSEA cluster, for Central, Southern, East African viruses) is in blue; the primarily Asian group is in red, and the CHIK viruses involved in Indian Ocean outbreaks since 2000 are represented in yellow. For each group, a box summarizes the knowledge acquired on CHIKV ecology and human infection risks

2.6 Viral dissemination into mosquito tissue and human tissue

Most of the emerging viral infections in humans, originate from known zoonosis. Pathogens have been engaged in long-standing and highly successful interactions with their hosts since their origins [94]. Many strategies have evolved that maximize invasion rate, ensuring timely replication and survival both within-host and between-host, thus facilitating reliable transmission to new susceptible hosts [86], is extremely important to better understand the evolutionary events that profile of genotype, phenotype of pathogens occurred [95]. For that, to determine how cells from human and non-human species resist to infection; so, ultimately novel molecules may be designed to protect permissive cells from viral infection. The importance molecules are described in Table 3 and 4 which relatively to viral infection.

2.6.1 Dengue and chikungunya virus infected mosquito vector and mosquito tissue (C6/36)

DENV dissemination into mosquito tissues is dependent on the ability of the virus to penetrate several barriers [96]: the midgut infection barrier (MIB), the midgut escape barrier (MEB) and the salivary glands. Infection of mosquito salivary glands and subsequent injection of infectious saliva into the human host are key events of DENV transmission cycle [95].

DENV and chikungunya virus are penetrated to *Aedes albopictus* (C6/36) cell [97-98] pass through protein receptor; two glycoprotein 40 and 45 kDa was found in DENV-4, Laminin-binding protein are importance protein like receptor for DENV-3 whereas protein 24, 45, 58, 60 and 62 kDa are related to chikungunya viral binding [99]. The other important protein binding receptor was described in *Aedes albopictus* (C6/36) cell line and in mosquito vector (Table 3 and 4)[100].

Table 3. Proposed dengue virus receptor proteins in C6/36 cell line.

Cell	Cell type	Serotype; strain	Receptor characteristics	References
C6/36	*Aedes albopictus* cell line	DEN-4; H-241	Two glycoprotein 40 and 45 kDa	Salas-Benito and del Angel (1997)
C6/36	*Aedes albopictus* cell line	DEN-2; NGC	80 and 67 kDa protein	Munoz et al. (1998)
C6/36	*Aedes albopictus* cell line	DEN-2; NGC	Tubulin protein or like-tubulin protein	Chee and AbuBakar (2004)
C6/36	*Aedes albopictus* cell line	DEN-3, 4	Laminin-binding protein	Sakoonwatanyo o *et al.* (2006)
C6/36	*Aedes albopictus* cell line	Chikungunya virus	24, 45, 58, 60 and 62 kDa	Mourya *et. al*., (1998)

Table 4. Proposed dengue virus receptor proteins in mosquitoes.

Cell	Cell type	Serotype; strain	Receptor characteristics	References
Ae. aegypti	Midgut, ovary and salivary gland cell, eggs, larvae and pupae cell extract	DENV-4; H-241	A 45 kDa glycoprotein	Yazi Mendoza *et al.* (2002)
Ae. aegypti	Salivary gland	DENV-1, 2, 3 and 4	37, 54, 58 and 77 kDa protein	Cao-Lormeau (2009)
Ae. polynesiensis	Salivary gland	DENV-1, 4	48, 50, 54, 56, and 77 kDa protein	Cao-Lormeau (2009)
Ae. aegypti	midgut brush border membrane	chikungunya	60 and 38 kDa	Mourya *et. al*., (1998)

2.6.2 Chikungunya virus infected human host cell

CHIKV was also found to bind and infect a number of cells derived from other tissues (Table 5) such as the cervical carcinoma epithelial cell line HeLa, the kidney epithelial cell line HEK-293T, the hepatocarcinoma epithelial cell line HUH7, and the neuroblastoma cell line SH-SY5Y are infected by CHIKV and show cytopathic effects and syncytia formation after CHIKV exposure. In addition, BEAS-2B, Hs 789.Sk skin cells, dividing and non-dividing MRC-5 lung cells and endothelial cell lines (TrHBMEC and hCMEC/D3) were also susceptible to CHIKV [71]. In muscular tissues, CHIKV infects and replicates in skeletal muscle progenitor cells, designed as satellite cells, but not in muscle fibers. More recently, it was reported that the syncytiotrophoblastic cell line BeWo is refractory to CHIKV[101]. In addition to this observation, we found that CHIKV is unable to replicate in the A549 alveolar epithelial cell line. Interestingly, the A549 alveolar epithelial cell line was reported to efficiently bind CHIKV. Given that a quite good correlation was reported between the efficiency of viral binding and sensitivity to infection [71], this may suggest that a co-receptor is lacking at the surface of A549 cell line or that this particular cell type is protected by a restriction factor, tissue-specific, acting post-entry. It is not known whether mechanisms of restriction similar to other well documented events of cellular resistance to viruses such as these generated by the tripartite motif proteins (TRIM/RBCC) and apolipoprotein B mRNA-editing enzymes (APOBEC) in HIV infected cells [102-103], exist for CHIKV, but it would not be surprising to find cells resistant to CHIKV by a restriction mechanism.

Table 5. Summary the sensitivity of CHIKV infected of different human cell lines.

Name	Cell type	Viral replication	Cytopathic effect		
			10^{-1}	10^{-2}	10^{-3}
THP-1	Monocytoid cell line	-	nd	nd	nd
U937	Monocytoid cell line	-	nd	nd	nd
Dendritic cells	Primary monocyte-derived cell	-	-	-	-
Macrophages	Primary monocyte-derived cell	+	+	+	+
H9	T-Lymphocyte-derived cell	-			
HUH7	Hepatocarcinoma cell line	++	++	+	-
SH-SY5Y	Neuroblastoma cell line	++	++	++	-
HeLa	Cervical carcinoma epithelial cell line	++	++	+	-
A549	Alveolar epithelial cell line	-	nd	nd	nd
HEK-293T	Kidney epithelial cell line	++	++	++	-

Viral replication was measured 24 h after exposure to the 5′CHIKV-EGFP virus at 25×TCID50. Cells were then fixed using paraformaldehyde and GFP positive cells were counted under epifluorescence using a Leica microscope. ++: at least 80% of GFP cells; +: less than 40% of GFP cells. The cytopathic effect was evaluated by MTT assay 48 h post infection. Cells were infected with the indicated TCID50 dilution (10^{-1} refers to 25×TCID50), nd: not determined. All results are representative of 3 independent experiments. CHIKV used is the West African strain 5′CHIKV-EGFP that encodes a GFP protein [69].

2.7 Virus vector relationship

Following the end of the Second World War, Asian cities experienced rapid growth and urban crowding, and with these came increased habitat for the vector mosquito of dengue, *Ae. aegypti*, which selectively breeds in water containers associated with human habitations. These breeding sites can include water jugs, blocked drain spouts, decorative plant containers, discarded refuse that may retain water, and many other items associated with modern life [104].

The same evolutionary pattern is now being repeated in the urban centers of Latin America[48, 105]. In the 1960s, a hemisphere-wide *Ae. aegypti* eradication programme was in existence, and nearly succeeded in total eradication of the vector. However, the decision was made to change the strategy from eradication to control. As a result, *Ae. aegypti* has now reinfested virtually all areas where it was once eliminated, and has even expanded to new habitats. As a result of this reinfestation, transmission of dengue has recently occurred in almost all parts of Latin America and the Caribbean. It is currently limited to increasingly frequent epidemics of dengue fever, often coupled with sporadic cases or outbreaks of DHF/DSS. The pattern is, however, identical to that experienced in Asia during the 1950s and 1960s, and it is clear that DHF/DSS will become increasingly common in the years to come [54, 106]. Indeed, in 1994 a major outbreak of dengue and DHF occurred in north-eastern Brazil, and some experts have suggested that as many as 300,000 cases might have occurred.

In Africa, similar conditions exist, but reporting has been fragmentary, and there are no reliable estimates of the number of persons infected or the virus serotypes in circulation. As a typical example, however, in 1993 an outbreak of dengue occurred in Comoros, during which an estimated 60,000 cases occurred (Pan American Health Organization 1993).

A pattern of dengue transmission then became evident, especially in major cities of South East Asia, first with increasingly frequent epidemics of classic dengue, then merging to continuous endemic transmission and appearance of DHF/DSS, initially sporadically but becoming more common within the last 20 years, until now [104]. This pattern has been seen in varying degrees throughout much of tropical Asia, from the southern provinces of China to the west coast of India, and south to northern Australia and many Pacific island nations.

CHIKV likely originated in Central/East Africa, where the virus has been found to circulate in a sylvatic cycle between forest-dwelling Aedes species mosquitoes and nonhuman primates. In these areas, sporadic human cases occur, but large human outbreaks are infrequent. However, in urban centers of Africa as well as throughout Asia, the virus can circulate between mosquitoes and naive human hosts in a cycle similar to that of dengue viruses. *Ae. aegypti* and *Ae. albopictus* mosquitoes are the main vectors responsible for urban transmission of CHIKV.

In addition, at least 18 countries throughout Asia, Europe, and North America documented imported cases of chikungunya fever, with a few of these countries developing local autochthonous transmission of the virus [78]. In addition to the spread of the Central/East African genotype of CHIKV among the Indian Ocean Islands, this genotype was imported to India where it had never been reported. The outbreak that ensued in India has continued for 13 years, resulting in millions of cases. The persistence of cases of infection in India is presumably attributable to vast numbers of immunologically naive people, who help sustain viral transmission. This is in contrast to other affected areas, primarily islands with limited populations, which did not report cases after the epidemic swept through, most likely because of the development of herd immunity.

CHIKV in India served as the source of viral introduction to Italy. A viremic traveler returning to his home after a visit to India was the index case that led to subsequent autocthonous transmission with local *Ae. albopictus* mosquitoes. A study of the epidemic curve of cases identified in Italy combined with detection of the virus in local *Ae. albopictus* mosquitoes established that CHIKV was maintained locally in a mosquito-human- mosquito cycle. The epidemics of CHIKV infections occurring from 2004 through 2008 demonstrated the ease with which this virus can spread and infect humans. Several factors likely contributed to the spread, including very high attack rates associated with the recurring epidemics, high levels (often 15 log10 plaque forming units per ml of blood) of viremia associated with infection in humans, and the worldwide distribution of the vectors responsible for transmitting CHIKV.

The extraordinary nature of this epidemic appears, with its unprecedented incidence rate in the Indian Ocean (as well as on La Reunion, relatively high incidences were seen in 2005 and 2006 on Maritius, the Seychelles, and the island of Mayotte), to have been driven by several features, including increased tourist numbers and the introduction of the virus into nonimmune populations. Perhaps though the most important factor in chikungunya's re-emergence in the region is its acquisition of an adaptive mutation allowing it to be efficiently vectored by *Ae. albopictus* (traditionally considered to have a low vector capacity) in addition to its traditional vector, *Ae. aegypti*.

This mutation (a single base-pair, valine are substitute to alanine at position 226 of the E1 gene, known as A226V) [107] appears to have occurred in 2005 or 2006 when chikungunya arrived in La Reunion[72] and other Indian Ocean islands where *Ae. albopictus* has displaced *Ae. aegypti*, and, as well as conferring fitness to be vectored by *Ae. albopictus*, may be associated with the more virulent nature of chikungunya infections with this variant, as well its ability to be transported by infected travelers to new areas where outbreaks occur. The A226V variant was also noted in the outbreak mentioned above in Italy, where transmission was also by *Ae. albopictus* [108]. This phenomenon, an independent adaptive mutation meeting a necessity of transmission by *Ae. albopictus*, is known as evolutionary convergence, and its observation in nature is considered extremely rare.

2.8 Dual infection of chikungunya and dengue virus

The first cases of chikungunya and dengue virus mixed infection were reported by [109]. This report determine blood sample which taken from a patient in the acute phase of a dengue like illness and the results present dual infection between dengue and chikungunya virus in the same patient. In Thailand and India also have been reported about dual infection in blood sample of the same patient[110]. Dual infection case with chikungunya virus and dengue serotype-1 (DENV-1) were also reported in India [111]. In Burma also found dengue and chikungunya infection in antibodies analysis that carrying from serological survey [112-113].

Moreover dual infection with 2 different serotypes of dengue virus have been reported in a single patient by [114-115]. Beside the point, dual infection between dengue virus and bacterium; *Salmonella typhi*, *Shigella sonnei* and *Leptospira spp*. were also reported [116-118]. Especially, the first report of mixed dengue and parasite (*P. falciparum*) has been reported by [119]. Additionally, first case infection of DEN-2 and *P. vivax* in a single patient was reported in Alappuzha District, Kerala, in India which surprising of virus and parasite mixed infection [120]. In Malaysia co-circulation in all 4 dengue virus serotypes was found but after the first outbreak in 1960's, only DENV-1, DENV-2 and DENV-3 were associated with DF/DHF outbreak [121].

RT-PCR and virus isolation was applied to confirm dengue infection during the period 2000 to 2001 in the Greater Metropolitan Region of the State of Rio de Janeiro. A total of 5324 serum samples from suspected cases of dengue were analysed. This study presents the co-circulation of three dengue serotypes: DENV-1, DENV-2 and DENV-3 [86]. The results confirmed 52.3% (42/79) of DENV-3 cases, showing the importance of acute serum samples in the virological surveillance of the disease. Despite the introduction of a new serotype, an outbreak due to DENV-1 was observed in the municipality of Niteroi. The restriction site-specific PCR (RSS-PCR) patterns obtained for DENV-1 and DENV-2 isolated in that period showed that those strains belonged to the subtypes previously circulating in the state. DENV-3 RSS-PCR patterns confirmed that these viruses belonged to subtype C (Sri Lanka/India

strains), represented by the strain circulating on the American continent. These data showed the importance of an active surveillance programme in countries where dengue is endemic [122].

An outbreak of dengue-like syndrome occurred in Toamasina on Madagascar's east coast. (Figure 20) from January through March 2006. Dengue type l or chikungunya viruses were detected in 38 of 55 patients sampled. *Ae. albopictus* was the only potential vector collected. Of 4,242 randomly selected representative residents interviewed retrospectively, 67.5% reported a dengue-like syndrome during this period [123].

Co-circulation of DENV-2 and DENV-3was responsible for the 2006 out-break in Karachi. Primary and secondary cases were seen in both groups. Viral RNA detection of 250 patients revealed positive results in 185 (74.0%) samples. DENV-2 was predominant genotype (n = 104, 56.2%) Dengue specific antibodies were detected in 139 samples of which 81 were classified as primary cases. DENV-2 accounted for these. Within secondary cases, 63.2% were due to DENV-2 (total 57), the rest were positive for DENV-3. DHF (p = 0.064) and abdominal pain (p = 0.059) were more frequently associated with DENV-2 as compared to DENV-3. None of the samples were positive for DENV-1 or DENV-4. Cases with DHF showed marginal association with DENV-2. Introduction of a new serotype (DENV-3) and or a genotypic shift of endemic serotype (DENV-2) are the probable factors for the recent out-break of DHF in this region [124].

An outbreak of febrile illness occurred in Gabon in 2007, with 20,000 suspected cases. Chikungunya or dengue-2 virus infections (Figure 19) were identified in 321 patients; 8 patients had documented co-infections. *Ae. albopictus* was identified as the principal vector for the transmission of both viruses[8]

Co-infections with dengue (DENV) and chikungunya (CHIKV) viruses are increasing from first described in humans in 1964. The mechanism of dual infection between dengue and chikungunya virus in mosquitoes are rarely reported, particularly after the emergence of chikungunya (CHIK) in the Indian Ocean in 2005-2006 due to a new variant highly transmitted by *Ae. albopictus*. In this geographic area, a dengue (DEN) outbreak transmitted by *Ae. albopictus* took place shortly before the emergence of CHIK and co-infections were reported in patients. A co-infection in humans can occur following the bite of two mosquitoes infected with one virus or to the bite of a mosquito infected with two viruses [122]. Co-infections in mosquitoes have never been demonstrated in the field or in the laboratory. Until 2010, Vazeille and her college demonstrated oral infection in mosquito. To determine the ability of *Ae. albopictus* co-infected with two different arboviruses. They exposed *Ae. albopictus* from La Reunion Island with DENV-1 and CHIKV isolated respectively during the 2004-2005 and the 2005-2006 outbreaks on this same island. They show that *Ae. albopictus* could disseminate both viruses and deliver both infectious viral particles concomitantly in its saliva. These reports are succeeded in inducing a secondary infection with CHIKV in mosquitoes previously inoculated with DENV-1. This finding is concern as *Ae. albopictus* is still expanding its geographical range in the tropical as well as in the temperate regions [92, 125].

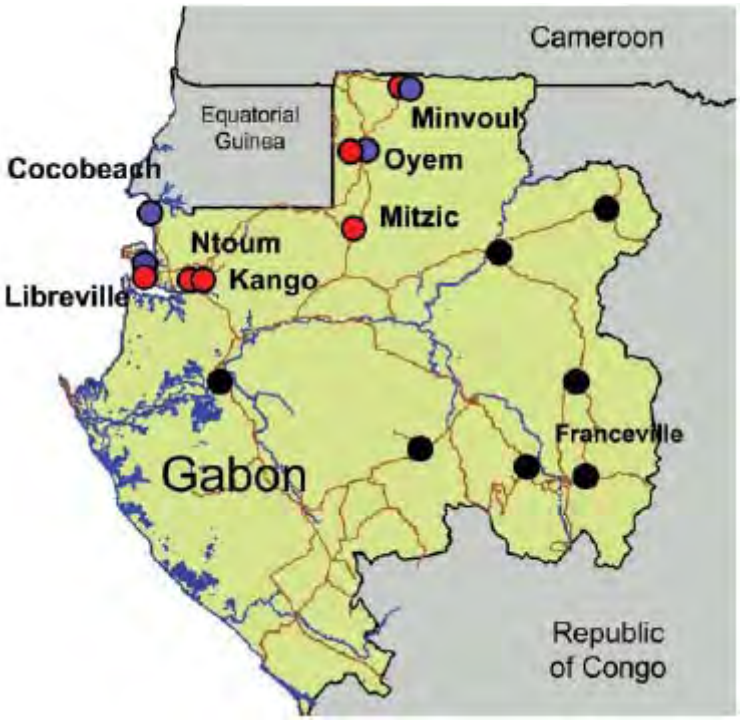


Figure 19. Chikungunya and dengue outbreak in Gabon, 2007. Distribution of the outbreak and location of the 7 towns where suspected cases were laboratory confirmed by using quantitative reverse transcription-PCR assay are shown. Chikungunya cases are represented by red circles, dengue cases by blue circles, and cases negative for both viruses by black circles[8].

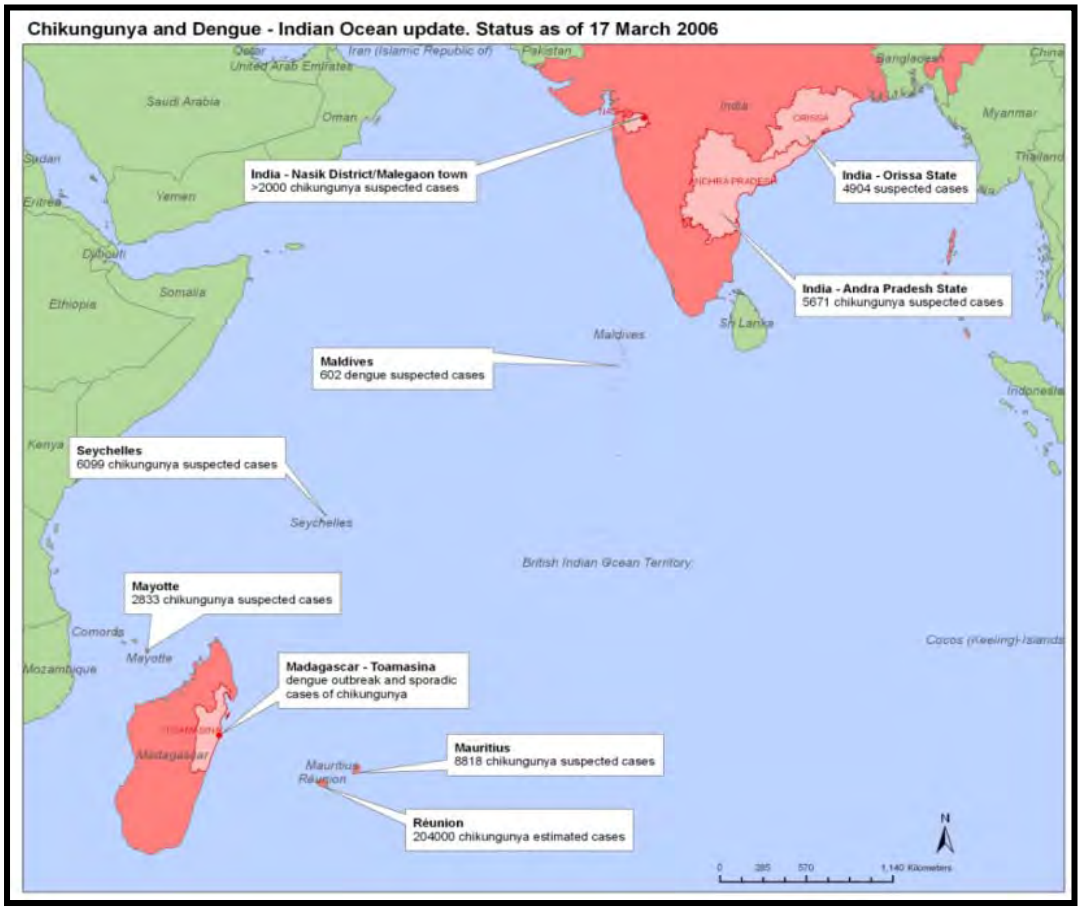


Figure 20. Co-infection of dengue and chikungunya virus distribution in Toamasna on Madagascar's east coast and Indian Ocean. red colour is country with occurrence of dengue and/or chikungunya, pink colour is affected area, red dot is affected city, green colour is country (WHO 2006)

Additionally, It is known that insects and crustaceans can carry simultaneous, active infections of two or more viruses without showing signs of disease, but it was not clear whether co-infecting viruses occupied the same cells or different cells in common target tissues. Previously work by Kanthong and her college showed that successive challenge of mosquito cell cultures followed by serial, split-passage resulted in stabilized cultures with 100% of the cells co-infected with Dengue virus (DENV-2) and an insect parvovirus (densovirus) (DNV) [126].

By addition of Japanese encephalitis virus (JE) were tested these hypothesis that stable, persistent, triple-virus co-infections could be obtained by the same process. Using immunocytochemistry by confocal microscopy, they found that JE super-challenge of cells dually infected with DENV and DNV resulted in stable cultures without signs of cytopathology, and with 99% of the cells producing antigens of the 3 viruses. Location of antigens for all 3 viruses in the triple co-infections was dominant in the cell nuclei. Except for DNV, this differed from the distribution in cells persistently infected with the individual viruses or co-infected with DNV and DENV-2 [127]. The dependence of viral antigen distribution on single infection or co-infection status suggested that host cells underwent an adaptive process to accommodate 2 or more viruses. Individual mosquito cells can accommodate at least 3 viruses simultaneously in an adaptive manner. The phenomenon provides an opportunity for genetic exchange between diverse viruses and it may have important medical and veterinary implications for arboviruses[126].

Although dual infection between arboviruses are illustrated but the symptoms of chikungunya are also difficult to distinguish and may be misdiagnosed from those of dengue fever especially in Thailand that dengue endemic [128-131]. Thus the differential diagnosis of these two viral infections is essential for clinical management and virus–vector relationships are very important to study.

Moreover, dual infection with dengue and chikungunya can possibly change clinical spectrum of the disease; consequently, specific treatment may also be affected [69]. Therefore, the aims of this project focus on dual infection between dengue and chikungunya viruses in laboratory and to study virus–vector relationships of *Ae.*

aegypti and *Ae. albopictus* mosquitoes. To support our studies, we used *Ae. aegypti and Ae. albopictus* mosquito and *Aedes albopictus* (C6/36) cell line as a model of viral infection and transmission *in vitro* and *in vivo* respectively.

D-RT-PCR, real-time RT-PCR, plaque assay and specifically antibody was applied for determine viral concentration and viral particle in *Aedes* spp. and Ae. albopictus (C6/36) cell culture. The results of this study will give the information about effect of vector-virus interactions and transmission which can be used to predict the outbreak and control of the diseases in the future.

CHAPTER III

MATERIALS AND METHODS

3.0. Source of Materials

Chemicals

Acetic acid	Merck, Darmstadt, Germany
Acetone	BDH AnalaR®, Poole, England
Bovine serum albumin fraction V (BSA)	Research Organics, U.S.A
Bromophenol blue (B8026)	Sigma, St.Louis, MO, U.S.A.
$CaCl_2 \cdot 2H_2O$	Fluka Chemica, Canada
Crystal violet	Bio Basic Inc., Ontario,
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
DMSO	Sigma, St.Louis, MO, U.S.A.
Ethanol	Merck, Darmstadt, Germany
Ethelynediamine trichloroacetic acid (EDTA)	Bio Basic Inc., Ontario,
Formaldehyde	Merck, Darmstadt, Germany
KCl	Sigma, St.Louis, MO, U.S.A.
KH_2PO_4	Merck, Darmstadt, Germany
Magnesium Chloride	Fluka Chemica, Sleeze, Germany
Methanol	Merck, Darmstadt, Germany
Methycellulose	Sigma, St.Louis, MO, U.S.A.
MgCl2·6H2O	Merck, Darmstadt, Germany
NaCl	BDH AnalaR®, Poole, England
NaH_2PO_4	Merck, Darmstadt, Germany
PBS	BDH AnalaR®, Poole, England
Tris-base	Sigma, St.Louis, MO, U.S.A.
Triton X-100	Research Organics, U.S.A
xylene cyanol(X4126)	Sigma, St.Louis, MO, U.S.A.

Culture Media and Reagents

Dulbecco's Modified Eagle's medium (DMEM)	Hyclone, Logan, Utah, USA
Fetal bovine serum	GIBCO BRL, Maryland, USA
HEPES	Hyclone, Logan, Utah, USA
HyQ® MEM/EBSS NEAA Modified (MEM)	Hyclone, Logan, Utah, USA
L-glutamine (100X)	Hyclone, Logan, Utah, USA
MEM alpha medium	Hyclone, Logan, Utah, USA
Penicillin / Streptomycin	Hyclone, Logan, Utah, USA
Sodium pyruvate	Hyclone, Logan, Utah, USA
Trypsin (1X)	Hyclone, Logan, Utah, USA

Miscellaneous Materials

ImmunoPure® Goat Anti-Mouse IgG, (H+L) peroxidase conjugated	Santa Cruz Biotechnology, California, USA
Propidium iodide (PI)	Santa Cruz Biotechnology, USA
100 bp DNA ladder marker	Fermentas, Maryland, USA
Seakem L.E. Agarose	SeaKem LE, BMA, USA
Trypan blue dye	GIBCO BRL, Maryland, USA
DAB	DAKO Corporation, CA
DAPI	DAKO Corporation, CA
Secondary antibody conjugated with FITC (F-2761)	DAKO Corporation, CA
Cell scraper	Hyclone, Logan, Utah, USA

Cell Lines

- C6/36 : cell line derived from whole body clone number C6/36 of *Aedes albopictus* (mosquito) larva
- LLC-MK_2 : kidney cell line from rhesus monkey epithelial cell line
- Vero : kidney cell line from Cercopithecus aethiops (African green monkey)

Virus

- DENV-3 virus strain
- CHIK virus strain

3.1 Culture Medium

C6/36 : HyQ® MEM/EBSS NEAA Modified (MEM∞), 10% FBS, 1% L-glutamine,100 units/ml penicillin and 100 μg/ml streptomycin

LLC-MK_2 : Dulbecco's Modified Eagle's medium (DMEM), 10% fetal bovine Serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin

Vero : DMEM, 5% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin

3.1.1 Freezing Medium

1x filtered MEM with 1% P/S and 1% L-glutamine	50%
FBS	40%
DMSO	10%

3.1.2 Crystal Violet 1% (w/v) in 10% Ethanol

3.1.3 Fixative for plaque assay 3.7% Formaldehyde in PBS

3.2 Agarose Gel Electrophoresis

The agarose powder was melted in TAE buffer (see Appendix A) until homogeneous at final concentration 1.0% agarose gel solution. The gel solution was set in an electrophoretic chamber and submerged in TAE buffer. DNA samples were mixed with loading dye and electrophoresis was performed at 100 V for 60 mines followed by staining with ethidium bromide before being photographed under UV illumination.

3.3 Buffer for viral infection

- PBS 0.15 M (pH7.4) For 1 liter

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g

These chemicals were mixed; well dissolve in distilled water, adjust to pH 7.2 with HCl and adjust to 1,000-ml final volume with distilled water. Sterilize by autoclaving for 15 min at 121°C, 15 lb/square inches.

- Acid Glycine For 500 ml

NaCl	4.00	g
KCl	0.19	g
$MgCl_2 \cdot 6H_2O$	0.05	g
$CaCl_2 \cdot 2H_2O$	0.05	g
Glycine	3.75	g

Adjust pH to 3.0 with HCl and autoclave

- BA-1 Diluent For 100 ml solution

10x M199E (filtered)	10	ml
1M Tris-HCl (pH7.6)	5	ml
BSA fraction V	1	g
100x Penicillin/Streptomycin	1	ml
7.5% NaHCO3	1	ml
Tissue culture graded dH_2O to	100	ml

3.4 Mosquito maintenances

The adults female *Ae. albopictus* and *Ae. aegypti* mosquitoes were collected from field and maintenance until F4 in insectariums at Medical Entomology department, Tropical Medicine, Mahidol University, Thailand. These female mosquitoes were collected after emerging 3-7 days. One thousand of female *Ae. albopictus* mosquito were starved overnight prior to blood feeding. The mosquitoes were collected by using insect aspirator to paper cup, 30 individuals per cups. The cup are covered with fine nylon netting and secured with rubber bands. After infection fully engorge mosquito collected into a new cup and give 10% sugars and maintenance at 25^{o}C until further studies. Each paper cup might keep in cage to protect ourselves.

3.5 Intrathoracic inoculation of mosquitoes

The *Ae. aegypti* lavae 4 stage was isolated and maintenances one by one in plastic cup. After mosquito emerged, female mosquitoes were identified and maintenances separately with male *Ae. aegypti*. Three to five days-old adults female *Ae. aegypti* were inoculate with DENV-3 and CHIKV by intrathoracic injection according to the method that described by Rosen and Gubler. Adults female were maintained after inoculums at 25°C and provided with 10% sucrose as nourishment. Live mosquitoes were frozen at -20°C on days 1, 3, 5, 7, and 9. A group of mosquitoes that had died 3 days post inoculation were frozen and determine viral propagation. Uninfected groups, female *Ae. aegypti* were reared at the same time of infected group and inoculated with culture media and maintenance parallel with trial group. These groups used for negative control.

3.6 Artificial membranes feeding or Oral feeding

Fresh blood are mixed with Chikungunya virus (infected culture fluid) or dengue virus together, total volume 2 ml at MOI = 0.1, 1.0 before presenting to the mosquito by using an artificial membrane feeding technique (Figure 3). Negative controls feeding were included as follows: Blood and clean C6/36 cell. DENV or Chikungunya viruses from C6/36 cell culture were the positive control (Figure 2). Only fully engorged females was collected into cage and maintained for 7-14 days at 25^{o}C for determine the virus replication in the body of mosquitoes by D-RT-PCR and IFA.

3.7 Dengue and chikungunya virus strains

Dengue virus strains that used in this study are maintenance in laboratory, Chulalongkorn University, Thailand. This species was supported by Dr. Kiat Ruxrungtham (Professor of Medicine. Head, Allergy and Clinical Immunology Division, Department of Medicine, Faculty of Medicine, Chulalongkorn University) and Dr. Wanla Kulwichit (Professor Assistant, M.D.). This species is originating from AFRIMS, Thailand.

Chikungunya virus that used in this study was originally obtained from the Division of Virology, NIH, Thailand and propagated in *Ae. albopictus* C6/36 cell cultures after passage every 14 day until viral production present a high titer by plaque assay. Viral production was collected and aliquot into 1.5 ml cryovial tube and kept in -80^{o}C until used.

3.8 Cell culture

Aedes albopictus mosquitoes (C6/36) cells were cultivated at 28^{o}C in MEMα supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone), 100 U/ml of penicillin-streptomycin (HyClone), 10% HEPES (HyClone), 20% L-glutamine (HyClone) and 0.1 mM non-essential amino acid (HyClone).

Vero cell line (African green monkey kidney cell) were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah) containing 10% heat-inactivated FBS, 10% HEPES (HyClone), 20% L-glutamine (HyClone), 110 mg/ml of sodium pyruvate (HyClone) and antibiotics under an atmosphere of 5% CO_2 at 37^{o}C.

LLC-MK_2 (Rhesus monkey kidney cell) were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, Utah) containing 10% heat-inactivated FBS, 10% HEPES (HyClone), 20% L-glutamine (HyClone), 110 mg/ml of sodium pyruvate (HyClone) and antibiotics under an atmosphere of 5% CO_2 at 37^{o}C.

3.9 Viral propagation

The *Aedes albopictus* mosquitoes (C6/36) cells line were seed in complete MEMα (pH= 6.4) medium at concentration 3.0 x 10^6 cell/flask that containing 10% heat inactivated FBS. The monolayer cultures were grown in plastic 75 cm^2/flasks, at 28°C, 72 h. Discard media and wash cell line with PBS (pH 7.4) three time. Numbers of total cell survival were calculated by tryphan blue (Appendix C). Each virus (dengue or chikungunya virus) were mixed with 3 ml MEMα (without FBS) and infected to the monolayer C6/36 cell line separately at MOI=1, 120 min (Shaking every 10 min), at 28°C. Added 22 ml of fresh complete MEMα (pH= 6.4) and maintained at 28°C for 7-14 days. Collected supernatant and kept at -80°C until used for identify viral titer by plaque assay

3.10 Viral titer

The LLC-MK_2 cell monolayer were grown in 6 well plates at 4 x 10^5 cell/ml in 3 ml of complete DMEM medium with 10% heat-inactivated FBS under an atmosphere of 5% CO_2 at 37^{o}C, 24 h. The media was discarded and washed cell with PBS three times. Each virus (dengue or chikungunya virus) was diluted with BA-1 solution and 200 µl of 10-fold serial dilution were infected on LLC-MK_2 cell separately, 90 min, at 37 ℃, 5% CO_2, (Shaking every 10 min). Overlay 1% methylcellulose in fresh DMEM media on each well. After 30-60 min until methylcellulose stable, incubated plate 7 days at 37 ℃ in 5% CO_2. Fixed cell with 3.7% paraformadehyde in PBS after 7 days later and staining with 2% crystal violet. Counting plaque number and calculation plaque performing unit (PFU/ml) after staining with crystal violate (see Appendix D).

3.11 Viral infection in C6/36 cell line

Confluent monolayers of C6/36 cell line were inoculated with DENV or CHIKV at either high or low multiplicities of infection (MOI) 0.1 to 1 or 1x10^4 or 1x10^5 PFU per cell, respectively. After 2 h of adsorption at 28℃, the inoculate were removed by acid glycine buffer (137 mM NaCl, 5 mM KCl, 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.68 mM $CaCl_2 \cdot 2H2O$, 99.84 mM glycine, pH3.0) (see Appendix B) for 1 min to inactivate any uninternalized extracellular viruses and cultures were washed three times with PBS buffer [94]. Maintenance media were replaced, and cultures were incubated at 28℃ in incubator. At 24 to 72 h intervals, triplicate samples of infected C6/36 cell line in culture medium were frozen at -80℃ until assay. All samples were assayed at the same time in 24-well panels with vero cell line monolayer by the plaque formation method.

3.12 Examination virus infection by 1- step duplex reverse transcription polymerase chain reaction (D-RT-PCR)

The genomic viral RNA was extracted from 150 µl of infected C6/36 cell culture fluid with an unknown viral titer by using the Nucleospin® RNA II kit (Macherey-Nagel) according to the manufacturer's protocols. The viral RNA was eluted from the Nucleospin® columns in a volume of 50 µl of elution buffer and stored at−80 ^{o}C until used. The viral RNA was use for one-step RT-PCR was performed by SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) in a volume of 25 µl containing 5 µl RNA template, 12.5 µl (2X) Reaction Mix, 0.5 µl sense (2.5 µM), 0.5 µl anti-sense (2.5 µM), 1 µl SuperScript III RT/ Platinum *Taq* Mix and 5.5 µl of ddH_2O.

Primers CHIKV were selected in the *E1* structural protein regions;

sense-F3 (CACCGCCGCAACTACCG) and

anti-sense B3 (GATTGGTGACCGCGGCA).

DENV-3 primer was used nucleotide sequence on *C-prM* gene

DEN-R: 5'-TCAATATGCTGAAACGCG(ACT)GAGAAACCG-3'

DEN-F: 5'-TTGCACCA(AG)CA(AG)TC(AT)ATGTC(AT)TCAGGTTC-3'

These primers were described by Dash et al., 2008. The amplification program included: a reverse transcription at 55^{o}C for 30 min, an activation step of the polymerase at 94^{o}C, 2 min followed by 40 cycles of 30 s at 94^{o}C and 1 min at 55^{o}C, 72^{o}C 30 s, a step at 72^{o}C 30 s, with a final amplifying of 72^{o}C 10 min. RT-PCR product were analyzed on 1.0% agarose gel electrophoresis.

The positive results from D-RT-PCR were purified and sequenced using specific primer for *E1* gene or *C-prM* gene of CHIKV and DENV-3 respectively. Database homology searching was done by using Blast 2.2.22. Multiple alignment sequence alignments were done using CLUSTAL W online software and compared the percentage of homologies between sequences by using Bioedit software.

3.13 Quantitative RT-PCR (real time RT-PCR)

Infected female mosquitoes were grind in 1.5 ml appendorf tube for RNA extraction in lysis buffer following Nucleospin® RNA II kit (Macherey-Nagel) manufacturer's protocol. The SYBR Green technology was used to quantify the amount of virus in each sample. The one-step RT-PCR was performed with a Power SYBR® Green RNA-to-CT™ one step kit (Applied Biosystem) in a volume of 25 µl containing 2 µl RNA template, 12.5 µl 2X Power SYBR® Green I RT-PCR Mix , 0.25 µl sense (2.5 µM), 0.25 µl anti-sense (2.5 µM), 0.2 µl RT enzyme mix and 9.8 µl of ddH_2O. Primers were selected in the E1 structural protein regions of sequences retrieved from the

GenBank database; sense-F3 (CACCGCCGCAACTACCG)

anti-sense B3 (GATTGGTGACCGCGGCA).

Specific primer to dengue virus were selected in *C-prM* gene;

DEN-R: 5'-TCAATATGCTGAAACGCG(ACT)GAGAAACCG-3'

DEN-F: 5'-TTGCACCA(AG)CA(AG)TC(AT)ATGTC(AT)TCAGGTTC-3'.

The amplification program in an Applied Biosystem 7700 real time PCR system included: a reverse transcription at 55°C for 30 min, an activation step of the polymerase at 94°C, 2 min followed by 40 cycles of 30 s at 94°C and 1 min at 55°C, 72°C 30 s, a step at 72°C 30 s, with a final ramping of 72°C 10 min. The size of the amplification product was 217 bp. A standard curve was generated using duplicates of 10-fold serial dilutions of RNA synthetic transcripts. Quantification of viral RNA was done by comparison of the threshold cycle (Ct) values of the samples to the standards according to the ΔCt analysis (see Appendix E).

3.14 Immunocytochemistry staining (ICC)

At designated time intervals after infection, monolayer (C6/36 cell line and vero cell line) on cover glass within 24-well panels were examined for 90% of cytophatic effect (CPE). Phosphate-buffered saline (PBS) were used for wash old media and then fixed cell line in freshly prepared cold acetone for 5-10 min. After being fixed, cultures were rinsed in PBS and then treated with 0.25% Triton X-100 in PBS for 10 min at room temperature. Monolayer were again rinsed in PBS, placed in 20% glycerol-PBS, and frozen at -80°C until staining.

Frozen samples of infected C6/36 cell line and vero cell line monolayer in 24-well panels of the same input multiplicity and time after infection were thawed at 37°C, rinsed three times with PBS, and assayed according to the procedure immunocytochemistry, we used specificity primary complex antibody to DENV to examine dengue virus position in C6/36 cell line. Uninfected or control were treated for ICC as described previously. Infected cell lines on cover glass were washed with PBS buffer and incubated with 2% bovine serum albumin at 4 ^{o}C for 30 min to protect non specific protein binding. After staining with primary antibody to dengue virus 1: 200, Fast 3,3' Diaminobenzidine (DAB) (Sigma) was used to reveal the staining and foci were counted staining and visualized under microscope.

3.15 Indirect immunofluorescence assay (IFA)

Fully engorged females mosquitoes were maintained at 25 ^{o}C for 14 day (p.i.) and keep in -80 ^{o}C until used. Head of female mosquitoes were tested for the presence DENV-3 by IFA on their head squashes. Head squashes being performed by fixing with cold acetone 5-10 min, washed with PBS and blocking non specific protein with 1% BSA, 1 h. DENV-3 antigens were detected with ratio 1:200 of primary specific antibody to dengue virus and 1: 50 of secondary anti-mouse labeling with FITC for 30 min, washed with PBS. After final wash in PBS 1X, DAPI was used to reveal the staining and foci were counted under Phase contrast Fluorescence microscope (Nikon, company). Mosquitoes inoculated with CHIKV and DENV-1 was used as positive controls, negative controls were inoculated with cell culture media.

3.16 Statistically analysis

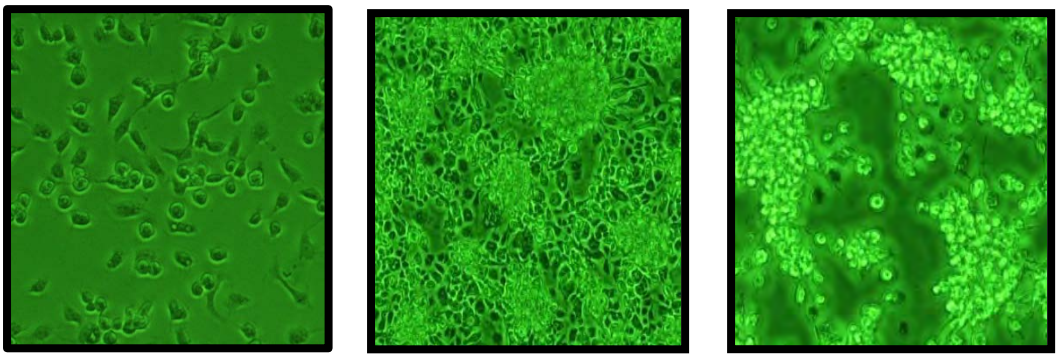
Comparison the viral replication in single infection relative to mixed infections and evaluate which viruses give a high titer rate in mixed infection, it would be suppressed each other. The viral titer in super infection used for predicted the effect of mixed infection in C6/36 cell line. The viral titer was evaluated by plaque assay and calculated number of plaque performing unit per milliliter (PFU/ml) by following the PFU formula.

CHAPTER IV

RESULTS

4.1 Virus propagation

Aedes albopictus (C3/36) is an insect cell line that was isolated clones from larvae *Ae. albopictus* mosquitoes by Igarashi A. in 1978. These cell lines are widely used for viral propagation such as Japanese encephalitis, Yellow fever virus, Bunyamwera virus, shrimp yellow head virus and shrimp Taura syndrome virus. In addition to C6/36 is sensitive to dengue or chikungunya viruses' infection and replication (Igarashi, 1978). The viral infection was showed in Figure 5 and collected the supernatant after present 90% of CPE. The supernatant was used for identify viral titer by plaque assay.



(A) Non infection (20X) (B) DENV infection (20X) (C) CHIKV infection (20X)

Figure 21. Virus infection in C6/36 cell line presented 90% CPE; (A) Non infection C6/36 cell line (20X), (B) DENV infection in C6/36 cell line (20X), (C) CHIKV infection in C6/36 cell line (20X)

4.2 Localization of dengue virus infection in C6/36 cell culture by Immonocytochemistry staining

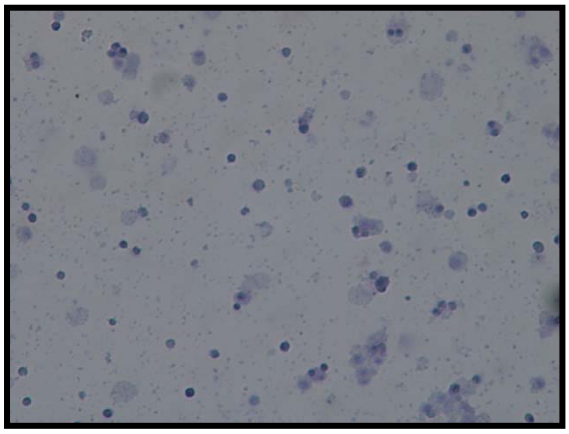
Dengue virus was infected into C6/36 for 1 h, washing and added with complete fresh media following incubation at 28 ^{o}C until collected. These cell lines were applied for dengue virus antibody according to procedure. Evaluate C6/36 cell lines under microscope, normal cell line was stained with blue colour by hematoxylin and lithium chloride and infected cell were present brown colour (Figure 22B and 23).

DENV-3 is infected C6/36 cell line and replicated in cytoplasm. Figure 23 was showed DENV-3 viral antigen binding with specifically antibody that derived from mouse. Secondary anti-mouse IgG is labeled with HRP (Horseradish peroxidase) that using DAB as a substrate. This substrate are developed brown colour that presented in Figure 22 and 23.

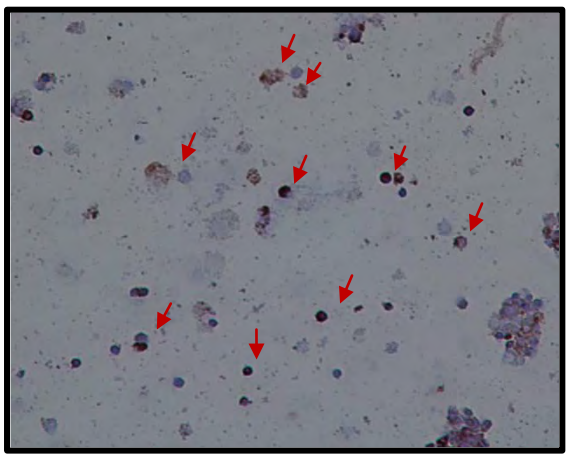
This technique uses HRP-labeled streptavidin. The secondary antibody is conjugated with biotin and the streptavidin-HRP complex reacts with the biotin on the secondary antibodies. The resulting biotin-avidin-HRP complex can react with the primary antibody bound to the specific epitope of the target antigen. The HRP enzymes of the streptavidin complex then catalyze the substrate/chromogen reaction to form a colored reaction product (brown to black when using DAB as the chromogen) at the antigen site. A biological stain is then used to visualize the DENV infection in C6/36 cell line.

In Figure 22, DENV-3 infection in C6/36 cell line presents brown colour in nuclear cellular and perinuclear cellular comparative to MOCK infection. These results conclude that DENV-3 infected C6/36 cell line by pass through cell membrane and replicated in inside nuclear membrane (Figure 23). These figures used acetone fixative 30 min. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents (such as paraformaldehyde) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require the addition of a permeabilization step, to allow

access of the antibody to the specimen. Fixation with both methods may denature protein antigens, and for this reason, antibodies prepared against denatured proteins may be more useful for cell staining.



(A) Mock



(B) C6/36 infected with DENV

Figure 22. The localization of dengue virus infected. (A) Negative control, (B) C6/36 infected with DENV; red arrow is positive DENV infection (20X)

DENV-3 replicated into *Aedes albopictus* C6/36 cell line

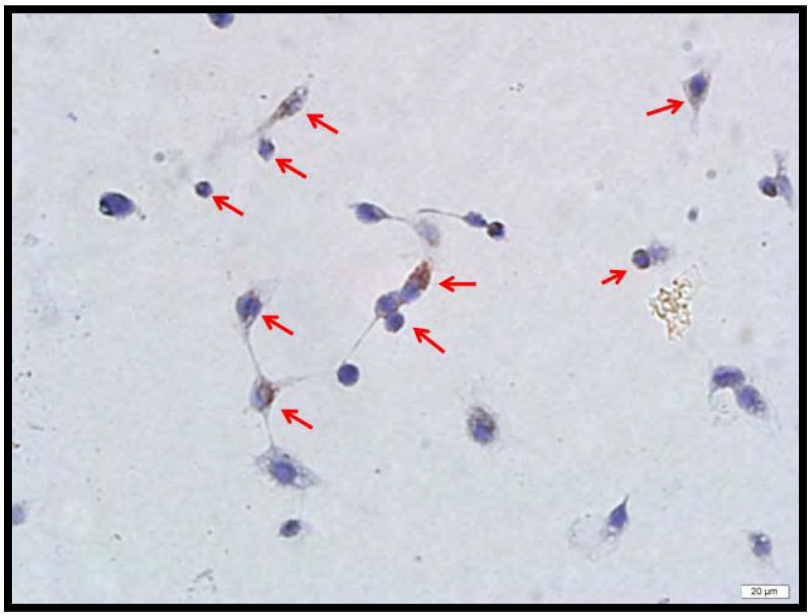


Figure 23. The localization of dengue virus is assembly in to cytoplasm. Red arrow is present DENV-3 replication in C6/36 cell line.

4.3 Plaque assay; calculation PFU/ml

Viruses were propagated in C6/36 cells, and after incubation at 28°C for 7 days, the cellular supernatant was clarified by centrifugation, supplemented were collected, and stored at -80°C until further studies of viral titer by plaque assay. For plaque assays, monolayers of LLC-MK_2 cells were grown to 90 to 95% confluence in twenty four-well plates and were inoculated in duplicate with 200 µl of cellular supernatant containing serial dilutions of the viral stock. After 2 h at 37°C, the cells were overlaid with MEM containing 1% Methycellulose (Sigma) and 2% FBS and were incubated for 7 days at 37°C in 5% CO_2. The cells were then fixed in 3.7% formadehyde for 2 h and stained with a solution containing 0.27% crystal violet. Calculate number of plaque forming unit followed the formula that describ as belows.

$$\text{PFU /ml} = \frac{\#\ \text{plaques}}{d \times V}$$

= number of plaque

d = dilution factor that found viral plaque

V = volume of diluted virus added to the well

The results of unknown concentration of DENV-3 was showed 42 plaques on the plate made from the 10^{-4} dilution. The titer of the virus stock is therefore 2.1 x 10^6 PFU/ml.

In the CHIKV sample was showed 34 plaques on the plate made from the 10^{-3} dilution. The titer of the virus stock is therefore 1.7 x 10^5 PFU/ml.

4.4 Determine DENV-3 and CHIKV infected C6/36 cell line in order to single infection, mixed infection and super-infection by D-RT-PCR

In these experiments, the supernatant of virus infection was collected following table 7. The result of dengue and chikungunya virus mixed infection equal titer was shown both of dengue and chikungunya virus particles in the same culture by D-RT-PCR analysis (Figure 25) and two viruses can be replicated in C6/36 cell line at the same time (Figure 25. Lane 6). According to mixed infection between DENV-3 (MOI=0.1) lower titer than CHIKV (MOI=1.0) was found positive both of dengue and chikungunya virus particle in the same sample (Figure 25. Lane 8). In contrast, mixed infection between DENV-3 (MOI=1.0) higher titer than CHIKV (MOI=0.1) were shown positive results only DENV-3 particle (Figure 25. Lane 7).

Serial infection (super-infection) with DENV-3 (MOI=1.0) was applied by infected virus in C6/36 cell line, 1 h and removed this virus by acid glycine buffer (pH 3.0) and washing with PBS buffer (pH 7.4) following infected with CHIKV (MOI=1.0) after 1 h of viral infection, removed virus and added fresh media following incubation at 28^{o}C until collected. After sample collected the viral was used for RNA extraction and the results was demonstrated positive signal of both dengue and chikungunya virus (Figure 25. Lane 9). Alternatively, using CHIKV (MOI=1.0) infection 1 h and washing with PBS buffer (pH 7.4), following DENV-3(MOI=1.0) infection but D-RT-PCR results also represented both of virus that similarity to mix infection with DENV-3 and CHIKV at the same time (Figure 25. Lane 10). We also increased the viral titer 10 fold at ratio 1:10 (MOI=1.0 and 10.0) to infection. D-RT-PCR results was displayed both positive results even though we mixed virus together before infectious or vary serial infection (Figure 24). These data was supported DENV-3 and CHIKV infection depend on amount of viral not related to serial infection thus two virus might be infected host cell at the same time if they have a highly virulent.

4.4.1 Determine viral RNA replication in culture media of C6/36 cell line in order to single infection, mixed infection and super-infection by D-RT-PCR at MOI 1: 10

Mixed-infection that use in this figure is 10-fold ratio and introduced to C6/36 cell line. The result was show two virus infection in cell culture at the same time. The viral concentration is increase compare with single infection control.

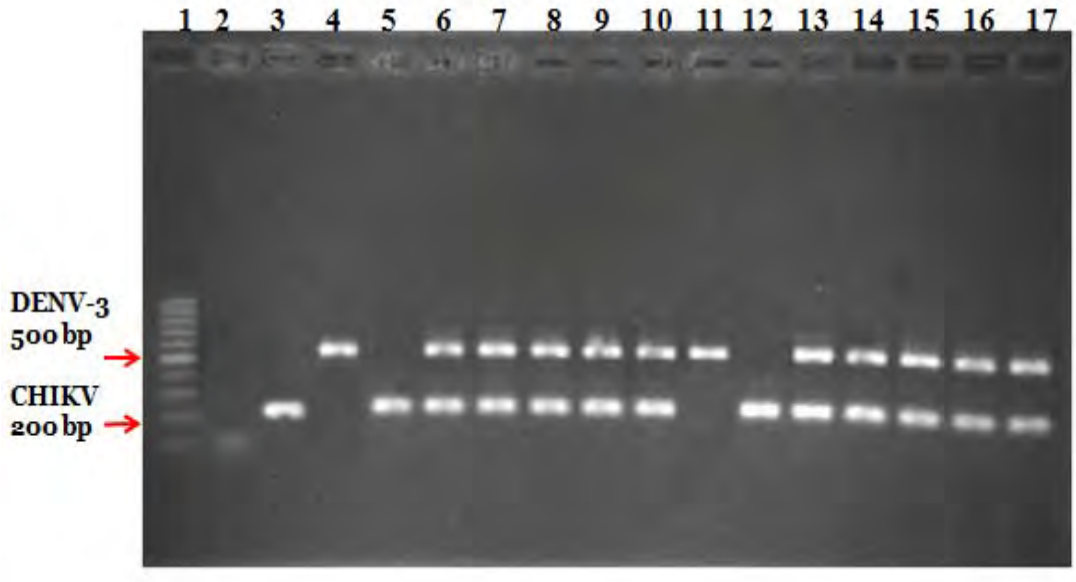


Figure 24 The suprainfection of D-RT-PCR amplicons. Lane 1, 100-bp DNA ladder (Fermentas, Glen Burnie, MD); lane 2, Negative control; lane 3, positive control CHIKV; lane 4, DEN-3 single infection (moi=1); lane 5, CHIKV single infection (moi=1); lane 6, mixed infected with CHIKV (moi=1) and DENV (moi=1); lane 7, mixed infected with CHIKV (moi=1) and DENV (moi=10); lane 8, mixed infected with CHIKV (moi=10) and DENV (moi=1); lane9, infected with DENV (moi=1) following CHIKV (moi=1) ;lane 10, infected with CHIKV (moi=1) following DENV (moi=1), lane 11-17 is duplicated sample similarity to lane4-10, respectively.

Table 6. The results of dengue and chikungunya virus infected in *Aedes albopictus* (C3/36) cell culture.

After 7 days post infections, the supernatant of each experiment were collected and evaluated by D-RT-PCR. The data show positive RT-PCR product from mixed-infection and super-infection trial. These results may be led to high concentration amount two viruses. We decided to reduce the virus concentration 10 fold at ratio MOI = 1:0.1, the conditions are present in table 7.

Viral infected in *Aedes albopictus* (C3/36) cell	**MOI***	**D-RT-PCR**	
		DENV	**CHIKV**
DENV-3	1.0	+	-
CHIKV	1.0	-	+
DENV-3 + CHIKV	1.0 + 1.0	+	+
DENV-3 higher than CHIKV	10 + 1	+	+
DENV-3 lower than CHIKV	1 + 10	+	+
DENV-3 infected following with CHIKV	1.0, 1.0	+	+
CHIKV infected following with DENV-3	1.0, 1.0	+	+

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

+ Positive results, - Negative results

Table 7. The results of dengue and chikungunya virus infected in *Aedes albopictus* (C3/36) cell culture.

The supernatant of each experiment were collected at 7 day post infection and evaluated by D-RT-PCR. The results explain the virus concentration at MOI = 1: 0.1, mixed-infection between DENV-3 lower than CHIKV and superinfection were clarified the positive RT-PCR product with DENV-3 and CHIKV infection. In contrast, mixed infection between DENV-3 higher than CHIKV present unexpectedly results which involve to competitive suppression by DENV-3 as a results of CHIKV cannot be replicated and evaluated in this sample. The data were described as below.

Viral infected in *Aedes albopictus* (C3/36) cell	**MOI***	**D-RT-PCR**	
		DENV	**CHIKV**
DENV-3	1.0	+	-
CHIKV	1.0	-	+
DENV-3 + CHIKV	1.0 + 1.0	+	+
DENV-3 higher than CHIKV	1.0 + 0.1	+	-
DENV-3 lower than CHIKV	0.1 + 1.0	+	+
DENV-3 infected following with CHIKV	1.0, 1.0	+	+
CHIKV infected following with DENV-3	1.0, 1.0	+	+

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

+ Positive results, - Negative results

D-RT-PCR was evaluated after 7 days post infections, the supernatant of each experiment were collected. The results show the competitive suppression of DENV-3 higher concentration with CHIKV mixed-infection trial (Figure 25 lane 7). Super-infection and other mixed-infection presented dual infection in all experiments.

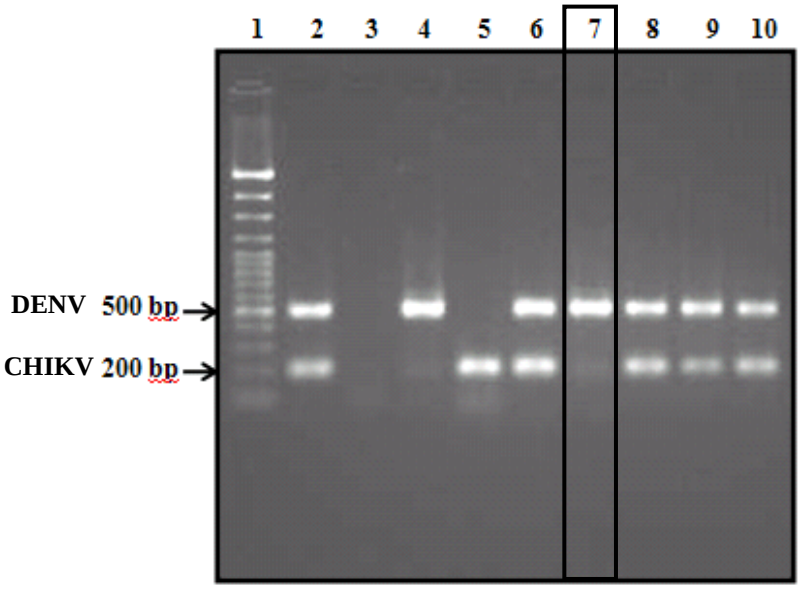


Figure 25. The positive results of dengue and chikungunya mixed-infection was evaluated by D-RT-PCR. Lane 1: 100 bp DNA ladder (Fermentus), Lane 2: Positive control dengue and chikungunya virus, Lane 3: negative control, Lane 4: single infection with DENV-3 virus, Lane 5: single infection with CHIKV, Lane 6: Mixed infection equal titer between DENV-3 and CHIKV, Lane 7: Mixed infection between dengue virus higher titer than CHIKV, Lane 8: Mixed infection between dengue virus lower titer than CHIKV, Lane 9: super-infection with DENV-3 following CHIKV, Lane 10: super-infection with CHIKV following DENV-3

4.5 Orally infection in *Ae. aegypti* and *Ae. albopictus* mosquitoes

1. Virus growth curve in *Ae. aegypti* by intra thoracic injection

Non-fertilized female *Ae. aegypti* mosquitoes were selected in the larvae stage and maintained until emerge. New emerged female five days olds were used for inoculation. Three replicate inoculums were done on separately sample. Three infected mosquito were collected follow the schedule and kept in -80 ^{o}C for further studies. Whole mosquitoes body was extract for viral RNA and D-RT-PCR was amplified the PCR product. 2.0% agarose gel electrophoresis used for separately PCR fragments. Quantity One® 1-D Analysis programs (Bio-rad) were applied to analysis % volume density and linear graph plot between % volume densities per day.

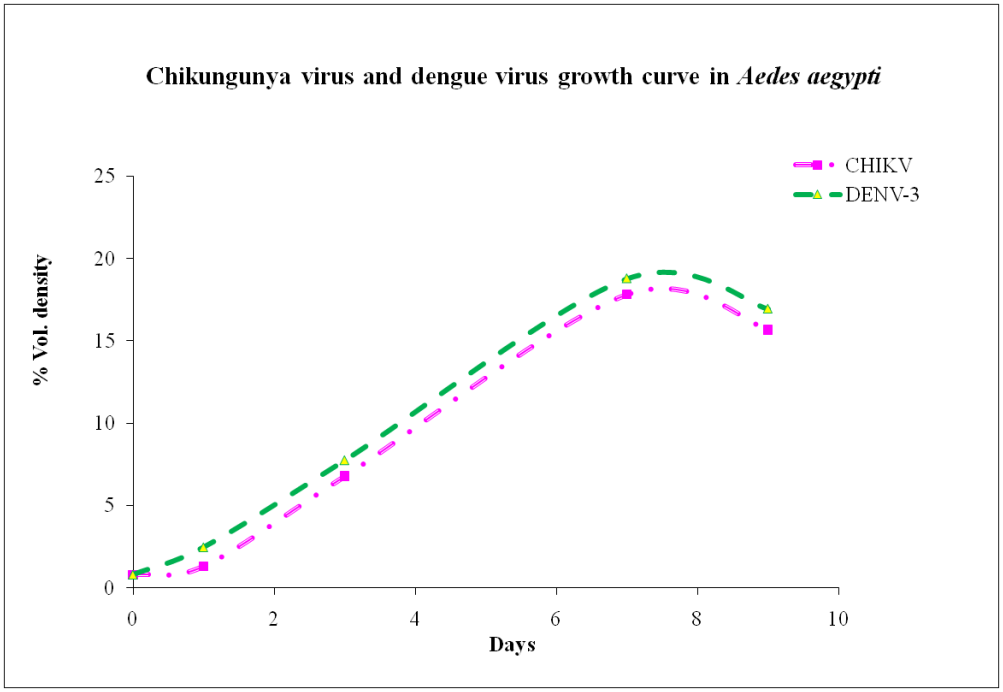


Figure 26. The percentage of dengue and chikungunya virus growth curve was present after calculation of D-RT-PCR product density by Quantity One® 1-D Analysis program (Bio-rad). Chikungunya virus was showed as pink color and green color is dengue virus serotype 3.

2. Co- infection in *Ae. aegypti* and *Ae. albopictus* by oral infection technique

Both of dengue and chikungunya viruses were mixed with blood meal and fed to *Ae. aegypti* and *Ae. albopictus* mosquito by oral infection technique following describe as below. The survival fully engorges mosquitoes were maintenance at 25^{o}C and determined viral replication by D-RT-PCR. Percentage of mosquito survival was calculated from survival rate with describe in Table 8 of *Ae. aegypti* mosquitoes survival 14 (p.i.) and Table 9 of *Ae. albopictus* mosquitoes survival 14 (p.i.).

$$\text{(\%) Survival rate} = \frac{\text{(number of mosquito survive)}}{\text{(number of mosquito tested)}} \times 100$$

D-RT-PCR product show positive result of DENV-3 infection on agarosegel electrophoresis, *C-prM* gene presents at 217 bp. Percentage of virus infection was calculated for comparative the number of virus infection in two mosquitoes. The Percentages of virus infection are derived from the formula that describe as below and the results of two virus co-infection was show in table 10 and 11.

$$\text{(\%) Infection rate} = \frac{\text{(number of virus positive mosquitoes)}}{\text{(number of mosquito infected)}} \times 100$$

The percentage of virus positive mixed infection results from *Ae. aegypti* and *Ae. albopictus* mosquito were present in figure 27 and 28 which comparison between single infection and mixed-infection. Mixed-infection sample was comparative by lower or higher dose infection. For super-infection the data cannot analysis because numbers of feeding mosquitoes with secondary virus are less than control and difficult to present secondary feeding.

Table 8. The number of *Ae. aegypti* mosquito survival after 14 day post infection (p.i.)

Virus feeding	Fully engorge	Number of mosquito survival	Number of death	(%) survival rate
1.Single infection				
-DENV-3	22	22	None	100
-CHIKV	15	14	1	93.33
2. Mixed infection of equivalent titer	52	52	None	100
3. Mixed infection of DENV-3 higher than CHIKV	10	9	1	90
4. Mixed infection of DENV-3 lower than CHIKV	10	10	None	100

The results of single infection showed CHIKV survival 93.33 percentage compare with DENV-3 and Mixed-infection higher DENV-3 than CHIKV are effect to *Ae. aegypti* because it was reduced number of mosquito survival. The mortality rate of mixed-infection in these samples is higher than Mixed infection of DENV-3 lower than CHIKV approximately 10 percent. Mixed-infection of equivalent titer and mixed-infection of DENV-3 lower than CHIKV were present 100 percents of mosquito survival. Data not showed the number of superinfection because rarely number of fully engorged mosquito and did not success to doing double infection.

Table 9. The number of *Ae. albopictus* mosquito survival after 14 day post infection (p.i.)

Virus feeding	Fully engorge	Number of mosquito survival	Number of death	(%) survival rate
1. Single infection				
-DENV-3	17	16	1	94.12
-CHIKV	13	11	2	84.62
2. Mixed infection of equivalent titer	34	34	None	100
3. Mixed infection of DENV-3 higher than CHIKV	13	13	None	100
4. Mixed infection of DENV-3 lower than CHIKV	10	10	None	100

The number of *Ae. albopictus* mosquito survival after 14 day post infection (p.i.) were present in table 9. The mortality rate was found only in single infection. DENV-3 infected *Ae. albopictus* mosquito showed 94.12 percentage of mosquito survival whereas CHIKV infection showed 84.62 percentage. The results was showed CHIKV infection are reduced the mosquito survive and the number of mosquito death are higher compare with DENV-3 infection. Data not showed the number of superinfection because rarely number of fully engorged mosquito and did not success to doing double infection.

Table 10. The number of co-infection in *Ae. aegypti* by D-RT-PCR

Viral infection	Total number of fully engorge	Number of viral Positive	Percentage of virus infection (%)	Number of co-infection in one mosquitoes
1. Single infection				
-DENV-3	22	11	50	None
-CHIKV	14	11	78.57	None
2. Mixed infection of equivalent titer	52			11
-DENV-3		15	28.85	
-CHIKV		39	75	
3. Mixed infection of DENV-3 higher than CHIKV	9			4
-DENV-3		6	66.66	
-CHIKV		4	44.44	
4. Mixed infection of DENV-3 lower than CHIKV	10			2
-DENV-3		3	30	
-CHIKV		6	60	

The results were showed co-infection between dengue and chikungunya virus in one mosquito. These results are led to virus-vector relationship and *Ae. aegypti* is a highly competence that compatible to carrying both of virus. Mixed-infection equal volumes of virus titer are higher number of co-infection round about 11 mosquitoes individual and if we used high virus concentration the number of co-infection are reduced.

Table 11. The number of co-infection in *Ae. albopictus* by D-RT-PCR

Viral infection	Total number of fully engorge	Number of viral Positive	Percentage of virus infection (%)	Number of co-infection in one mosquitoes
1. Single infection				
-DEN-3	16	9	56.25	None
-CHIKV	11	7	63.64	None
2. Mixed infection of equivalent titer	34			None
-DEN-3	-	2	5.88	
-CHIKV	-	9	26.47	
3. Mixed infection of DENV-3 higher than CHIKV	13			None
-DEN-3		4	30.77	
-CHIKV		1	7.69	
4. Mixed infection of DENV-3 lower than CHIKV	10			None
-DEN-3		5	50	
-CHIKV		3	30	

After 14 day post infection did not found the number of co-infection in *Ae. albopictus* by D-RT-PCR. The results may be related to viral-vector competence and the importance mechanism that mosquito used for protect themselves.

This figure 27 showed the number of percentage of viral post infection in *Ae. aegypti* mosquitoes, comparison of single infection, mixed infection and super-infection after 14 day post infection (14 p.i.)

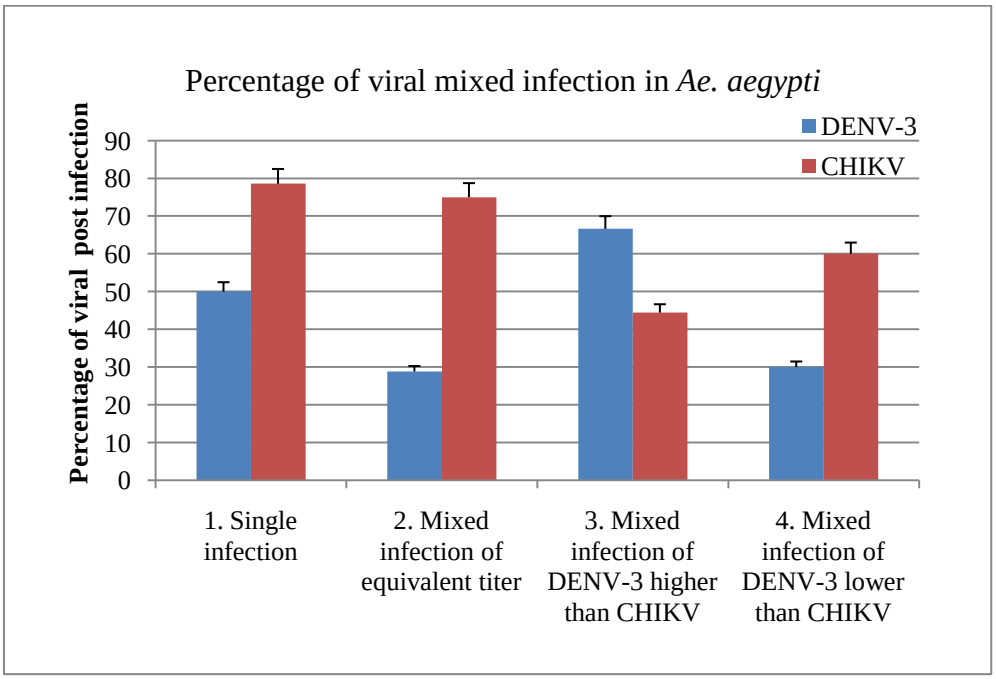


Figure 27. The single infection, mixed infection and super-infection of *Ae. aegypti* mosquito are described under these 2D columns1- 4 , respectively; blue column color is DENV-3 infection and red column color is CHIKV infection.

This figure 28 showed the number of percentage of viral post infection in *Ae. albopictus* mosquitoes, comparison of single infection, mixed infection and super-infection after 14 day post infection (14 p.i.)

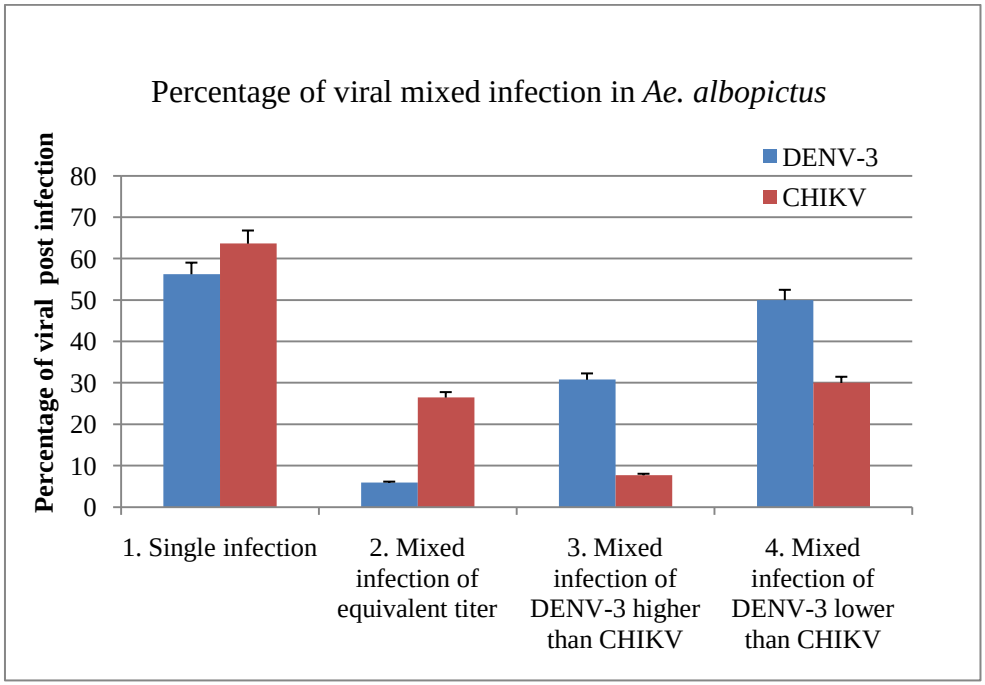


Figure 28. The single infection, mixed infection and super-infection of *Ae. albopictus* are described under these 2D columns 1- 4 , respectively; blue column color is DENV-3 infection and red column color is CHIKV infection.

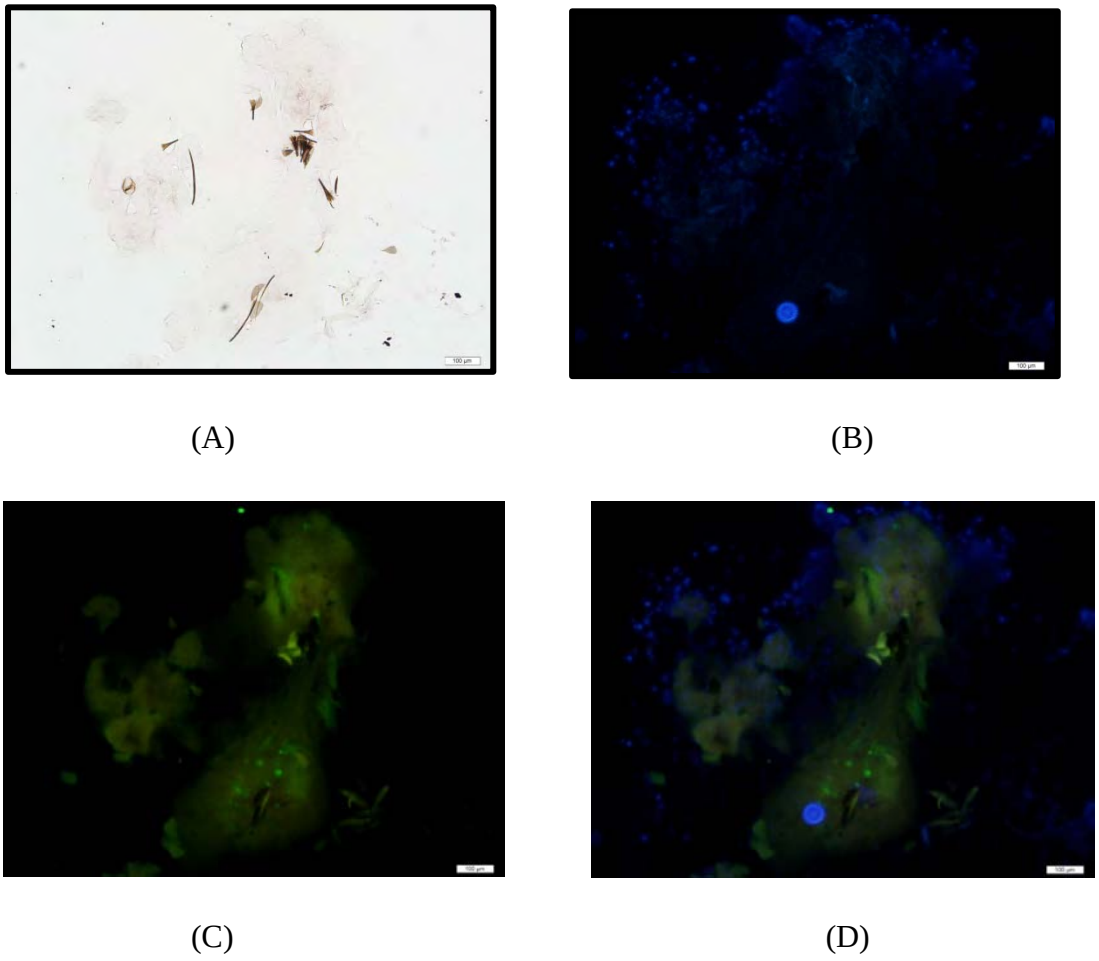
Table 12. The percentage of mosquitoes susceptible to dengue and chikungunya virus

This table was showed the percentage of mosquitoes susceptible to single infection and mixed infection of dengue and chikungunya virus.

Viral infection	***Ae. aegypti***		***Ae. albopictus***	
	DENV-3	CHIKV	DENV-3	CHIKV
1. Single infection	50(22)	78.57(14)	56.25(16)	63.64(11)
2. Mixed infection of equivalent titer	28.85(52)	75(52)	5.88(34)	26.47(34)
3. Mixed infection of DENV-3 higher than CHIKV	66.66(9)	44.44(9)	30.77(13)	7.69(13)
4. Mixed infection of DENV-3 lower than CHIKV	30(10)	60(10)	50(10)	30(10)

4.6 IFA on head squash of *Ae. aegypti* and *Ae. albopictus* mosquitoes

Head of fully engorged female mosquito after 14 day (p.i.) was squash on the slide and applied to IFA staining by randomly. The results IFA was show viral infection on the head parts; thus, we can be concluded that the virus can be replicated and transmitted to another host pass through saliva when they were taken blood meal. These figure 29 showed the position of viral infection on head squash at 20x under Phase Contrast Fluorescence microscope.



(A) (B)

(C) (D)

Figure 29. IFA staining on head squash of fully engorged female mosquito; (A) Bright field (20X), (B) Counter staining of nuclei by DAPI (20X), (C) IFA was presented in green colour (20X), (D) Merged of picture (B) and (C) to reveal viral infectious position (20X).

4.7 Standard curves and amplification efficiencies for real time RT-PCR

4.7.1 Construction plasmid of standard curves for copy number determination

To clone the dengue virus amplicons for internal standard control, dengue viral RNA was amplified with 5’ UTR primers containing the restriction sites *Eco*RI and *Bam*HI, respectively. The products were treated with proteinase K, digested with the appropriate restriction enzymes, gel purified, and ligated with pGEM-T easy Kit following the manufacturing’s procedure. pGEM-T easy vector (Promega) is transform into *E*.coli and blue/white colonies selective marker were applied. The construct a control plasmid with a dengue-3 fragment; 149 bp were extract with DNA extraction kit. The concentration of the plasmid was measured using a fluorometer and the corresponding copy number was calculated using the following equation [132]:

$$\text{DNA (copy)} = \frac{6.02 \times 1023(\text{copy/mol}) \times \text{DNA amount(g)}}{\text{DNA length(dp)} \times 660(\text{g/mol/dp})} \quad \ldots\ldots(1)$$

Ct values in each dilution were measured in duplicate using a real-time QPCR with the *E1* and *C-prM* gene to generate the standard curves for CHIKV and DENV-3 respectively. The Ct values were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by a linear regression of the plotted points. From the slope of each standard curve, PCR amplification efficiency (*E*) was calculated according to the equation [133-134]:

$$E = 10{-}1/\text{slope}^{-1} \quad \ldots\ldots(2)$$

All of viral RNA was extracted and evaluated by One step RT-PCR and quantitative viral RNA by real time RT-PCR. These experiments were detected viral RNA copy following ABI procedure. The masters mixed SYBR from ABI Company were used in each sample. The data was collected by ABI thermocycling and analyzed these data by ABI 7500 software v 2.01. The comparison were calculated from ΔCt value and normalized by standard deviation.

The amplification specificity was checked by both melting curve analysis and gel electrophoresis. Figure 32 shows the melting peaks and the corresponding gel band pictures for the Real-time quantitative PCR (QPCR) products with the DENV-3.

Figure 30 shows the standard equation from QPCR that used for *C-prM* gene calculation

$$Y = 4E+08e^{-0.661x}$$

$$R^2 = 0.9977$$

Figure 31 shows the standard equation from QPCR that used for *E1* gene calculation

$$Y = 1E+07e^{-0.538x}$$

$$R^2 = 0.9953$$

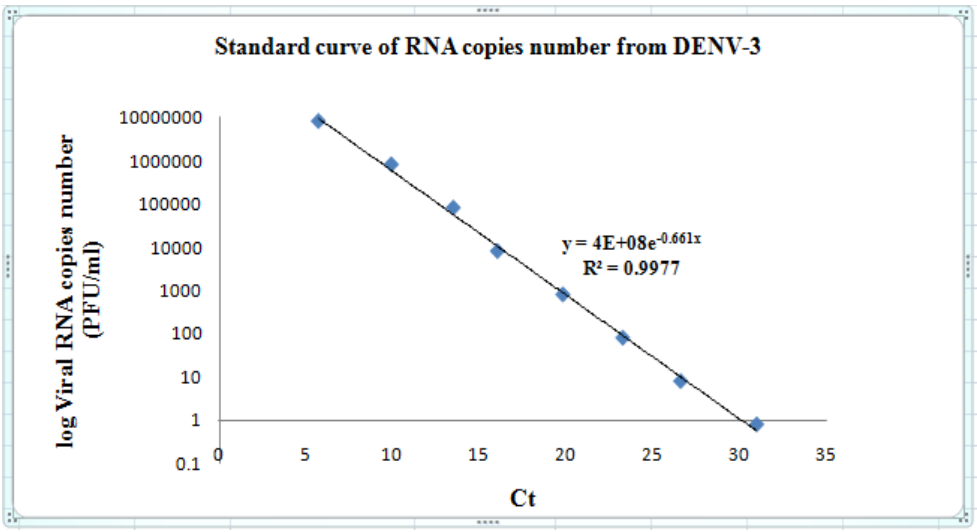


Figure 30. The standard curve and linear formula for DENV-3 calculation

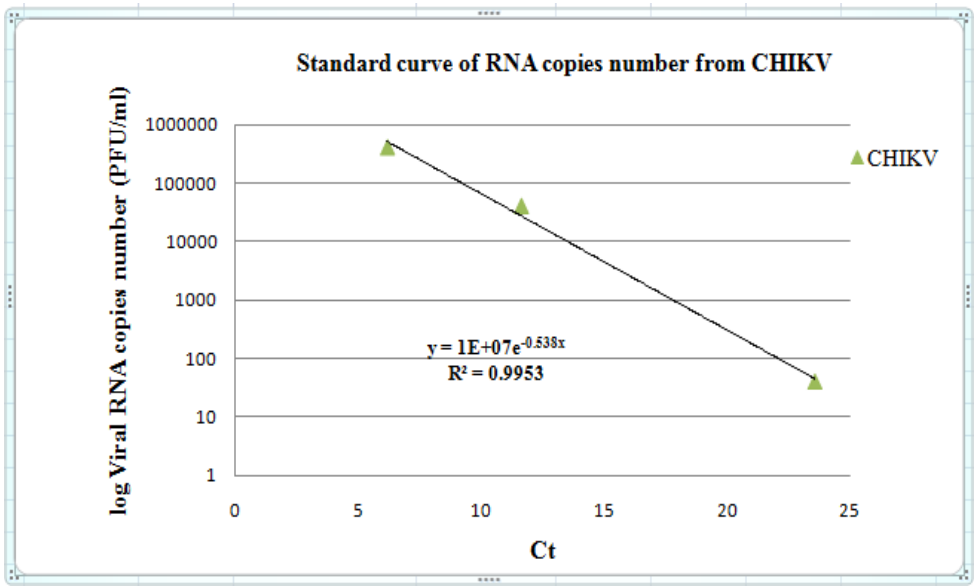


Figure 31. The standard curve and linear formula for CHIKV calculation

4.7.2 Melting curve

SYBR Green I dye binds to double-stranded DNA in a sequence-independent way and emits fluorescence; thus specific and non-specific PCR products are both detected [135]. Therefore, amplification of non-specific PCR products must be avoided for exact quantification [136]. A melting curve analysis is frequently performed to rule out nonspecific PCR [137]. The melting process of double-stranded DNA causes a sharp reduction in the fluorescence signal around the melting temperature (*T*m) of the PCR product, resulting in a clear peak in the negative derivative of the melting curve (−d*F*/d*T*). Therefore, different fragments with different melting temperatures appear as separate peaks [137]. Our results was showed the significally only one peak of melting temperatures at 80.24 ^{o}C (Figure 32)

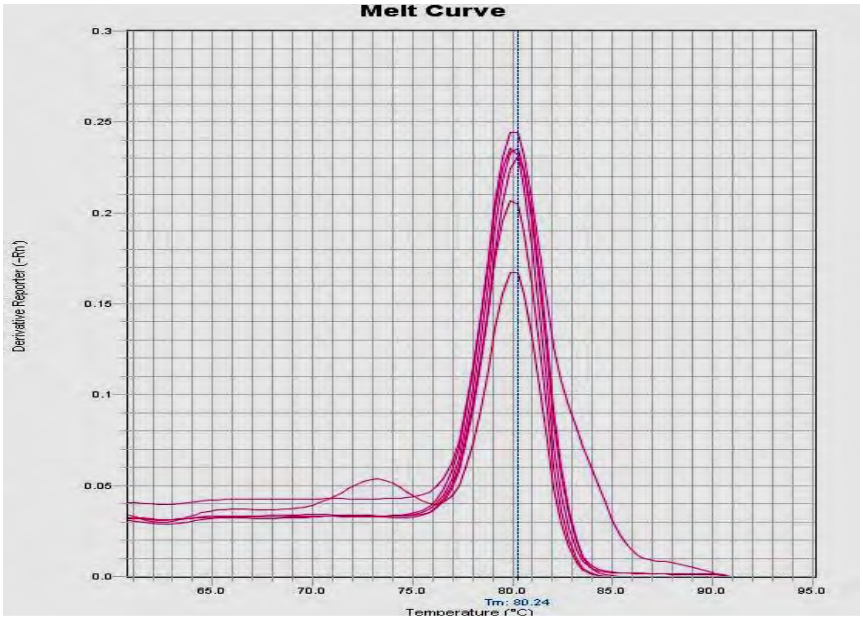


Figure 32. The standard melting curve showed the significally of melting temperature at 80.24 ^{o}C

4.7.3 The standard curves

The standard curves for *C-prM* , each ranging from $1x10^2$ to $1x10^8$ copies/µl, are shown in Figure 33. Both curves were linear in the range tested ($R^2 > 0.99$) by the duplicate reactions. From the slopes, a high amplification efficiency of 0.99 was determined for both *E1* and *C-prM* in the investigated range and was used for relative quantification.

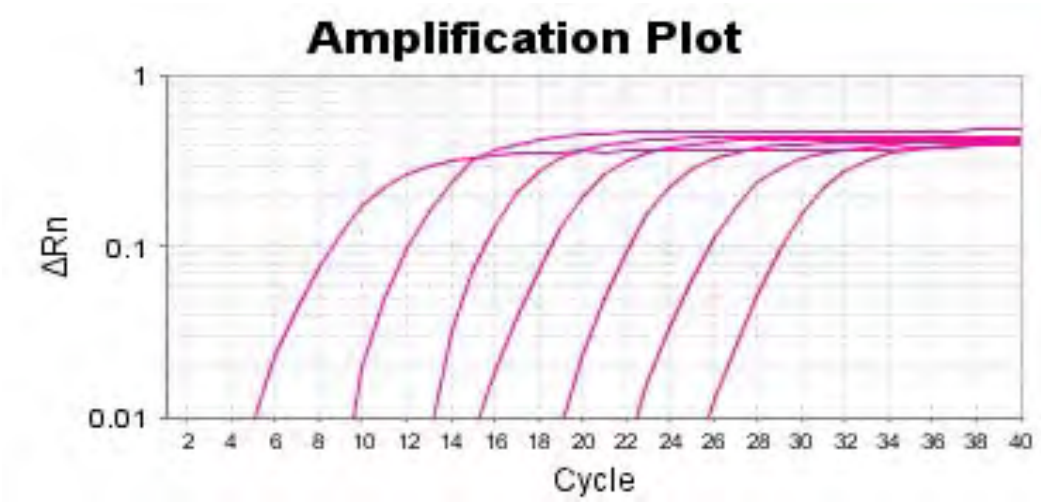


Figure 33. The Amplification Plot between ΔRn and initiate cycle number showed the threshold cycle (ΔCt) of standard trial dilution.

4.7.4 ΔCt calculation

The ΔCt was calculated using each of the 10-fold serial dilutions of the pGEM-T easy insert clone of DENV-3. A plot of the logarithm of initial copy number versus ΔCt was made and the slope of the regression line was close to zero (0.0028). This indicates that the amplification efficiencies of *E1* and *C-prM* are identical enough for the ΔCt calculation [138]. The results for ΔCt value from *Ae. aegypti* and *Ae. albopictus* mosquitoes was show in Figure 34.

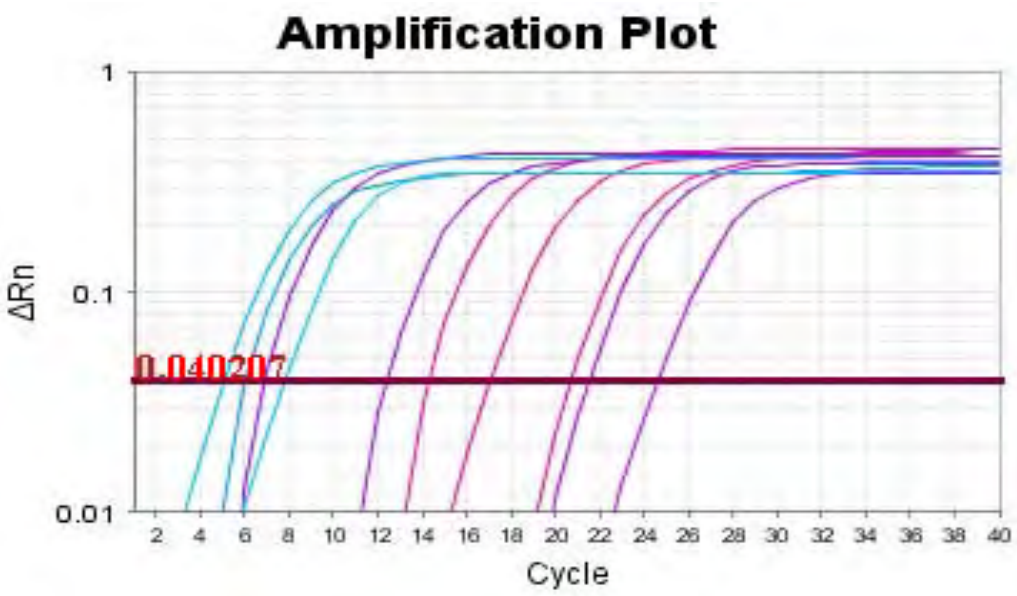


Figure 34. The example of ΔCt value of virus infection from *Ae. aegypti* and *Ae. albopictus* mosquitoes extraction.

CHAPTER V

CONCLUSION AND DISCUSSIONS

Aedes albopictus (C6/36) monolayer are inoculated with a DENV-3 or CHIKV, and then observed for cytological changes that indicate virus growth. Cytopathic changes are occurred in infected cell line that also known as the term "cytopathic effect" (CPE). CPE is frequently applied to virus-induced cellular changes that are visible by light microscopy (Figure 21). DENV-3 and CHIKV are show the different morphology such as irregular outline and nuclear membrane begins to disintegrate. Some nucleoli are difficult to be seen. The cytoplasm appears as a mottled, lumps diffuse. These changes include swelling or shrinkage of cells, the formation of multinucleated giant cells (syncytia), and the production of "inclusions" (made visible by staining) in the nucleus or cytoplasm of the infected cell [139]. The most efficient way to demonstrate cellular changes is by staining with chromatic dyes. Cell monolayer are fixed and then exposed to basic and acidic dyes that accentuate the nature and location of the changes. In our experiment we used of haemotoxylin (basic dye) and eosin (acidic dye) to staining infected cell line. These methods are often referred to as H&E staining. We also presents virus invasion in cell line by Immunocytochemistry (ICC). In this procedure are present brown colours when primary antibody binding to viral antigen. A secondary antibody is coupled with streptavidin-horseradish peroxidase which reacted with 3,3' Diamino-benzidine (DAB) to produce a brown staining wherever primary and secondary antibodies are attached in a process known as DAB staining. These brown colours are present inside the cell infection after 48 h post infection (p.i.) (Figure 22 and 23).

In these experiments, the supernatant of virus infection was collected following table 6 and 7. The result of dengue and chikungunya virus mixed infection equal titer was shown both of dengue and chikungunya virus particles in the same culture by D-RT-PCR analysis (Figure 24). These results are presented co-infection between DENV-3 and CHIKV in one culture and two viruses can be replicated in C6/36 cell line at the same time (Figure 25. Lane 6). According to mixed infection between DENV-3 (MOI=0.1) lower titer than CHIKV (MOI=1.0) was found positive both of dengue and chikungunya virus particle in the same sample (Figure 25. Lane

8). In contrast, mixed infection between DENV-3 (MOI=1.0) higher titer than CHIKV (MOI=0.1) were shown positive results only DENV-3 particle (Figure 25. Lane 7). The result is show competitive infection in same culture because low viral titer cannot replicate in mixed-infection sample. Super-infection trial with DENV-3 (MOI=1.0) was applied by infected virus in C6/36 cell line with primary virus, 1 h and removed this virus by acid glycine buffer (pH 3.0) and PBS (pH 7.4) following infected with CHIKV (MOI=1.0), demonstrated positive signal of both dengue and chikungunya virus (Figure 25. Lane 9); otherwise, using CHIKV (MOI=1.0) following DENV-3(MOI=1.0) infection the D-RT-PCR results also represented two positive PCR product at *E1* gene of CHIKV and *C-prM* gene of DENV-3(Figure 25. Lane 10). We also increased the viral titer 10 fold at ratio 1:10 (MOI=1.0 and 10.0) to infection. D-RT-PCR results was displayed both positive results even though we mixed virus together before infectious or vary serial infection (Figure 24). These data was supported DENV-3 and CHIKV infection depend on amount of viral not related to serial infection thus two virus might be infected host cell at the same time if they have a high volume.

Co-infection and superinfection were evaluated of dengue virus in C3/36 cell line. Previously reported in 2008 by Pepin, found asymmetric competitive suppression between DENV-2 and DEN-4 and they found DENV-2 infection was more suppressed than DENV-4 in mixed infections [2]. In our experiment mixed infection of DENV-3 and CHIKV equal titer was shown positive both of *C-prM* gene at 490 bp of DENV-3 and 201 bp of *E1* gene of CHIKV, respectively. In addition, mixed infection variable ratio by CHIKV (MOI=1.0) higher concentration than DENV-3 (MOI=0.1) also presented two positive viral production (Figure 25. Lane 8) but not showed in DENV-3(MOI=1.0) higher titer than CHIKV (MOI=0.1). Furthermore, DENV-3 higher than CHIKV at MOI 1.0:0.1 was shown only DENV-3 positive these results may be leading to DENV-3 is stronger than CHIKV and competitive infection may be occurred. In super-infection experiment was shown significantly positive but CHIKV have a lower yielded compared with co-infection or single infection treatments (Figure 25. Lane 9 and 10). From these results DENV-3 may be presented a highly competitive infection or it can be suppressed CHIKV infection as a result of CHIKV was decreased yield concentration and cannot be identified viral production,

compared with single infection treatment. Otherwise DENV-3 might be a highly competitive strain than CHIKV when we mixed together with CHIKV or it has been a specific receptor that could be binding to insect cell line [98]. These results are similar to Pepin, 2008 that they found super-infection between DENV-2 and DENV-4 is lower yield compare with single infection control.

The primer pair B3 and F3 could amplify cDNA fragment of CHIKV. Database homology search of the sequence showed that the fragment sequenced was part of E1 gene and marked 10273 to 10477 (gb|AF490259.2) with reference to standard Ross strain full genome sequence, standard control but mixed-infection sample presented different sequences. CHIKV infected C6/36 cell line show chikungunya virus strain IND-00-MH4, complete genome, length 11814 at the position 10295 to 10460 (gb|EF027139.1). The sequence is reference for Indian strain that distribute in Asia countries. For superinfection also presents a different sequence compare with positive control (Ross strain) which gb|EU244823.2| Chikungunya virus strain ITA07-RA1, complete genome, length 11788 at the position 10349 to 10468 show the compatible sequences with our results (see Appendix F). All positive results of DENV-3 is used the generic primer for amplifying and sequencing; thus, the results are demonstrate conserve region between dengue virus 1-4 serotype. These data cannot compare with positive control and need more than 1 primer to evaluate on another gene. However, while unlikely, genetic exchanges between the 2 viruses, either by recombination or complementation, are not definitively excluded. The results would provide the new knowledge in order to understand the effect of vector-virus interactions on dengue and chikungunya transmission which can be used to predict the outbreak and control of the diseases in the future. This may be one of the supporting factors in predicting dengue outbreaks in a specific area.

All of viral RNA was extracted and evaluated by One step RT-PCR and quantitative viral cDNA by real time RT-PCR. These experiments were detected viral RNA copy following ABI procedure. The masters mixed SYBR from ABI Company were used in each sample. The data was collected and analyzed by ABI 7500 software v 2.01. The comparison were calculated from ΔCt value and normalized by standard deviation. In our experiments were show ΔCt value at low cycle of real-time RT-PCR

and higher copy number of RNA viral in each sample compare with normalized standard and control (Figure 33). These methods can be applied to identify number of virus infection in C6/36 cell line and in mosquito vector but need to known the viral RNA concentration standard and cDNA negative control for normalize standard curve [140].

Ae. albopictus plays a relatively minor role compared to *Ae. aegypti* in DENV transmission, at least in part due to differences in host preferences and reduced vector competence. For fully engorged mosquitoes, we apply CHIKV and DENV-3 to *Ae. albopictus* and *Ae. aegypti* mosquitoes by oral feeding technique. These experiments was introduced two virus to mosquito like mosquito uptake a blood meal; thus, the virus can be replicated and multiplied into mosquito body similar to mosquitoes uptake the virus in natural. After 14 day post infection, these virus are penetrated to saliva gland which we show in figure 29 and the position of viral entry into mosquitos' cell line was show in figure 22. Thus, our experiments have been evaluated two viral entries into mosquito cell line and both viruses can be multiplied in C6/36 cell line with the cell line are produce viral progeny and secretion into the media fluid that we maintenances the cell line, described in figure 24 by D-RT-PCR. Furthermore, we also demonstrated single infection, mixed infection and super-infection in *Ae. albopictus* compare with *Ae. aegypti*. We found that co-infection was occurred in only sampling of *Ae. aegypti*, and *Ae. aegypti* are susceptible to these virus more than *Ae. albopictus*. *Ae. albopictus* was uptake the blood mixed-infection at the same time of *Ae. aegypti* but co-infection are not present, only single virus strain was found in *Ae. albopictus* trial. These results may be related to viability of vector competent and relation between virus and vector compartment.

For superinfection in the mosquitoes, the unfortunately was found because fully engorge mosquito don't need to uptake a blood at second time. These results are supposed by another article that they also cannot do the superinfection by oral feeding. Another way we may be applied intrathoracic injection to introduce primary virus to the mosquito and secondary virus may be used oral feeding but the amount of virus concentration and feeding blood pass through midgut like blood sucking should be concern [125, 141].

In 2010, *Ae. albopictus* mosquito in Reunion, France was demonstrated orally infection between DENV-2 and chikungunya virus (mutation strain at A226V). They found *Ae. albopictus* is a major vector of DENV-3 and CHIKV (A226V) in La Reunion Island, Indian Ocean and it can carry both dengue and chikungunya virus particles in their saliva by IFA, each sample were added to monolayers of C6/36 cells in 24 wells plaque to detect infectious particles by the foci forming technique using an immune-peroxydase assay. Cells were incubated 3 days for CHIKV and 5 days for DENV-1 at 28^{o}C under an overlay consisting of 50% of Leibovitz L-15 medium supplemented with 10% FBS and 50% of carboxyl methyl cellulose. Cells were then fixed with 3.6% formaldehyde at room temperature (RT) for 20 min and a immunoperoxydase assay staining was performed to detect foci. After a first incubation of 4 min with PBS 0.1% Triton X-100 (Sigma) at RT, cells were incubated 20 min at 37 ^{o}C with a mouse ascitic fluid at a dilution of 1:1000 for CHIKV and 1:100 for DENV-1 (both ascetic fluids were provided by the French National Reference Center for Arbovirus at the Institut Pasteur). After a wash in PBS 1X, cells were incubated at 37 ^{o}C for 45 min with peroxydase-conjugated goat anti-mouse IgG antibody (Pierce biotechnology, Rockford, USA) at a 1:100 dilution in PBS 1X. After final wash in PBS 1X, Fast 3,3' Diaminobenzidine (Sigma) was used to reveal the staining and foci were counted. The titer of infectious particles per saliva was expressed as FFU/ml (foci forming unit)/ml) and they reported 10^{6} FFU/ml of CHIKV infection and $10^{4,5,9}$ FFU/ml for DENV-1 infection. In the other hand, they used DENV-1 but we used DENV-3 and CHIKV that they applied to these mosquitoes are higher than our experiment and they also suggested doing in cell culture [125].

Rohani and her friend was investigated mixed infection between DENV-2 and chikungunya virus in *Ae. aegypti* (a major mosquito vector in Malaysia) by artificial feeding at 14 day post infections (pi). The mosquito was applied to fresh blood that mixed with virus in equal titer. After feeding 1 h the fully engorged mosquitoes were collected to another cases and maintenances until 14 days and detected viral RNA in 10 pools mosquitoes by RT-PCR. They cannot found mixed infection, although they used a high titration of viral at 10^{8} PFU/ml and they also suggested identifying the compatible titer that induces to cell line and mosquito vector [142].

Persistence of DENV-3 through transovarial transmission passage in successive generations of *Ae. aegypti* mosquito was reported. The seventh generation obtained from the infected parental mosquitoes showed that virus could persist in mosquitoes in successive generations through transovarial passage. The rate of vertical transmission initially increased in the few generations (F1-F2), but in subsequent generations it was found to be steady. Parental mosquitoes inoculated with virus showed higher mortality than the diluent-inoculated controls [84-85, 96, 143]. The rate of transovarial transmission may depend on the serotype and strain of virus and on the species or the geographic strain of mosquito. Therefore it is important to evaluate the competence of local vectors in each geographic area [96].

However, co-infection in C6/36 cell line between DENV-3 and CHIKV have never been reported but in further design also need to replete and applied to other relative dengue virus serotype because DENV-3 have a different domain compare with other relative that might be used to specific binding with host cell receptor [144-145]. Thus, further experiment should be characterized viral protein binding that suitable for viral and host cell interaction to applied for drug treatments and in our experiment was shown dengue and chikungunya virus can be infected and replicated in the same culture which these results may be occurred in mosquitoes vector [2-3] or mosquito vector have a chance to transmission two virus by one mosquito, thus to prevent viral transmission we should be avoid mosquito bites and elimination of mosquito breeding sites because no available vaccination of two virus currently.

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APPENDICES

APPENDIX A

Loading buffers

The loading buffer gives colour and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The most common dyes are bromophenol blue (Sigma B8026) and xylene cyanol (Sigma X4126). Density is provided by glycerol or sucrose.

Typical recipe

bromophenol blue or xylene cyanol	25	mg
sucrose	4	g
H_2O to	10	ml

The exact amount of dye is not important. Store at 4°C to avoid mould growing in the sucrose. 10 mL of loading buffer will last for years.

- Bromophenol blue migrates at a rate equivalent to 200–400bp DNA. If you want to see fragments anywhere near this size (ie. anything smaller than 600bp) then use the other dye because the bromophenol blue will obscure the visibility of the small fragments.

- Xylene cyanol migrates at approximately 4 kb equivalence. So do not use this if you want to visualise fragments of 4 kb.

APPENDIX B

TAE buffer

TAE buffer is a buffer solution containing a mixture of Tris base, acetic acid and EDTA. In molecular biology it is used in agarose electrophoresis typically for the separation of nucleic acids such as DNA and RNA. It is made up of Tris-acetate buffer, usually at pH 8.0, and EDTA, which sequesters divalent cations. TAE has a lower buffer capacity than TBE and can easily become exhausted, but linear, double stranded DNA runs faster in TAE. The soluble of (10X) TAE buffer was described as below.

• Tris-base	48.44	g
• Sodium acetate trihydrate ($NaCH_3$)	27.22	g
• Disodiumsal dihydrat EDTA (Na_2EDTA)	7.44	g
• Glacial acetic acid (adjust pH=7.8)	17	ml
• Add dH_2O until	1,000	ml

Autoclaved before kept at room temperature.

Ethidium Bromide

The most commonly used stain for detecting DNA/RNA is Ethidium Bromide (EtBr). EtBr is a DNA intercalator, inserting itself into the spaces between the base pairs of the double helix. EtBr possesses UV absorbance maxima at 300 and 360 nm. Additionally, it can absorb energy from nucleotides excited by absorbance of 260 nm radiation. Ethidium re-emits this energy as yellow/orange light centered at 590 nm. Ethidium Bromide is a sensitive, easy stain for DNA. It yields low background and a detection limit of 1-5 ng /band. The major drawback to EtBr is that it is a potent mutagen. Solutions must be handled with extreme caution, and decontaminated prior to disposal. Nonetheless, the sensitivity, simplicity (the dye may be run in the gel with the DNA if desired, eliminating a separate staining/destaining process) and nondestructive nature of EtBr staining have made it the standard stain for double stranded DNA.

It is important to note that Ethidium staining is strongly enhanced by the double stranded structure of native DNA. Staining of denatured, ss DNA or RNA is relatively insensitive, requiring some 10 fold more nucleic acid for equivalent detection. Another limitation is that the fluorescence of Ethidium is quenched by polyacrylamide, reducing sensitivity by 10-20 folds in PAGE gels.

Detection of DNA/RNA using Ethidium Bromide

Caution: Ethidium Bromide Is A Potent Mutagen. Handle Only With Gloves And Proper Precautions.

Method I - Including ethidium bromide in the gel and buffer

Add EtBr to 0.5 µg/ml final concentration. (Stocks are generally 10 mg/ml, and require 5µl stock/100ml gel).

Run Gel: Upon completion of run, place gel in plastic wrap on a UV light box. Bands will appear bright orange on a faint orange background. This method will detect approximately 5ng of DNA. Destaining in water or 1 mM MgSO4 may be required to achieve full sensitivity.

As an alternative, Ethidium may be included in the gel, but not the buffer. Ethidium is positively charged, and will migrate in the opposite direction from the DNA. In general, sufficient Ethidium will remain bound to the DNA even at the cathode end of the gel. Such gels will have an area of high background where the Ethidium has not yet migrated out of the gel. They are, however, sufficient for many purposes, and do not generate as much Ethidium waste (Your safety office will know what level of Ethidium causes a buffer to be declared a hazard).

Method II - Post Run Staining

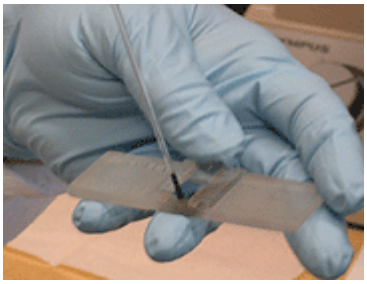
Prepare enough 0.5µg/ml EtBr in water or buffer to completely submerge the gel. This solution is stable for 1-2 months at room temperature in the dark. After the run submerge the gel in the staining solution for 15-30 minutes (depending upon gel thickness). Place the gel on plastic wrap on a UV light box and observe under 300nm illumination. Bands will appear bright orange on a pale orange background.

Notes: This protocol minimizes the amount of EtBr waste created with each gel run. Sensitivity is the same as method I, and may require destaining in water or 1mM $MgSO_4$ to achieve the best sensitivity.

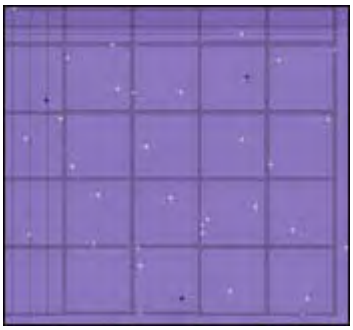
APPENDIX C

Tryphan Blue staining

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is a diazo dye. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue are not absorbed; however, it traverses the membrane in a dead cell. Thus, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. This dye may be a cause of certain birth defects such as encephalocele.



(A) Loading cell staining into Hematcytometer (B) Count number of cell survival and cell death



(C) Hematcytometer grid under microscope (D) cell survival and cell death under microscope

Equipment & Reagents

(A) - Haemocytometer plus a supply of cover-slips
- 0.4% Trypan Blue stain (fresh & filtered) in phosphate buffered saline
- Tally Counter
- Cell Suspension
- Gilson pipettes or similar

(B)- Inverted microscope (preferably phase contrast)

Materials:

Trypan Blue: dilute at 0.8 mM in PBS. Store at room temperature. Stable for 1 month.

Procedure

1. Ensure the cover-slip and haemocytometer are clean and grease-free (use alcohol to clean).
2. Collected cell type following scraper for C6/36 cell line or 0.25% trypsin for Vero cell and LLC-MK_2
3. Centrifuged cell pellet at 1250 rpm, 5 min
4. Mix equal volumes of 0.4% trypan blue stain and a well mixed cell suspension (not too vigorous) e.g. mix 100µl trypan blue stain with 100 µl cell suspension.
5. Pipette trypan blue/cell mix (approximately 10µl) at the edge of the cover-slip and allow to run under the cover slip.
6. Visualise the haemocytometer grid under the microscope, refer to figure D for layout of grid. Please note:

i. Trypan Blue is a "vital stain"; it is excluded from live cells.
ii. Live cells appear colourless and bright (refractile) under phase contrast.
iii. Dead cells stain blue and are non-refractile.
iv. To aid accuracy and consistency of cell counts use counting system illustrated

Dead cells stain blue, while live cells exclude trypan blue. Cannot distinguish necrotic vs. apoptotic cells.

7. Count viable (live) and dead cells in one or more large corner squares and record cell counts.
8. It is advisable to count around 40 to 70 cells to obtain an accurate cell count - therefore it may be necessary to count more than one large corner square.
9. To calculate cell concentration per ml:

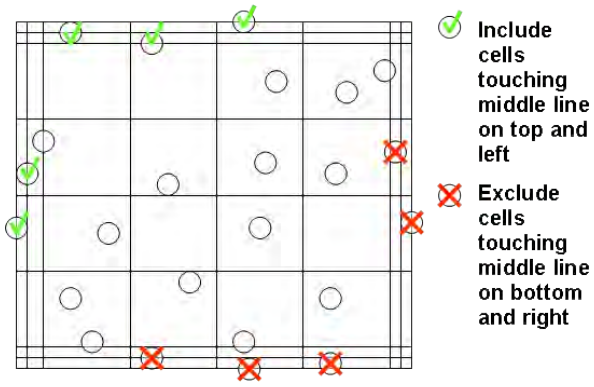
Average number of cells in one large square x dilution factor* x 10^4

*dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) cell suspensions.

10^4 = conversion factor to convert 10^{-4} ml to 1ml

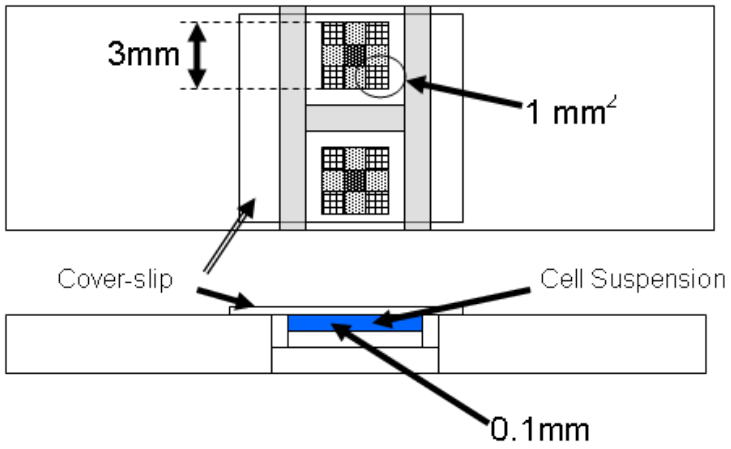
Calculation of Cell Viability:

$$\frac{\text{No. of Viable Cells Counted}}{\text{Total Cells Counted (viable + dead)}} \times 100 = \% \text{ viable cells}$$

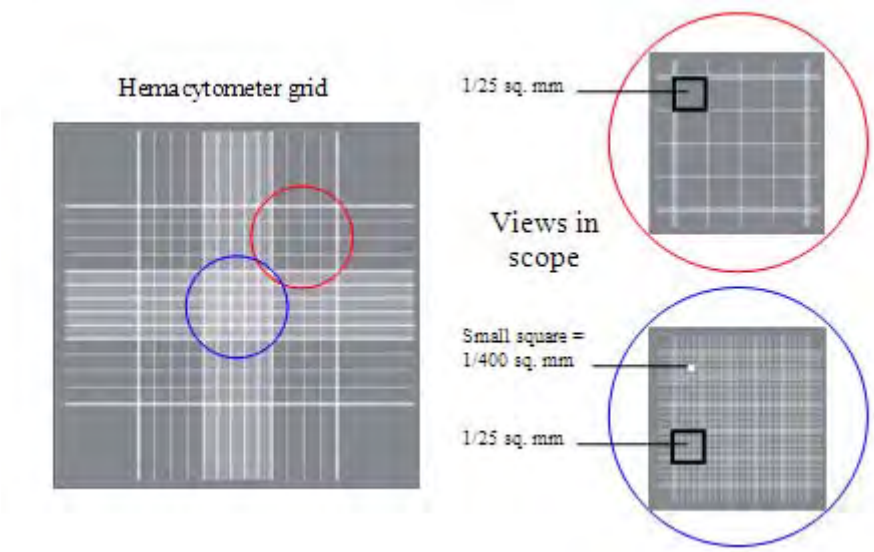


Counting system to ensure accuracy and consistency. Count the cells within the large square and those crossing the edge on two out of the four sides.

Haemocytometer arrangement and dimensions



Therefore volume of 1 large corner square =1 mm^2 x 0.1 mm = 0.1 cm x 0.1 cm x 0.01= 10^{-4} cm^3 or 10^{-4} ml



Comments:

Avoid the exposure of cells to trypan blue for a period longer than 10 minutes. In this case it is possible to observe an increase in the dead cell population (trypan blue positive) due to the trypan toxicity.

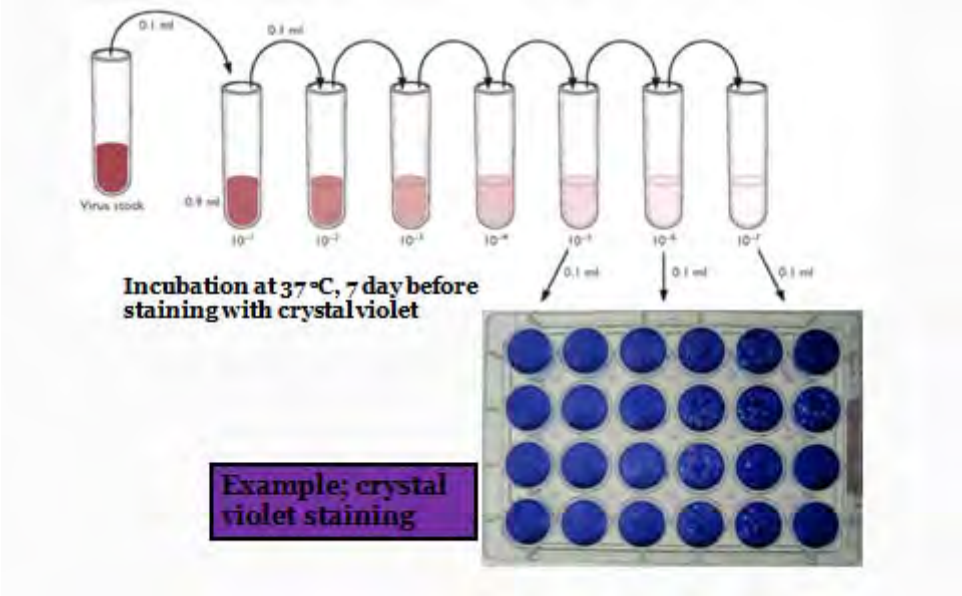
APPENDIX D

Standard plaque assay

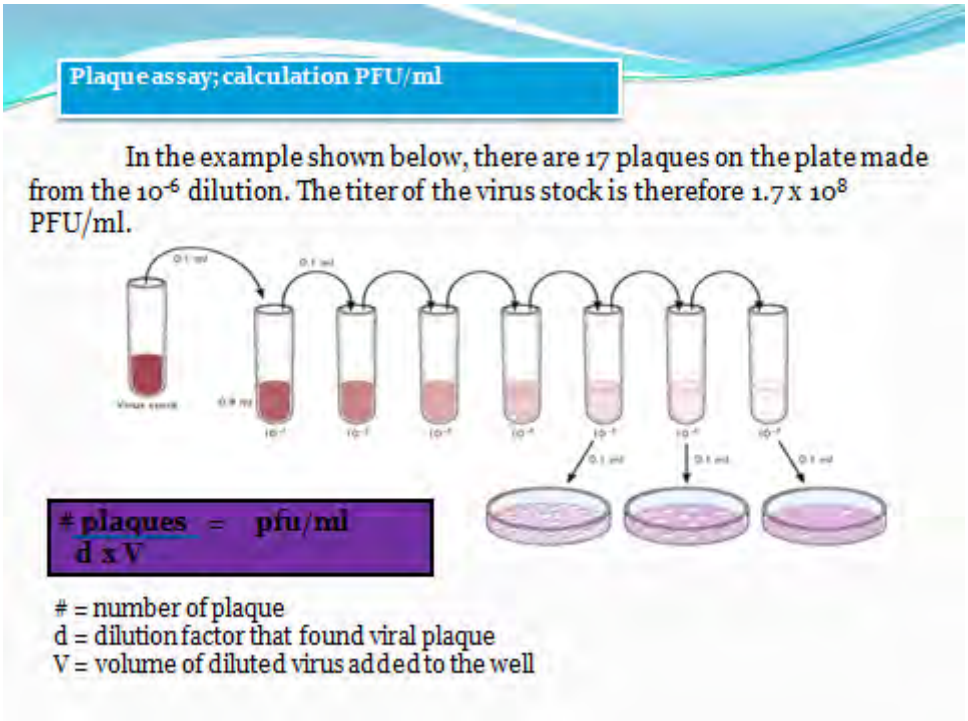
The plaque assay is originally a virological assay developed to count and measure infectivity of bacteriophages. Later, it was applied to count mammalian viruses and other viruses as well. The plaque assay remains to be the most widely used technique for virus isolation and purification, and to determine viral titers. The basis of the technique is to measure the ability of a single infectious virus to form a "plaque" on a confluent monolayer culture of cells. A plaque is formed as a result of infection of one cell by a single virus particle followed by the replication of that virus, and eventually, the death of the cell. The newly replicated virus particles will then infect and kill surrounding cells. The culture will then be stained with a dye; crytal violet, which stains only viable cells but not the dead cells. Hence, the dead cells in the plaque will appear unstained against the colored background

Ten-fold dilutions of a virus stock are prepared prior to performing a plaque assay and 0.2 ml aliquiots are put on to the cell monolayers such as Vero cell or LLC-MK_2. This mixture is incubated for a period of time so that the virus has time to attack the cells. Furthermore, the monolayers are covered by a nutrient medium that contains a substance (usually agar). The nutrient medium forms a gel and after the culture is incubated, the original infected cells release viral progeny. The gel restricts the spread of the virus to surrounding cells, and as a result, the infectious particle produces a circular zone called a plaque. With time, the plaque grows to a size visible with the naked eye and dyes are used to enhance the viewing. The example for plaque assay method according to 10-fold dilution and viral calculation was show in step 1 and 2. In this image, the cells have been stained with crystal violet, and the plaques are readily visible where the cells have been destroyed by viral infection. The titer of a virus stock can be calculated in plaque-forming units (PFU) per milliliter. To determine the virus titer, the plaques are counted. To minimize error, only plates containing between 10 and 100 plaques are counted, depending on the size of the cell culture plate that is used. Statistical principles dictate that when 100 plaques are counted, the sample titer will vary by plus or minus 10%. Each dilution is plated in duplicate to enhance accuracy.

Step 1. Plaque assay method



Step 2. Calculation PFU/ml



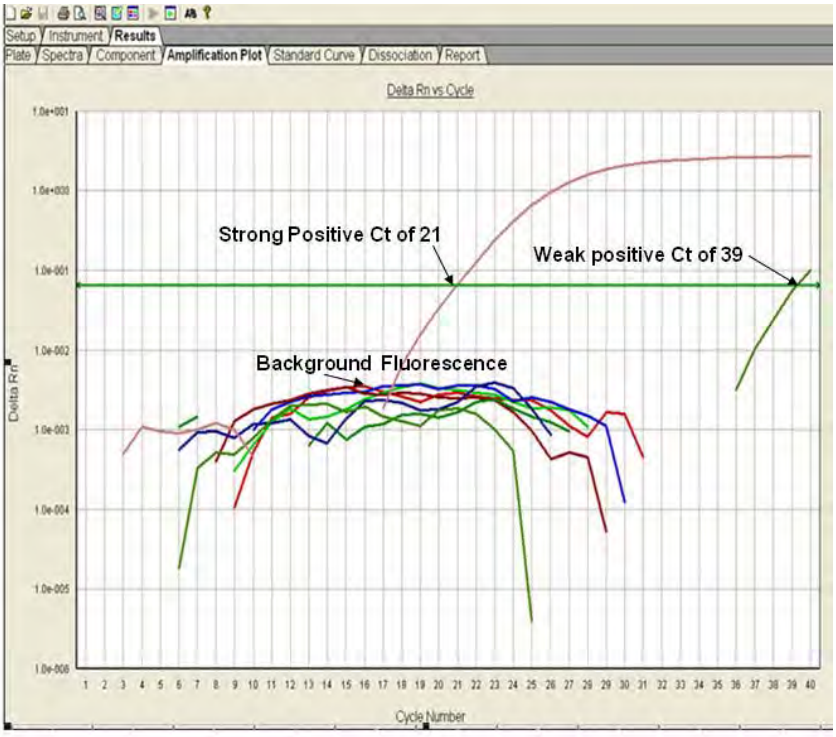
APPENDIX E

Real Time PCR Ct Values

What does ΔCt mean?

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The ΔCt (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). ΔCt levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). ABI real time assays undergo 40 cycles of amplification.

- Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample
- Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid
- Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.



APPENDIX F

1. Basic Local Alignment Search Tool for Chikungunya virus strain (Positive control)

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:

(Click headers to sort columns)

AF490259.2	Chikungunya virus strain Ross, complete genome	379	379	99%	3e-102	100%
AF339485.1	Chikungunya virus strain S27 nonstructural protein 4 gene, partial cds; and structural polyprotein precursor, gene, complete cds	379	379	99%	3e-102	100%
AF369024.2	Chikungunya virus strain S27-African prototype, complete genome	379	379	99%	3e-102	100% G
AF192906.1	Chikungunya virus isolate CAR256 structural polyprotein (E1) mRNA, partial cds	379	379	99%	3e-102	100%
AF192905.1	Chikungunya virus isolate Ross structural polyprotein (E1) mRNA, partial cds	379	379	99%	3e-102	100%
EU244823.2	Chikungunya virus strain ITA07-RA1, complete genome	374	374	99%	1e-100	99%
EU272133.1	Chikungunya virus isolate ITA8_VE E1 protein gene, partial cds	374	374	99%	1e-100	99%
EU272132.1	Chikungunya virus isolate ITA7_BI E1 protein gene, partial cds	374	374	99%	1e-100	99%
EF027139.1	Chikungunya virus strain IND-00-MH4, complete genome	374	374	99%	1e-100	99%
DQ520753.1	Chikungunya virus isolate IND00MH4 E1 protein gene, partial cds	374	374	99%	1e-100	99%
AF192907.1	Chikungunya virus isolate Ag41855 E1 gene, partial sequence	374	374	99%	1e-100	99%
EU862807.1	Chikungunya virus isolate KTM/RRII/07 polyprotein gene, partial cds	370	370	98%	2e-99	99%
EU372006.1	Chikungunya virus isolate DRDE-07, complete genome	368	368	99%	6e-99	99%
FJ445511.1	Chikungunya virus isolate SGEHICHD13508 E1 envelope protein gene, partial cds	368	368	99%	6e-99	99%
FJ445510.1	Chikungunya virus isolate SGEHICHS277108 E1 envelope protein gene, partial cds	368	368	99%	6e-99	99%
FJ445487.1	Chikungunya virus isolate SGEHICHS107408 E1 envelope protein gene, partial cds	368	368	99%	6e-99	99%

2. Multiple alignment of Chikungunya virus strain (Positive control)

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 Identities = 205/205 (100%), Gaps = 0/205 (0%)
 Strand=Plus/Plus

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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10393  AACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAA  10452

Query  182    TTCATTGTGGGGCCAATGTCTTCAG  206
              |||||||||||||||||||||||||
Sbjct  10453  TTCATTGTGGGGCCAATGTCTTCAG  10477


>gb|AF339485.1|  Chikungunya virus strain S27 nonstructural protein 4 gene, partial
cds; and structural polyprotein precursor, gene, complete
cds
Length=4885

 Score =  379 bits (205),  Expect = 3e-102
 Identities = 205/205 (100%), Gaps = 0/205 (0%)
 Strand=Plus/Plus

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Sbjct  3486   AACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAA  3545

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              |||||||||||||||||||||||||
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 Strand=Plus/Plus

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Query  182    TTCATTGTGGGGCCAATGTCTTCAG  206
              |||||||||||||||||||||||||
Sbjct  10474  TTCATTGTGGGGCCAATGTCTTCAG  10498
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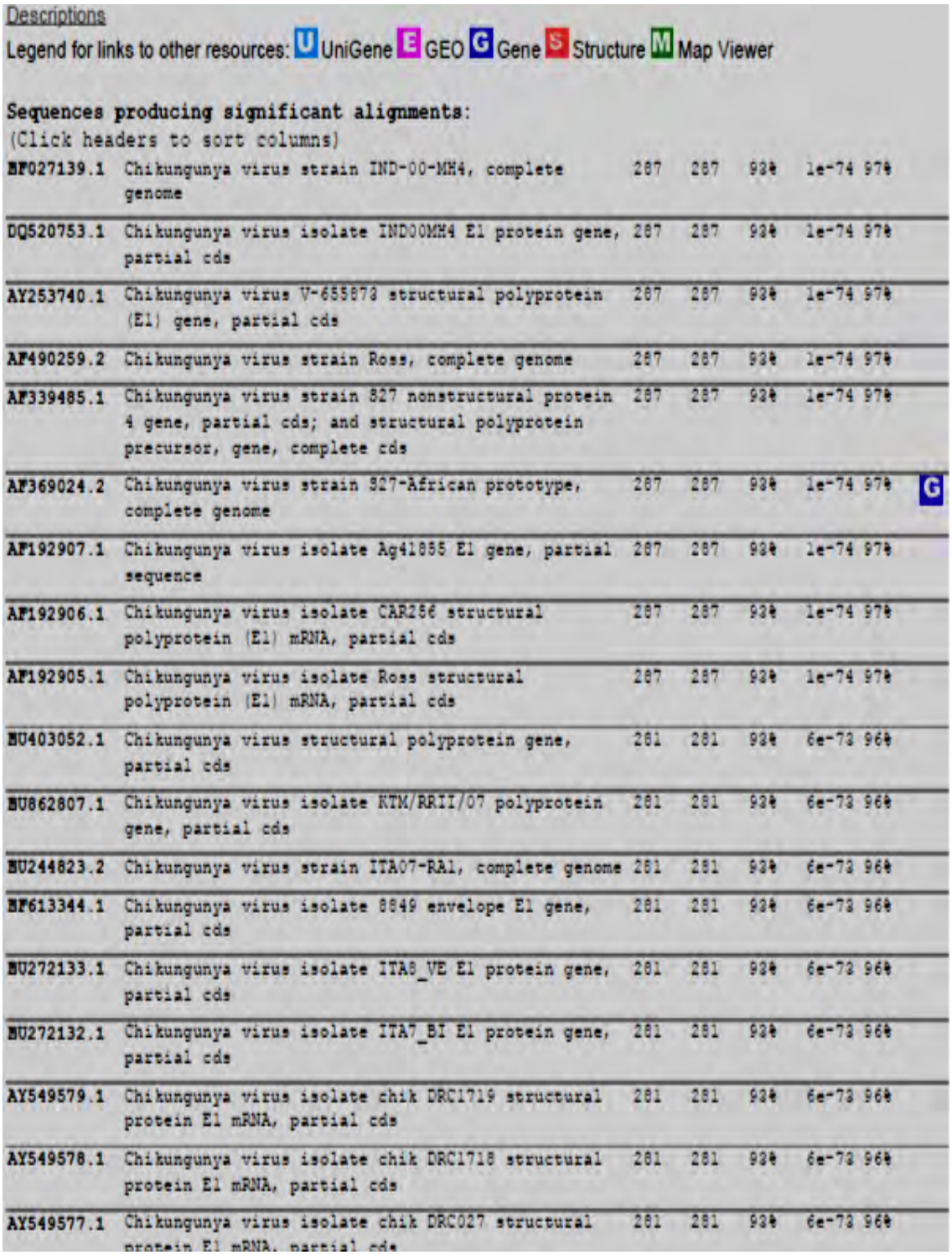
3. Basic Local Alignment Search Tool for Chikungunya virus strain (mixed-infection sample)



Descriptions

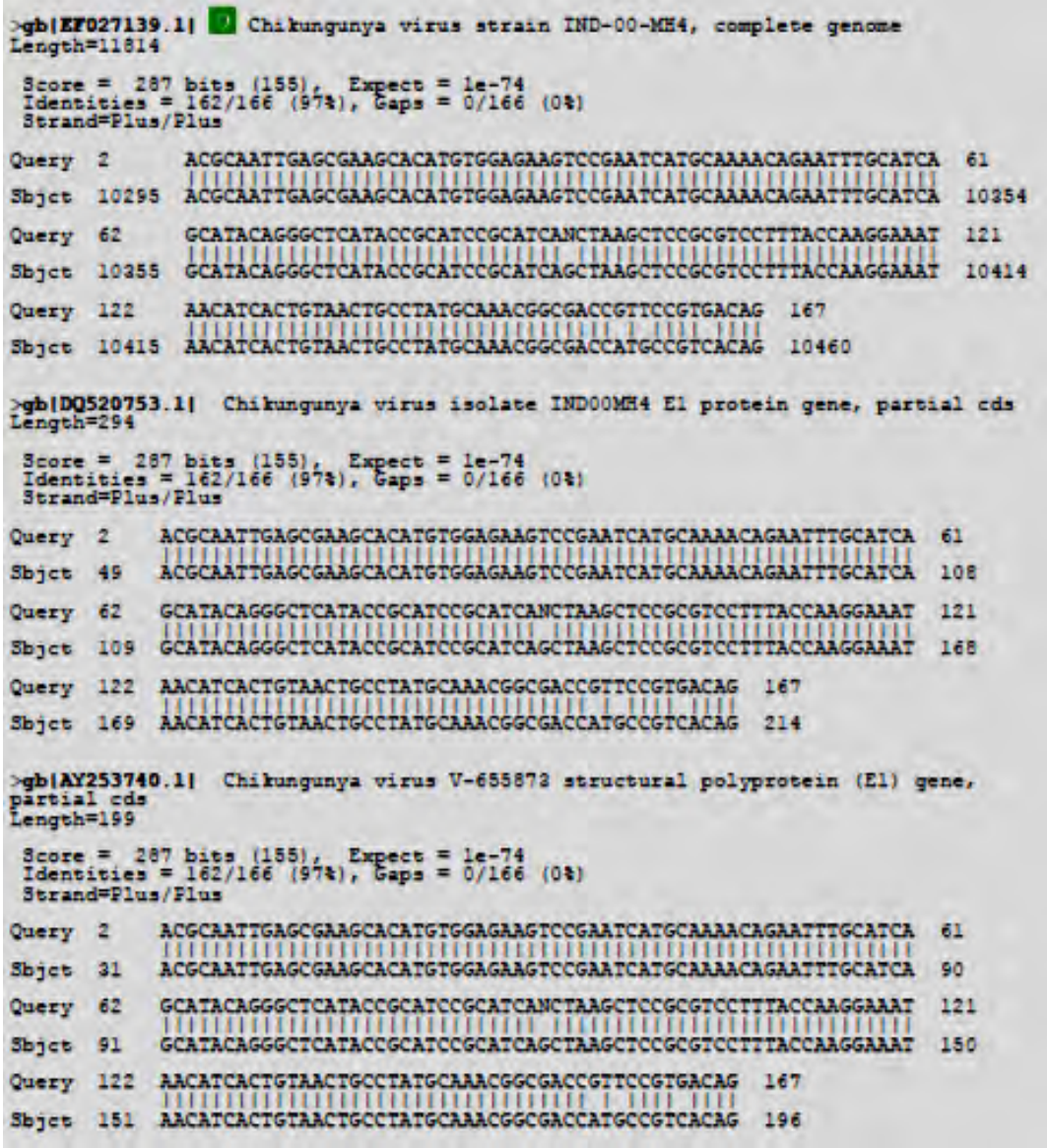
Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:

(Click headers to sort columns)

BF027139.1	Chikungunya virus strain IND-00-MH4, complete genome	287	287	93%	1e-74	97%	
DQ520753.1	Chikungunya virus isolate IND00MH4 E1 protein gene, partial cds	287	287	93%	1e-74	97%	
AY253740.1	Chikungunya virus V-655873 structural polyprotein (E1) gene, partial cds	287	287	93%	1e-74	97%	
AF490259.2	Chikungunya virus strain Ross, complete genome	287	287	93%	1e-74	97%	
AF339485.1	Chikungunya virus strain S27 nonstructural protein 4 gene, partial cds; and structural polyprotein precursor, gene, complete cds	287	287	93%	1e-74	97%	
AF369024.2	Chikungunya virus strain S27-African prototype, complete genome	287	287	93%	1e-74	97%	G
AF192907.1	Chikungunya virus isolate Ag41855 E1 gene, partial sequence	287	287	93%	1e-74	97%	
AF192906.1	Chikungunya virus isolate CAR256 structural polyprotein (E1) mRNA, partial cds	287	287	93%	1e-74	97%	
AF192905.1	Chikungunya virus isolate Ross structural polyprotein (E1) mRNA, partial cds	287	287	93%	1e-74	97%	
EU403052.1	Chikungunya virus structural polyprotein gene, partial cds	281	281	93%	6e-73	96%	
EU862807.1	Chikungunya virus isolate KTM/RRII/07 polyprotein gene, partial cds	281	281	93%	6e-73	96%	
EU244823.2	Chikungunya virus strain ITA07-RA1, complete genome	281	281	93%	6e-73	96%	
EF613344.1	Chikungunya virus isolate 8849 envelope E1 gene, partial cds	281	281	93%	6e-73	96%	
EU272133.1	Chikungunya virus isolate ITA8_VE E1 protein gene, partial cds	281	281	93%	6e-73	96%	
EU272132.1	Chikungunya virus isolate ITA7_BI E1 protein gene, partial cds	281	281	93%	6e-73	96%	
AY549579.1	Chikungunya virus isolate chik DRC1719 structural protein E1 mRNA, partial cds	281	281	93%	6e-73	96%	
AY549578.1	Chikungunya virus isolate chik DRC1718 structural protein E1 mRNA, partial cds	281	281	93%	6e-73	96%	
AY549577.1	Chikungunya virus isolate chik DRC027 structural protein E1 mRNA, partial cds	281	281	93%	6e-73	96%	

4. Multiple alignment of Chikungunya virus strain (Mixed-infection sample)



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 Identities = 162/166 (97%), Gaps = 0/166 (0%)
 Strand=Plus/Plus

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Query  62     GCATACAGGGCTCATACCGCATCCGCATCANCTAAGCTCCGCGTCCTTTACCAAGGAAAT  121
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Sbjct  10415  AACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAG  10460


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 Strand=Plus/Plus

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            |||||||||||||||||||||||||||||| | |||| ||||
Sbjct  169  AACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAG  214


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Length=199

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 Identities = 162/166 (97%), Gaps = 0/166 (0%)
 Strand=Plus/Plus

Query  2    ACGCAATTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCA  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31   ACGCAATTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCA  90

Query  62   GCATACAGGGCTCATACCGCATCCGCATCANCTAAGCTCCGCGTCCTTTACCAAGGAAAT  121
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  91   GCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAAT  150

Query  122  AACATCACTGTAACTGCCTATGCAAACGGCGACCGTTCCGTGACAG  167
            |||||||||||||||||||||||||||||| | |||| ||||
Sbjct  151  AACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAG  196
```

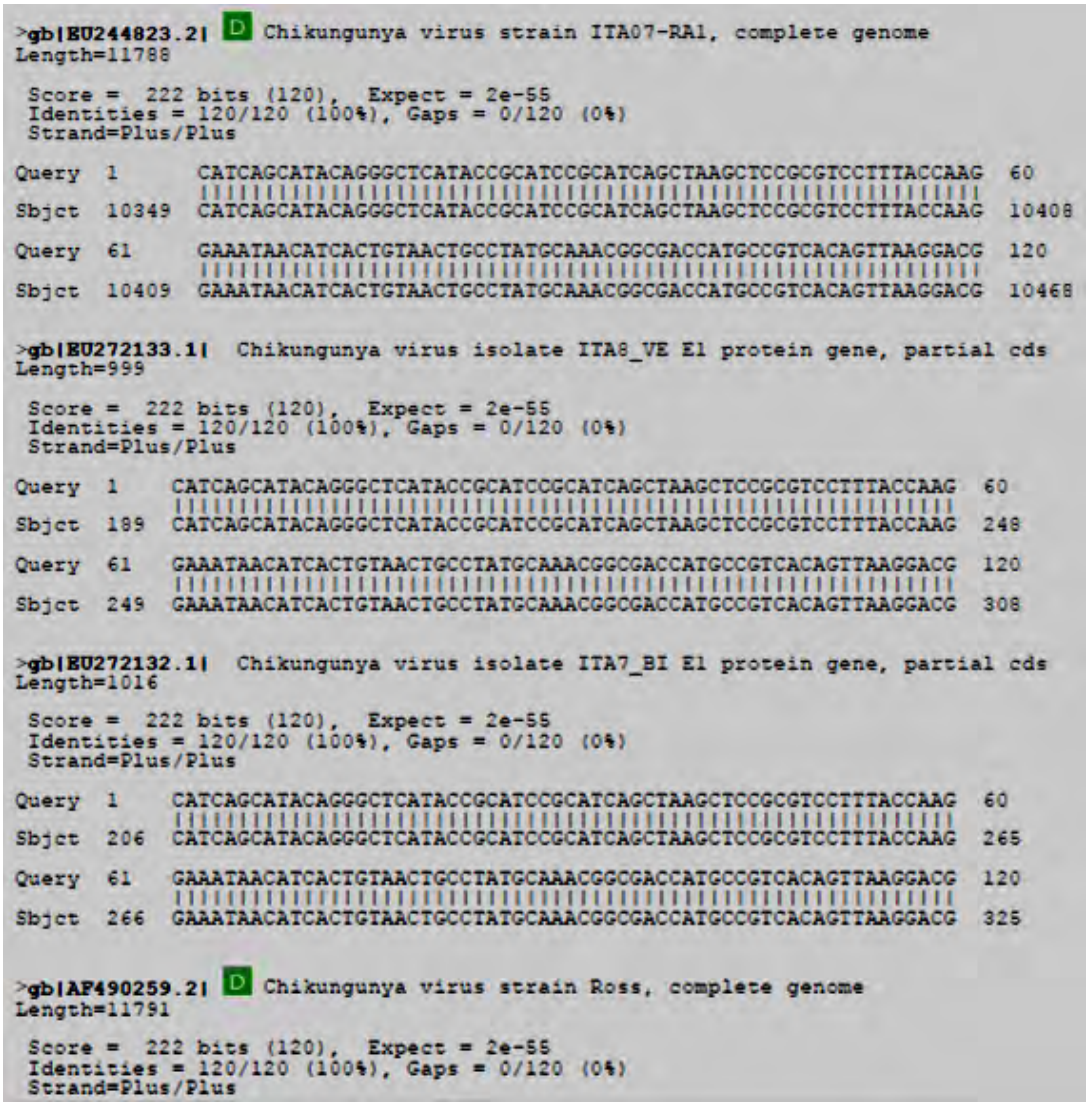
5. BLAST of chikungunya virus from superinfection sample

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:
(Click headers to sort columns)

EU244823.2	Chikungunya virus strain ITA07-RA1, complete genome	222	222	100%	2e-55	100%
EU272133.1	Chikungunya virus isolate ITA8_VE E1 protein gene, partial cds	222	222	100%	2e-55	100%
EU272132.1	Chikungunya virus isolate ITA7_BI E1 protein gene, partial cds	222	222	100%	2e-55	100%
AF490259.2	Chikungunya virus strain Ross, complete genome	222	222	100%	2e-55	100%
AF339485.1	Chikungunya virus strain 827 nonstructural protein 4 gene, partial cds; and structural polyprotein precursor, gene, complete cds	222	222	100%	2e-55	100%
AF369024.2	Chikungunya virus strain 827-African prototype, complete genome	222	222	100%	2e-55	100% G
AF192906.1	Chikungunya virus isolate CAR256 structural polyprotein (E1) mRNA, partial cds	222	222	100%	2e-55	100%
AF192905.1	Chikungunya virus isolate Ross structural polyprotein (E1) mRNA, partial cds	222	222	100%	2e-55	100%
FJ617290.1	Chikungunya virus isolate DK-2 envelope glycoprotein 1 gene, partial cds	217	217	100%	1e-53	99%
FJ617289.1	Chikungunya virus isolate DK-1 envelope glycoprotein 1 gene, partial cds	217	217	100%	1e-53	99%
FJ617288.1	Chikungunya virus isolate K8GD-8 envelope glycoprotein 1 gene, partial cds	217	217	100%	1e-53	99%
FJ617286.1	Chikungunya virus isolate K8GD-6 envelope glycoprotein 1 gene, partial cds	217	217	100%	1e-53	99%
FJ617283.1	Chikungunya virus isolate K8GD-2 envelope glycoprotein 1 gene, partial cds	217	217	100%	1e-53	99%
GU199351.1	Chikungunya virus strain SD08Pan, complete genome	217	217	100%	1e-53	99%
GU199350.1	Chikungunya virus strain FD080008, complete genome	217	217	100%	1e-53	99%
GU013530.1	Chikungunya virus isolate LK(EH)chik19708 E1 envelope protein gene, partial cds	217	217	100%	1e-53	99%
GU013529.1	Chikungunya virus isolate LK(PB)chik6008 E1 envelope protein gene, partial cds	217	217	100%	1e-53	99%
GU013528.1	Chikungunya virus isolate LK(PB)chik3408 E1 envelope protein gene, partial cds	217	217	100%	1e-53	99%

6. Multiple alignment of chikungunya virus from superinfection sample



```
>gb|EU244823.2|  Chikungunya virus strain ITA07-RA1, complete genome
Length=11788

 Score =  222 bits (120),  Expect = 2e-55
 Identities = 120/120 (100%), Gaps = 0/120 (0%)
 Strand=Plus/Plus

Query  1      CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10349  CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  10408

Query  61     GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10409  GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  10468


>gb|EU272133.1|  Chikungunya virus isolate ITA8_VE E1 protein gene, partial cds
Length=999

 Score =  222 bits (120),  Expect = 2e-55
 Identities = 120/120 (100%), Gaps = 0/120 (0%)
 Strand=Plus/Plus

Query  1    CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189  CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  248

Query  61   GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  308


>gb|EU272132.1|  Chikungunya virus isolate ITA7_BI E1 protein gene, partial cds
Length=1016

 Score =  222 bits (120),  Expect = 2e-55
 Identities = 120/120 (100%), Gaps = 0/120 (0%)
 Strand=Plus/Plus

Query  1    CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  206  CATCAGCATACAGGGCTCATACCGCATCCGCATCAGCTAAGCTCCGCGTCCTTTACCAAG  265

Query  61   GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  266  GAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACG  325


>gb|AF490259.2|  Chikungunya virus strain Ross, complete genome
Length=11791

 Score =  222 bits (120),  Expect = 2e-55
 Identities = 120/120 (100%), Gaps = 0/120 (0%)
 Strand=Plus/Plus
```

7. BLAST of DENV-3 (Positive control)

These results is DENV-3 sequence from generic complex primer which could be amplified all of 4 serotype. These primers can be applied for RT-PCR but did not suitable for sequencing because the results are compatible to all serotype of dengue virus.

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:
(Click headers to sort columns)

AY744150.1	Dengue virus type 2 vector p4(delta30)-4995, complete sequence	678	678	53%	0.0	99%
AY744149.1	Dengue virus type 2 vector p2(delta30), complete sequence	678	678	53%	0.0	99%
AY744148.1	Dengue virus type 2 vector p2, complete sequence	678	678	53%	0.0	99%
AY243469.1	Chimeric Dengue virus vector p4(Delta30)-D2-CME, complete sequence	678	678	53%	0.0	99%
AY243468.1	Chimeric Dengue virus vector p4-D2-CME, complete sequence	678	678	53%	0.0	99%
AY243467.1	Chimeric Dengue virus vector p4(Delta30)-D2-ME, complete sequence	678	678	53%	0.0	99%
AY243466.1	Chimeric Dengue virus vector p4-D2-ME, complete sequence	678	678	53%	0.0	99%
AY376438.1	Dengue virus vector p4(Delta30), complete sequence	678	678	53%	0.0	99%
AY656170.1	Dengue virus type 3 vector p3(delta30), complete sequence	678	678	53%	0.0	99%
AY656169.1	Dengue virus type 3 vector p3, complete sequence	678	678	53%	0.0	99%
AY656168.1	Chimeric dengue virus vector p4(delta30)-D3L-ME, complete sequence	678	678	53%	0.0	99%
AY656167.1	Chimeric dengue virus vector p4-D3L-ME, complete sequence	678	678	53%	0.0	99%
AY648301.1	Dengue virus type 4 vector p4, complete sequence	678	678	53%	0.0	99%

BIOGRAPHY

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- Bachelor degree of Sciences (Biology), Khonkaen University, Thailand, 1996-2000
- Master degree of Sciences (Genetics Engineering), Kasatsart University, Thailand, 2000-2003
- Diploma of Applied Parasitology and Entomology 2006 (DAP&E 2006), Institute of Medical Research (IMR), Kuala Lumpur, Malaysia

CURRENT WORK POSITION:

- 2003, Researcher of Center for Protein Structure and Function, Faculty of Sciences,Mahidol, University, Thailand
- 2004, The lecturer of the Faculty of Sciences, Burirum Rajabhat, University, Thailand
- 2005-present Researcher, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Thailand

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