



Institute of Health and Community Medicine

**Construction of Recombinant Baculoviruses Expressing Structural
Proteins of Chikungunya Virus**

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Construction of Recombinant Baculoviruses Expressing Structural Proteins of Chikungunya Virus

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. It is original and is the result of my work, unless otherwise indicated or acknowledged as referenced work. This thesis has not been submitted at Universiti Malaysia Sarawak or to any other academic institution or non-academic institution for any other degree or qualification.

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ABSTRACT

Chikungunya virus (CHIKV) is a reemerging human pathogen that causes a debilitating arthritic-like disease in humans. Although numerous strategies have been adopted to combat CHIKV infection, transmission of CHIKV continues to increase globally. The most effective measure to reduce the CHIKV burden is the development of a vaccine. However, there is no licensed CHIKV vaccine available currently for human use. CHIKV VLPs have the potential to be used as a prophylactic vaccine and currently there are several CHIKV VLPs candidates (generated via mammalian expression system) being evaluated for human use in Phase I clinical trials. Accordingly, this study was proposed to construct CHIKV recombinant structural proteins which could be potentially used as a source of CHIK VLPs using an insect cell-baculovirus expression system and to test the antigenicity of the expressed structural proteins against known CHIKV patient's sera. The selected gene region encoding CHIKV structural polyprotein (E3-E2-6K-EctoE1) was amplified as a BamHI-HindIII fragment and cloned into bac-to-bac[®] baculovirus expression vector. The resulting construct was used to transfect *Sf9* cells to generate a recombinant baculovirus (AcMNPV-E3E26KEctoE1). Western blot analysis of the expressed recombinant protein, confirmed the presence of a 120 kDa expected size product. Western blot results demonstrated that the expressed CHIKV proteins were detected by sera from patients previously exposed to CHIKV infection. CHIKV positive patient sera also detected a lower than expected recombinant proteins of 38 and 39 kDa respectively in the western blot analysis. These may possibly indicate incomplete or unassembled component proteins of the VLP.

Keywords: Chikungunya virus (CHIKV), Virus-like particles (VLPs), insect cells-baculovirus expression system, bac-to-bac[®] baculovirus expression system

Penghasilan Rekombinan Baculovirus yang Mengekspresikan Protein Struktur Virus Chikungunya

ABSTRAK

Virus Chikungunya adalah pathogen manusia yang akan menyebabkan penyakit arthritis dalam kalangan manusia. Walaupun banyak strategi telah diambil untuk menghapuskan jangkitan virus Chikungunya, penyebaran virus Chikungunya masih berkembang secara global. Cara yang paling berkesan untuk mengurangkan beban virus Chikungunya ialah pembangunan vaksin. Pada masa ini, tiada vaksin virus Chikungunya yang berlesen tersedia untuk kegunaan manusia. Zarah seperti virus Chikungunya mempunyai potensi untuk digunakan sebagai vaksin profilaksis. Pada masa ini terdapat calon-calon Zarah seperti virus Chikungunya (dihasilkan melalui sistem ekspresi mamalia) yang dinilai untuk kegunaan manusia dalam percubaan klinikal Fasa I. Oleh itu, kajian ini bertujuan untuk membina protein rekombinan virus Chikungunya yang mungkin berpotensi digunakan sebagai bahan-bahan untuk membuat zarah seperti virus Chikungunya menggunakan sistem ekspresi sel serangga-baculovirus dan untuk menguji reaksi antigenik protein yang dihasilkan terhadap sera pesakit. Gen yang mengodkan poliprotein struktural virus Chikungunya (E3-E3-6K-EctoE1) telah diamplifikasi sebagai fragmen BamHI-HindIII dan diklon ke dalam vector ekspresi bac-to-bac[®] baculovirus. Konstruk yang terhasil digunakan untuk mentransfeksi ke dalam sel Sf9 untuk menghasilkan baculovirus rekombinan. Analisis dari blot barat ke atas hasil ekspresi protein rekombinan mengesahkan kewujudan produk bersaiz 120 kDa. Hasil blot barat menunjukkan ekspresi potensi protein virus Chikungunya yang mempunyai reaksi antigenik terhadap antibodi virus. Sera pesakit positif virus Chikungunya juga mengesan protein rekombinan yang lebih

rendah daripada 38 kDa and 39 kDa masing-masing dalam analisis blot barat. Keadaan ini mungkin menandakan protein komponen VLP yang tidak lengkap atau tidak dipasang.

Kata kunci: *Virus Chikungunya, zarah seperti virus, sistem ekspresi sel serangga-baculovirus, sistem ekspresi, bac-to-bac[®] baculovirus*

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against CHIKV negative sera

LIST OF ABBREVIATIONS

aa	Amino acids
AcMNPV	<i>Autographa Carlifornia</i> multiple nuclear polyhedrosis virus
ATP	Adenosine 5'-triphosphate
BCB	BigDye® Cleaning Bead
BLAST	Basic local alignment search tool
bp	Base pairs
cm ²	Centimeter square
C	Capsid protein
CBB	Coomassie brilliant blue
cDNA	Complementary deoxyribonucleic acid
CHIK	Chikungunya
CHIKV	Chikungunya virus
CME	Clathrin-mediated endocytosis
CPE	Cytopathic effect
Cryo-EM	Cryogenic electron microscopy
CsCl	Cesium chloride
DALYs	Disability adjusted life years, a measure of debilitating disease burden
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
g	Gram
IC-BEVS	Insect cell-baculovirus expression system
IPTG	Isopropyl β -D-1-thiogalactopyranoside
kb	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
LB	Luria Bertani
M	Molar
Mabs	Monoclonal antibodies
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulfate
ml	Millilitre
mM	Milli molar
M-MuLV	Moloney murine leukaemia virus
NaCl	Sodium chloride
(NH ₄) ₂ SO ₄	Ammonium sulfate
ng/ μ l	Nanogram/microlitre
nsP1	Non-structural protein 1
nsP2	Non-structural protein 2
nsP3	Non-structural protein 3
nsP4	Non-structural protein 4

nt	Nucleotide
NTR	Non-translated region
NW	New world
ORF	Open reading frame
OW	Old world
PCR	Polymerase chain reaction
pFBD	pFastBac TM Dual
RNA	<i>Ribonucleic acid</i>
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TBE	Tris-borate EDTA
TEMED	Tetramethylethylenediamine
TPB	Tryptose Phosphate Broth
Tris-HCl	Tris-hydrochloride
UHQ	Ultra-high quality
U/μl	Unit/microliter
VLPs	Virus-like particles
v/v	Volume/volume
w/v	Weight/volume

$\mu\text{g}/\mu\text{l}$	Microgram/microliters
$\mu\text{g}/\text{ml}$	Microgram/milliliters
μl	Microliters
μM	Micro molar

CHAPTER 1

LITERATURE REVIEW

1.1 An overview of *Alphavirus*

Alphavirus is a genus of small, enveloped, single-stranded positive RNA viruses, classified under the family *Togaviridae*. They are typically transmitted to primates, rodents, or birds through the bite of an infected insect vector. However, it has been reported that a few of these viruses are able to infect fish and seals (Weaver & Smith, 2011). Alphaviruses are basically referred to as New World (NW) *Alphavirus* or Old World (OW) *Alphavirus* depending on the location where the alphaviruses emerged (Paredes et al., 2005). NW alphaviruses (e.g. Eastern, Venezuelan, and Western Equine Encephalitis viruses) are generally associated with potentially fatal encephalitic disease in Americas, while OW alphaviruses (e.g. Sindbis, Ross River, Barmah Forest, O'nyong-nyong and Chikungunya viruses) are predominantly associated with rheumatic disease in humans in Africa, Europe, Asia and Australia (Suhrbier et al., 2012).

Alphavirus comprises of 30 virus species distributed throughout the world (Brown et al., 2018). They are all arthropod-borne viruses with exceptions of two aquatic alphaviruses: southern elephant seal virus and salmon pancreatic disease virus (Giry et al., 2017). According to Weaver & Smith (2011), the species of genus *Alphavirus* are antigenically classified into 8 complexes as shown in Table 1.1.

The *Alphavirus* virion is 70 nm in diameter and comprises of a T=4 icosahedral symmetry of nucleocapsid, surrounded by a host cell-derived lipid envelope embedded with glycoprotein spikes (As shown in Figure 1.1). The RNA genome is 11.4-12 kb in length, containing a 5'methylguanylate cap and 3' polyadenylate tail. The 5' two-thirds of the genome is translated into the viral non-structural proteins (nsP1, nsP2, nsP3 and nsP4),

whereas the 3' one-third of the genome or subgenomic RNA is translated into the viral structural proteins (C, E3, E2, 6K and E1) (Weaver & Smith, 2011). Figure 1.2 shows the genome organization of *Alphavirus*.

Table 1.1: Alphaviruses

Antigenic Complexes	Virus species
Barmah Forest	Barmah Forest virus
Middelburg	Middelburg virus
Ndumu	Ndumu virus
Semliki Forest	Semliki Forest virus
	Chikungunya virus
	O'nyong-nyong virus
	Getah virus
	Bebaru virus
	Ross River virus
	Mayaro virus
	Una virus
Eastern Equine Encephalitis	Eastern equine encephalitis virus
Venezuelan Equine Encephalitis	Venezuelan equine encephalitis virus
	Mosso das Pedras virus
	Everglades virus
	Mucambo virus
	Tonate virus
	Pixuna virus
	Cabassou virus
	Rio Negro virus
Western Equine Encephalitis	Western equine encephalitis virus
	Sindbis virus
	Whataroa virus
	Aura virus
	Highlands J virus
	Fort Morgan virus
Trocará	Trocará virus

Note: Alphaviruses isolated from fish and seals are not included.
(Source: Adapted from Weaver & Smith, 2011)

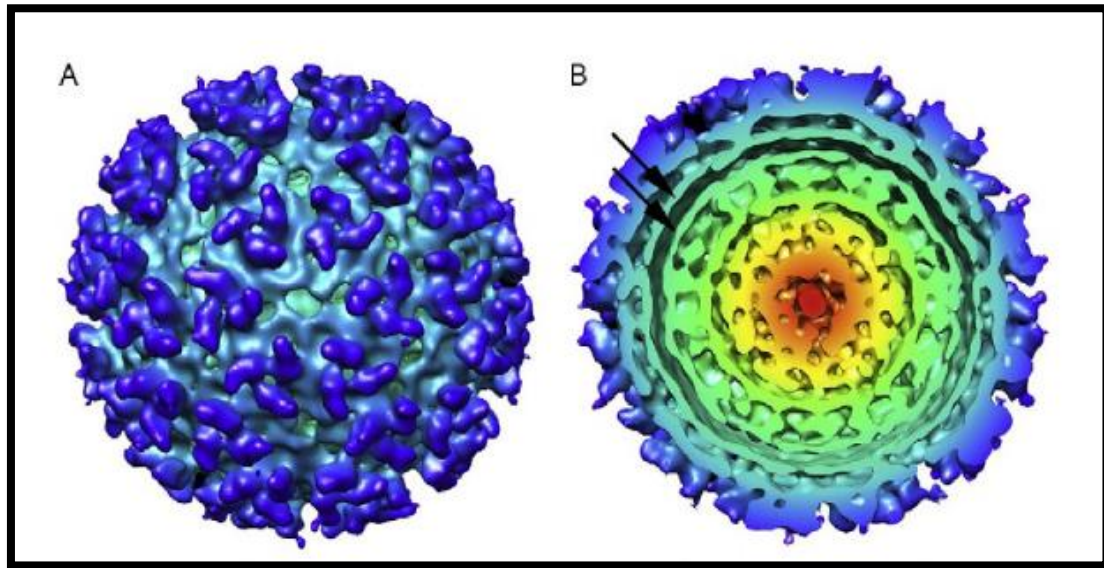


Figure 1.1: A schematic diagram of *Alphavirus* structure (A) Cryo-EM reconstruction of alphavirus. (B) Cutaway view of the structure, the outer most layer containing glycoprotein spikes is shown in blue, the inner most nucleocapsid shell of the virus particle is shown in green, yellow and red. The host cell-derived lipid bilayer is sandwiched between the layers (arrows).

(Source: Vancini et al., 2015)

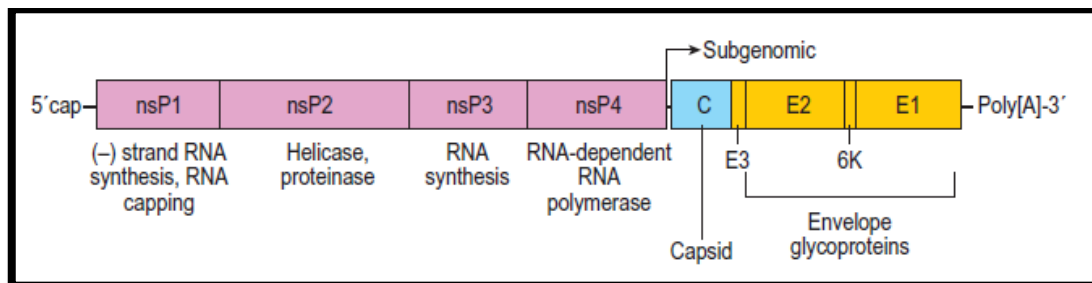


Figure 1.2: A schematic representation of *Alphavirus* genome organization (Source: Weaver & Smith, 2011)

1.2 An overview of Chikungunya virus (CHIKV) infection

CHIKV infection is an important arthropod-borne virus (arbovirus) disease caused by CHIKV, which is mainly transmitted through the bite of infected *Aedes* mosquitoes. The word Chikungunya means “to walk bent over” in Makonde word, which is a Bantu language spoken by an ethnic group in south-east Tanzania and northern Mozambique, in reference to the effect of incapacitating arthralgia suffered by infected individuals (Pialoux et al., 2007).

Human CHIKV infection is typically symptomatic, with a sudden onset of fever, followed by headache, maculopapular rash, and incapacitating arthralgia in major cases (Thiboutot et al., 2010). Normally the patients are quite sick as arthralgia is especially painful and debilitating, although the acute illness only lasts about 3-5 days, with recovery usually in 5-7 days (Weaver & Smith, 2011). In India, the national burden of CHIKV infection was estimated to be 25,588 DALYs (disability adjusted life years; a measure of debilitating disease burden) lost during 2006 CHIKV epidemic, in which persistent arthralgia was found to impose 69 % of the total DALYs. This resulted in heavy epidemiological burden and productivity loss to the community (Krishnamoorthy et al., 2009).

Although CHIKV infection is a self-limiting disease, severe neurological and hepatological complications of CHIKV infection can sometimes occur and may be life-threatening (Mohan et al., 2010; Presti et al., 2016). Fatalities following CHIKV infection have been reported in Mauritius, La Reunion Island and India (Weaver & Lecuit, 2015). A total of 254 excess deaths occurred during the 2005-2006 epidemic in La Reunion Island. On the other hand, in India (Ahmedabad), a total of 2,944 excess deaths were reported from August to November 2016 when compared with the average number of deaths in the same months for the previous 4 years. Most of the excess deaths occurred in patients with underlying chronic medical problems and patients older than 75 years old, as well as, rarely, in neonates.

1.3 Chikungunya virus (CHIKV)

1.3.1 Virus structure and genome organization

Like other members of the genus *Alphavirus*, CHIKV is relatively small (~70 nm in diameter), icosahedral-shaped, enveloped virus. Its RNA genome is surrounded by capsids,

and together form a complex called “nucleocapsid” (Yap et al., 2017). The nucleocapsid is surrounded by a close-fitting, host cell-derived lipid membrane which is enriched with cholesterol and sphingolipid. This cholesterol and sphingolipid-enriched membrane is essential for entry and budding process of virus particles (van Duijl-Richter et al., 2015). A small amount of membrane-associated protein 6K is also reported to enhance membrane permeability, which facilitates virus entry and budding (Guo et al., 2014). The membrane surface of the virus is covered by an arrangement of almost continuous layer of glycoprotein spikes, each comprises envelope proteins E1 and E2 in an icosahedral lattice with T=4 symmetry. The envelope proteins E1 and E2 together form 240 copies of heterodimers and arranged as 80 trimeric spikes (Sun et al., 2013). Both E1 and E2 are transmembrane proteins with C terminal cytoplasmic regions embedded within the membrane and interact with the nucleocapsid (Leung et al., 2011). Figure 1.3 shows the cryo-EM structure of the CHIKV. CHIKV contains an approximately 11.8 kb long, positive-sense single stranded RNA genome with a cap-structure at 5' end and polyadenosine tail at 3' end. The RNA genome composed of two large open reading frames (ORFs), in which the N-terminal ORF (7422 nucleotide) encodes the non-structural proteins (nsP1, nsP2, nsP3 and nsP4) while the C-terminal ORF (3744 nucleotide) encodes structural proteins (C, E3, E2, 6K and E1) (Leung et al., 2011). Figure 1.4 shows the genomic organization of CHIKV.

There is a non-translated junction region (J) between two ORFs, which contains an internal promoter and start site responsible for the transcription of 26S sub-genomic RNA (Murugan & Sathishkumar, 2016). Translation of 49S genomic RNA generates 2474 aa of non-structural polyprotein precursor, which is further cleaved into 4 non-structural proteins. On the other hand, a 1244 aa of structural polyprotein precursor is synthesized from the translation of the 26S sub-genomic RNA. The structural polypeptide undergoes cleavage

and post-translation modifications to form three major proteins (C, E1, E2) and two minor proteins (E3, 6K) (Leung et al., 2011; Murugan & Sathishkumar, 2016).

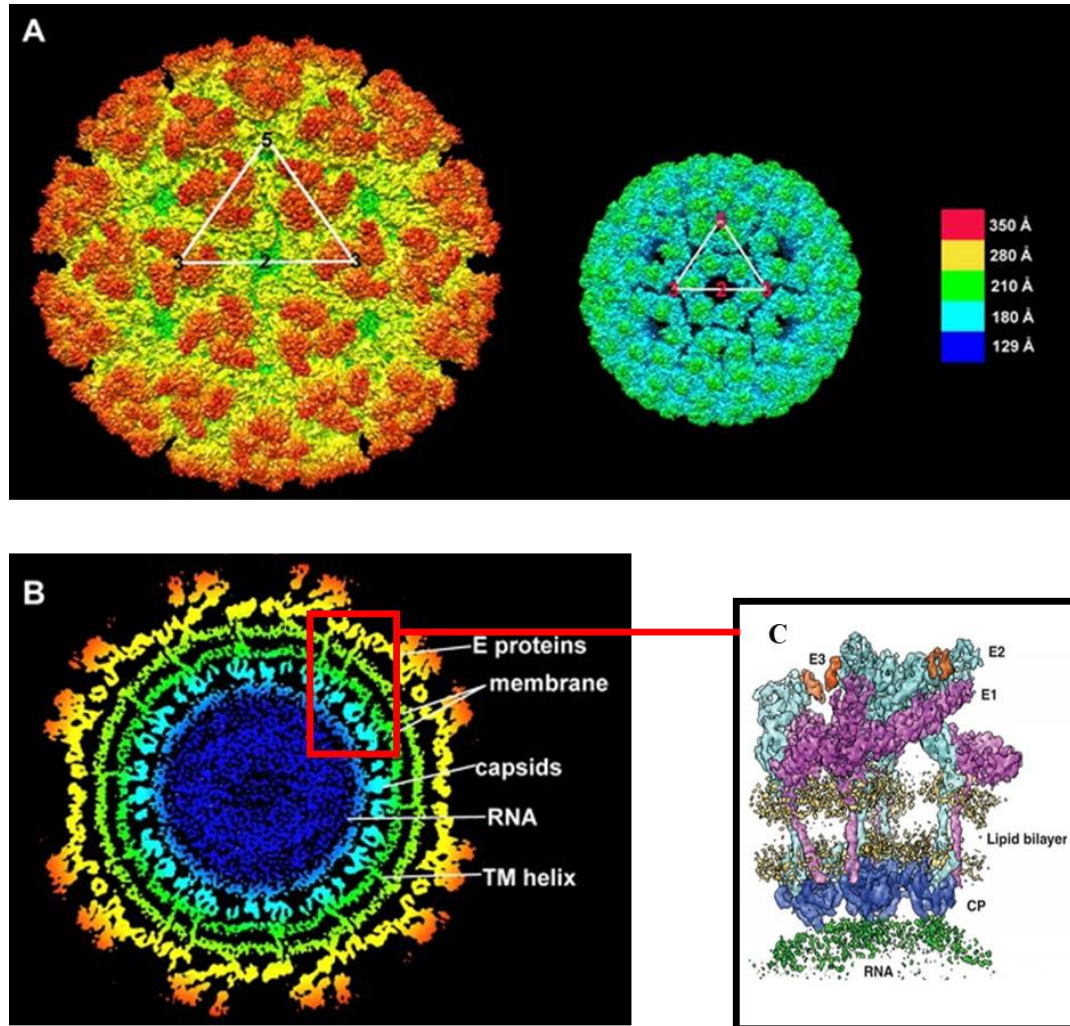


Figure 1.3: Cryo-EM reconstruction of CHIKV. (A) 3D cryo-EM map of CHIKV ectodomain (left), internal capsid protein shell of the CHIKV (right), icosahedral asymmetric unit is marked by a white triangle. (B) Central cross-sections of the immature CHIKV. (C) Enlarged view of the region outlined by the red rectangle in Figure B.

(Source: Adapted from Weaver et al., 2012; Sun et al., 2013)

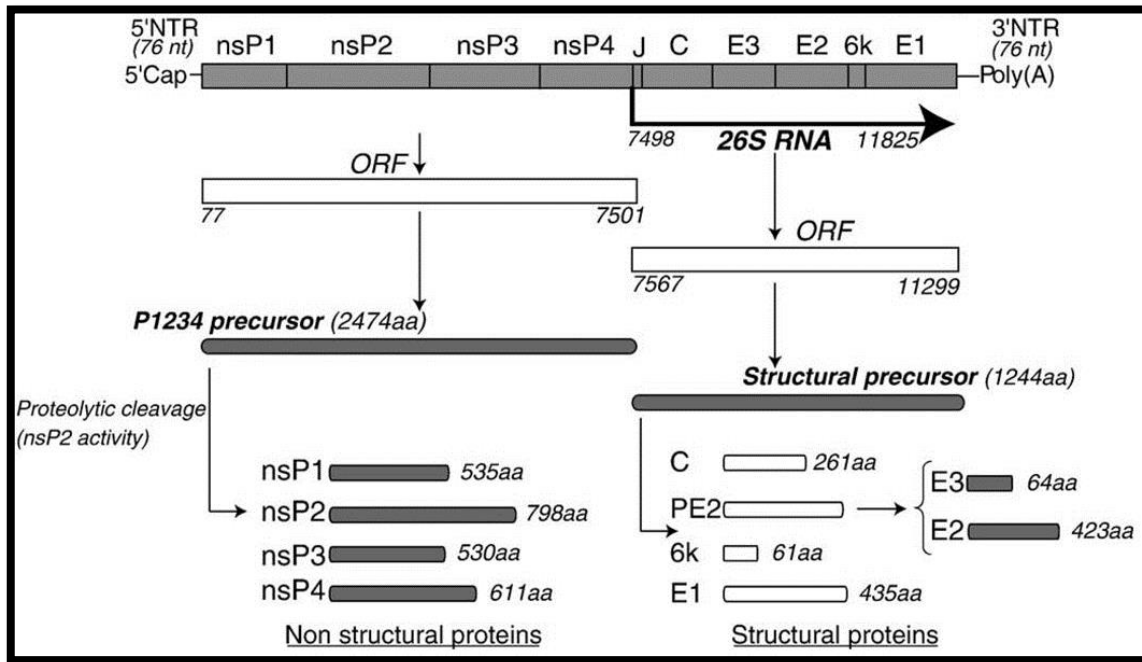


Figure 1.4: A schematic representation of the CHIKV genome and gene products. The genome organization is arranged as with other alphaviruses. The 5' ORF is translated from genomic RNA and encodes four non-structural proteins (nsP1, nsP2, nsP3, nsP4). The 3' ORF is translated from a sub genomic 26S RNA and encodes the structural proteins (capsid, E3, E2, 6K and E1).

(Source: Solignat et al., 2009)

1.3.2 Viral replication cycle

According to Hoornweg et al. (2016), the entry of CHIKV into host cells occurs via a clathrin-mediated endocytosis (CME) following the attachment of E2 protein to the specific receptor on the host cell surface and membrane fusion of the CHIKV and host cell, which is triggered by E1 protein.

The putative receptor binding sites are located on both domains A and B of E2 protein, in which domain B attach only to GAG expressing cells while domain A attach additionally to GAG-deficient cells (Weber et al., 2017). During the host cell receptor binding, CHIKV undergoes a rapid internalization by clathrin-coated pits to form a clathrin-coated vesicle in the presence of dynamin. Dynamin is an approximately 100 kDa protein. It assists the budding of clathrin-coated pits, which are responsible for the coated vesicles

formation (Leung et al., 2011). The clathrin-coated vesicle containing CHIKV is transported through the cytoplasm. During the transportation, the clathrin molecules begin to detach from vesicle and the virus is delivered to the early endosome (Hoornweg et al., 2016). Acidic environment within the early endosome mediates conformational changes of E1/E2 spikes by dissociating E1 protein from E2 protein, then exposes the fusion loop to the endosomal membrane (Solignat et al., 2009; Leung et al., 2011; Tang, 2012). Subsequently, the fusion loop is inserted into the endosomal membrane and this forces the two opposing membranes together until the membranes are merged together. Fusion of the two opposing membranes leads to the release of the nucleocapsid into the cytoplasm through the fusion pore (Murugan & Sathishkumar, 2016).

Within the cytoplasm, the nucleocapsid disassembles to release viral genome. The viral RNA genome is then translated to produce viral non-structural proteins (nsP1-nsP4). These non-structural proteins are assembled to form a viral replicase complex, which catalyses the synthesis of a negative-sense RNA strand. The negative-sense RNA strand serves as a template for synthesis of the full-length positive-sense genome (49S) and sub-genomic (26S) RNA (Tang, 2012).

The sub-genomic (26S) RNA is translated into a polypeptide precursor, C-pE2-6K-E1. C is then cleaved off by an autoproteolytic serine protease and associated with the RNA genome to form the nucleocapsid. Meanwhile, the cleavage of C reveals a signal sequence in pE2, which triggers the translocation of the remaining polyprotein (pE2-6K-E1) to the endoplasmic reticulum (ER). Once the polyprotein enters the ER, host signalases cleave 6K at the N- and C- terminal end (as shown in Figure 1.6), yielding pE2, 6K and E1. The E1 protein then forms a heterodimer together with pE2 and the pE2-E1 heterodimer is delivered to the cell surface via the golgi complex. During this movement, pE2 is cleaved by host furin protease to form mature E2 and E3 proteins. The mature E2-E1 heterotrimers are formed

and displayed at the cell-surface as trimeric spikes. Thereafter, the newly synthesized nucleocapsid together with the mature heterotrimers complete the viral assembly process (Solignat et al., 2009; Tang, 2012). The fully assembled virion particles are then enclosed by host cell-derived lipid bilayer and released from the host cell via budding. The CHIKV replication cycle is shown in Figure 1.5.

1.3.3 Viral proteins

1.3.3.1 Non-structural proteins

CHIKV non-structural proteins (nsP1-4) are resulting from the maturation of non-structural polyprotein. They are critical components of the viral replicase complex in viral replication (Schwartz & Albert, 2010). They are also essential in protein modification and immune antagonism (Sun et al., 2013).

nsP1: CHIKV nsP1 is a 535 aa long protein responsible for the negative strand RNA synthesis initiation, methylation and capping of newly synthesized viral RNA (Leung et al., 2011). Upon the RNA replication, nsP1 embeds the replication complexes in cellular membrane.

nsP2: CHIKV nsP2 is a 798 aa long protein exhibits RNA helicase, triphosphatase activities as well as protease activities which catalyse the cleavage of the precursor polypeptide to yield the mature non-structural proteins.

nsP3: A 530 aa long of nsP3 is part of the replicase unit of CHIKV which displays an ADP-ribose binding capacity that could facilitates the assembly of RNA by non-structural proteins. This RNA-binding property is due to the presence of positively charged amino acid at the surface of the protein (Solignat et al., 2009).

nsP4: CHIKV nsP4, a 611 aa long of structural protein which functions as viral RNA polymerase, work together with nsP1 to catalyse the initiation of negative strand RNA synthesis (Khan et al., 2002).

1.3.3.2 Structural proteins

Co- and post-translational modification of the structural polyprotein precursor (C-pE2-6K-E1) leads to the formation of 3 major structural proteins (C, E2 and E1) and 2 minor proteins (E3 and 6K).

C: C protein of CHIKV performs as a multifunctional protein in different stages of viral life cycle, which functions as a serine protease for self-cleavage, encapsulates viral genome and interacts with E1/E2 glycoproteins during virion formation (Goh et al., 2015). C protein is 261 aa in length, with a molecular weight of approximately 30 kDa, which is small enough to pass through nuclear pores (Solignat et al., 2009). It comprises two domains: N-terminal RNA binding domain rich in positively charged amino acids arginine, lysine and proline and C-terminal serine protease domain (Hong et al., 2006).

E3: E3 protein is a small and cysteine rich peptide which contains 64 aa, with a molecular weight of approximately 11 kDa. In viral life cycle, E3 functions as the signal sequence for translocation of the pE2-6K-E1 polyprotein precursor into ER (Snyder & Mukhopadhyay, 2012). E3 also forms a part of virus spikes, mediates proper folding of pE2 and stabilizes the interaction between E2 and E1 during viral particle assembly (Jose et al., 2009). E3 makes most of the contacts with E2 via a long β -ribbon connector, thereby ensuring the correct folding of E1/E2 heterodimer and prevent the disassembly of premature E2 and E3 (Snyder & Mukhopadhyay, 2012).

E2: E2 protein is a 50 kDa type I transmembrane glycoprotein. It is 423 aa in length, including 364 aa of N-terminal ectodomain, 26 aa of transmembrane (TM) helix and 33 aa of cytoplasmic endodomain (Li et al., 2010). The N-linked glycosylation sites of E2 are located at residues 263 and 345. The ectodomain of E2 is divided into three domains, namely domain A, B and C. Domain C is located towards the viral membrane and followed by a stem region which linked to TM region. Domain B is located at the tip of protein protruding

from the viral surface and it is linked to domain A via a long β –ribbon connector (Voss et al., 2010; van Duijl-Richter et al., 2015). On a mature virion, 240 copies of E1 and E2 are associated to form 80 trimeric spikes in which a single spike containing three E1/E2 heterodimers. The spikes are arranged in icosahedral T=4 symmetry and form a protein shell to surround the nucleocapsid (Metz et al., 2013).

6K: 6K protein is a small, hydrophobic peptide with a length of about 61 aa. 6K protein is acylated with fatty acids and forms a constitutive membrane protein. The role of 6K in viral replication is not fully understood, it appears to be involved in virus glycoprotein processing, cell membrane permeabilization and budding process (Jose et al., 2009).

E1: E1 protein is classified as a 45 kDa of class II viral fusion protein. It is 439 aa long, including 404 aa of N-terminal ectodomain, 30 aa of transmembrane (TM) helix and 5 aa of cytoplasmic domain at the C-terminal end. There is an N-linked glycosylation site situated at residue 141. The N-terminal ectodomain is folded into three β -sheet-rich domains, namely domain I, II and III. Domain III is located at the C-terminus of the protein which is the closest to the viral membrane, domain I is located at the central, and domain II is located at the tip of the protein. Domain II contains a hydrophobic fusion loop, which is essential for the fusion of viral and endosomal membranes (Sun et al., 2013; van Duijl-Richter et al., 2015).

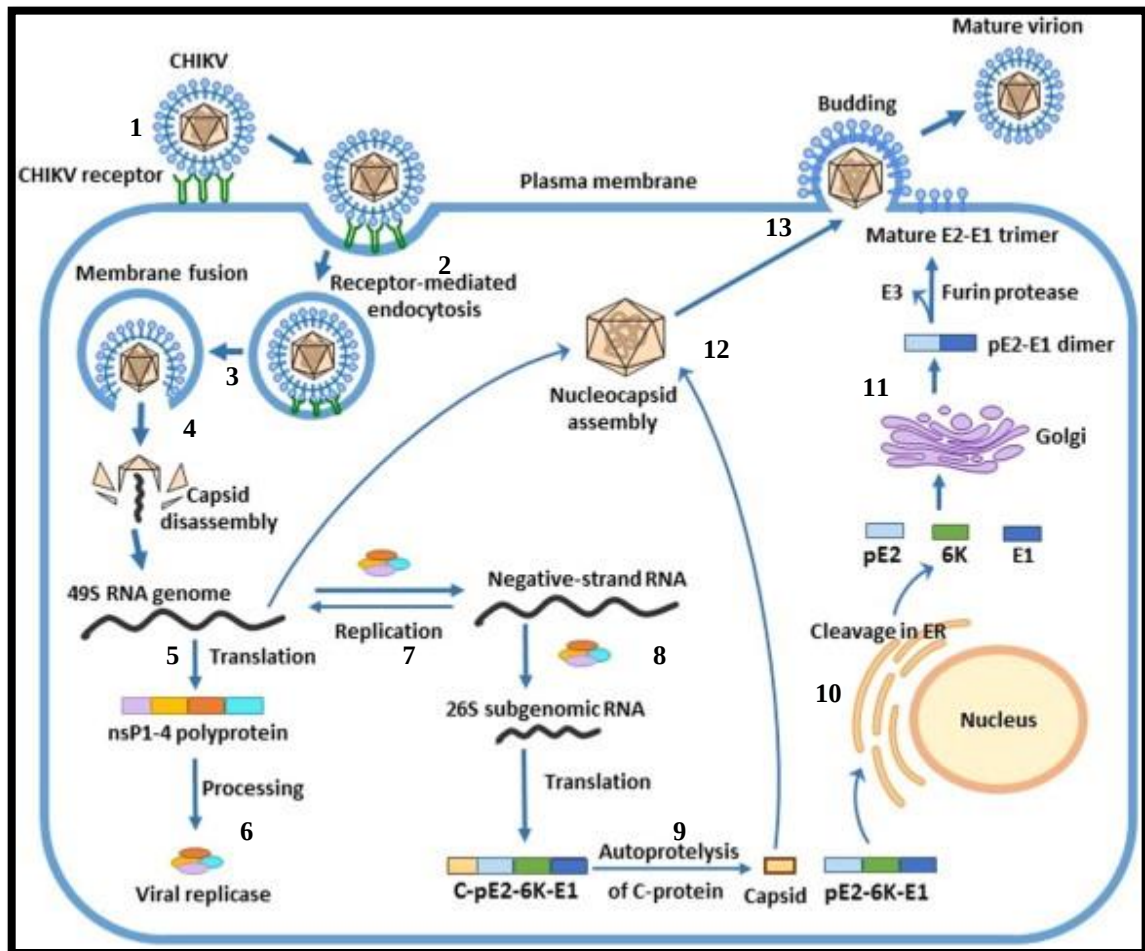


Figure 1.5: A schematic diagram of the CHIKV replication cycle. 1) Attachment of viral E2 to the receptors on host cell surface. 2) The virus enters the cells via clathrin-mediated endocytosis. 3) The acidic environment triggers the fusion of viral and host cell membrane and leads to the liberation of nucleocapsid to cytoplasm. 4) The nucleocapsid disassembles to release the viral genome into cytoplasm. 5) Non-structural proteins are translated from 49S RNA genome. 6) & 7) Non-structural proteins assemble together to form a viral replicase complex, which is used to synthesize a full-length negative-strand RNA intermediate. 8) The negative-sense RNA strand serves as a template for the production of 26S RNA. The 26S RNA drives the expression of the structural polyprotein precursor (C-pE2-6K-E1). 9) The polyprotein precursor is autoproteolytically cleaved to release C from the pE2-6K-E1 polyprotein. 10) The pE2-6K-E1 polyproteins are translocated into the ER to continue processing, the pE2, 6K and E1 proteins are generated by further processing through ER. 11) Both pE2 and E1 proteins are assembled in Golgi to form pE2-E1 heterodimer and delivered to plasma membrane, where pE2 is cleaved into E2 and E3 by furin protease. 12) C interact with the genomic RNA to form the nucleocapsid. 13) The viral components are recruited to form a virus particle and the assembled virus particle is liberated by budding through plasma membrane.

(Source: Adapted from Abdelnabi et al., 2015)

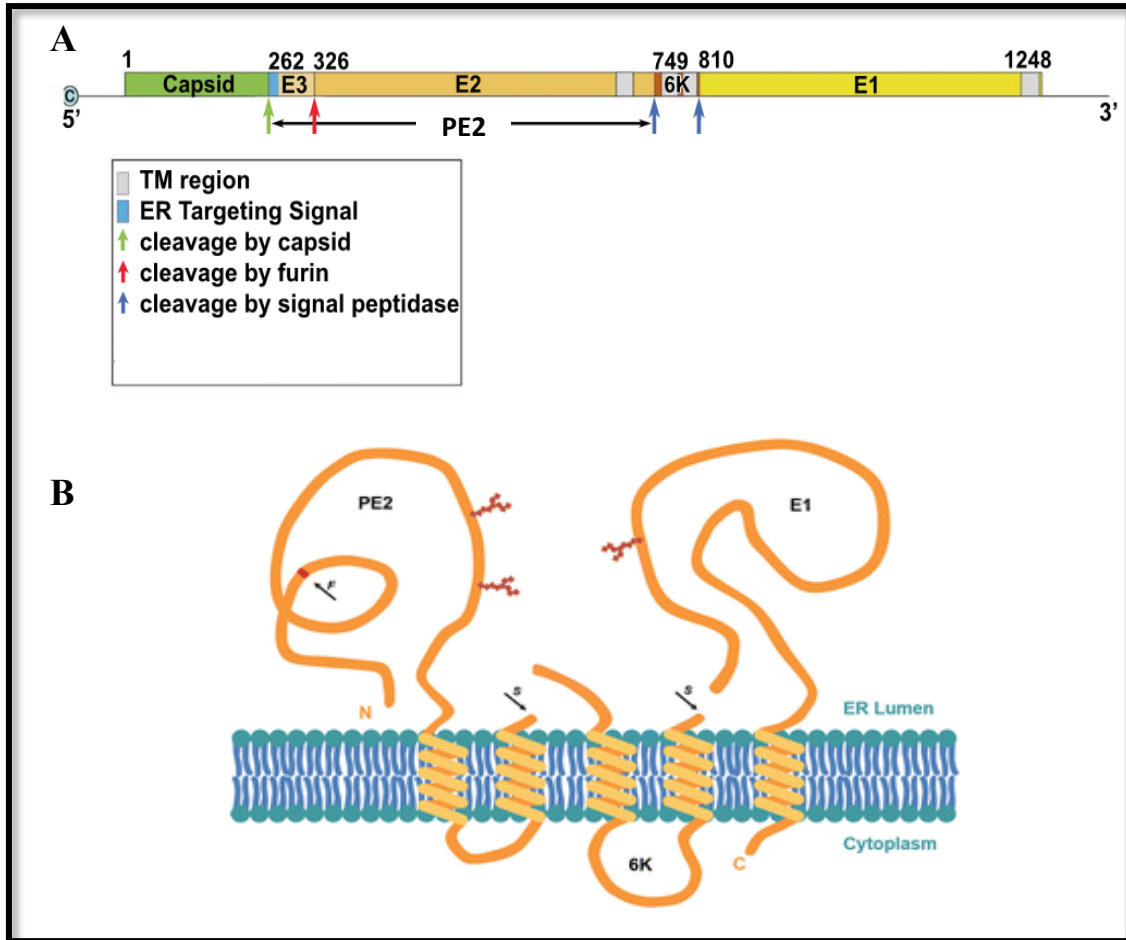


Figure 1.6: The organization of the CHIKV structural polyprotein and its topology with respect to the ER membrane. A) A linear diagram of CHIKV structural polyprotein. Symbols for important features and processing are shown in the legend box. B) A schematic representation of ER-membrane organization of the CHIKV envelope polyprotein during folding in the ER membrane of the infected cell. N- and C- terminal ends are indicated. The cleavage sites of signalases (S) and furin (F) are indicated by arrows.

(Source: Adapted from Voss et al., 2010; Metz & Pijlman, 2011)

1.3.4 Viral host range and transmission cycle

In Africa, the sylvatic cycle (animal-mosquito-animal) exists primarily in forest environment, where the virus circulates between non-human primates (chimpanzees, monkeys and baboons) and forest dwelling *Aedes* species (such as *Aedes africanus*, *Aedes furcifer-taylori*, *Aedes dalzieli*, *Aedes luteocephalus*) and non-*Aedes* species (*Mansonia*,

Culex) (Thiboutot et al., 2010). Sylvatic cycle has not been identified in Asia, however, it is probably present due to human activity.

In the rural village areas, human outbreaks tend to be small and sporadic. One of the factors contributed to these outbreaks is environmental condition, such as increased rainfall (Petersen & Powers, 2016). During heavy rain falls, the sylvatic mosquitoes can proliferate explosively, particularly of the *Aedes furcifer-taylori* group. They surpass the forest boundaries, enter the nearby villages and introduce the CHIKV infection among the villagers. Forest dwelling *Aedes furcifer* has been described as a major bridge vector responsible for the spillover of sylvatic CHIKV to humans as it is clearly demonstrated that *Aedes furcifer* landed frequently on humans in villages (Diallo et al., 2012). In the village cycle, the domesticated mosquitoes *Aedes aegypti* become infected and participate in the CHIKV transmission. CHIKV infection is then translocated into urban cycle via the human migration towards large urban area, where the urban mosquitoes *Aedes aegypti* and *albopictus* are predominant vectors (Slavov et al., 2015). Infection of *Aedes aegypti* results in rapid human-mosquito-human transmission cycles in urban areas, which is ideal for epidemic transmission.

Aedes aegypti is considered as a dominant vector in the tropics and sub-tropics (i.e. endemic areas of Africa and Asia). It is a highly efficient vector due to the habit of preferentially feeds on human blood over that of other animals and it usually feeds on different humans in order to obtain a complete, single blood meal (Galán-Huerta et al., 2015). *Aedes aegypti* predominantly breeds in stored fresh water found around human habitation in urban and semi-urban area, such as flower vase, water storage vessels and concrete water tanks in bathrooms (Mohan et al., 2010). This often brings the vector in close proximity to human hosts which increases the efficiency of human-mosquito-human transmission cycles in urban areas.

In addition to *Aedes aegypti*, *Aedes albopictus* (Asian tiger mosquito) is an aggressive, human-biting mosquito that is globally distributed, most likely via the shipment of timber and used car tires from Asia throughout the world in the early 1980s. Unlike *Aedes aegypti*, which only exists in the tropic and sub-tropic regions, *Aedes albopictus* has also emerged as a vital vector in temperate and even cold temperate regions, thus potentially introducing CHIKV to new ecological niches (Mohan et al., 2010; Petersen & Powers, 2016). *Aedes albopictus* has a wide range of breeding sites, such as coconut husks, cocoa pods, bamboo stumps, tree holes, rock pools as well as the artificial containers. It has a longer lifespan than other species of *Aedes* mosquitoes and its eggs are highly resistant to adverse environmental conditions (Galán-Huerta et al., 2015). *Aedes albopictus* is also able to fly for a relatively long distance with a flight radius of approximately 400-600 m (Kucharz & Cebula-Byrska, 2012). These biology features make *Aedes albopictus* an efficient vector for CHIKV transmission among humans. Based on the genetic investigation of CHIKV strains circulating in the areas where *Aedes albopictus* serves as primary vectors, a specific mutation (E1-A226V) was identified in the glycoprotein E1 of CHIKV. This mutation enhances CHIKV infectivity to *Aedes albopictus*, which facilitates CHIKV transmission and likely facilitated CHIKV infections globally (Tsetsarkin et al., 2007). Figure 1.7 shows the epidemiologic cycles of CHIKV.

1.3.5 Virus genotypes

Based on the phylogenetic analysis of CHIKV E1 sequences, three CHIKV distinct genotypes have been defined: West African, East/Central/South African (ECSA), and Asian (As shown in Figure 1.8).

The geographical distribution of these genotypes has been restricted to certain regions with the West African and ECSA genotypes responsible for all the outbreaks in Africa, while the Asian genotype was responsible for outbreaks in Asia (Horwood & Buchy,

2015). In 2005, the ECSA genotype spread from Kenya eastward across several islands in the Indian Ocean and most of the countries in Asia. The re-emergence of the ECSA genotype is related to an adaptive mutation in the E1 glycoprotein gene, which enhances the transmissibility of this virus in *Ae.albopictus* mosquitoes. These adaptive mutated viruses were then grouped into ECSA Indian Ocean Lineage (IOL) (Auerswald et al., 2018).

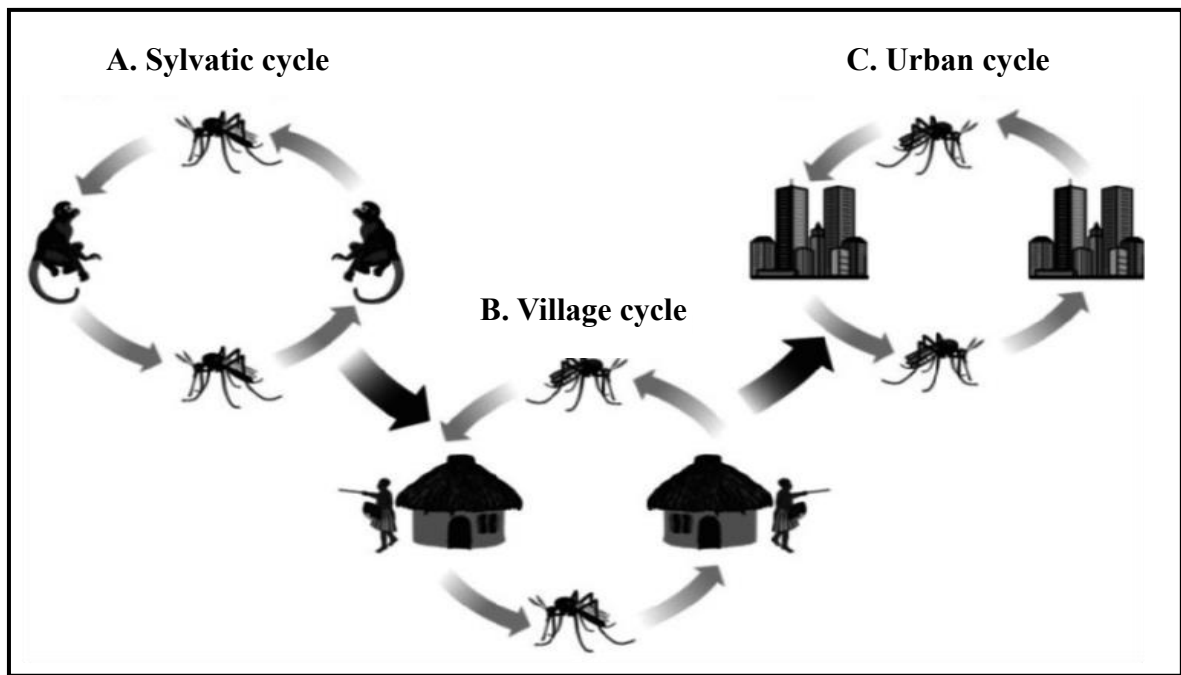


Figure 1.7: Epidemiologic cycles of CHIKV. A) Sylvatic cycle. The virus is transmitted between non-human primates and different species of forest dwelling mosquitoes including *Aedes* mosquitoes (*Aedes africanus*, *Aedes furcifer-taylori*, *Aedes dalzieli*, etc.) and non *Aedes* mosquitoes (*Mansonia*, *Culex*, etc.). B) Village cycle. During heavy rainfalls, the forest dwelling mosquitoes surpass the forest barriers and introduce the infection in the nearby village. In this cycle, the domesticated mosquitoes *Aedes aegypti* become infected and involve in CHIKV transmission. C) Urban cycle. The infection is translocated towards large urban centers via the human migration. The transmission in urban cycle is performed by urban mosquitoes *Aedes aegypti* and *Aedes albopictus*.

(Source: Adapted from Slavov et al., 2015)

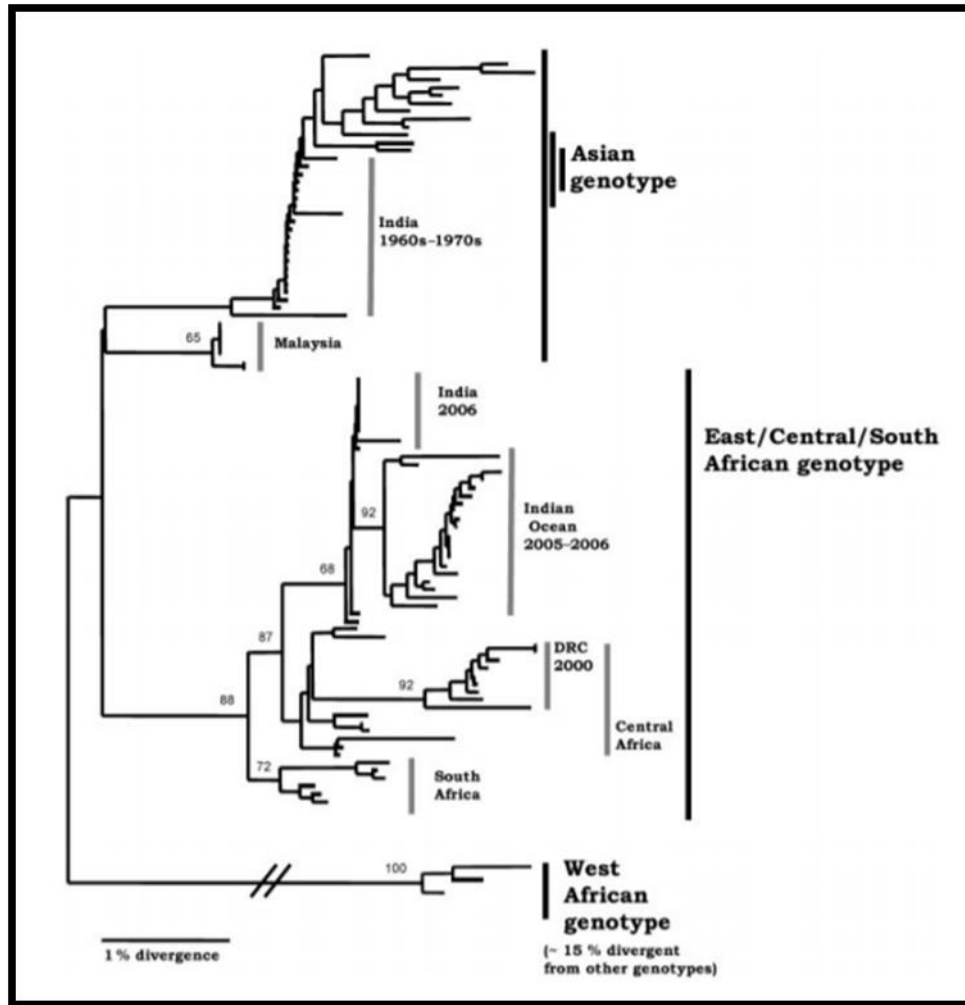


Figure 1.8: A phylogram of CHIKV E1 sequences. Three CHIKV genotypes and close relationships among the lineages from each genotype based upon geography and time of the outbreak.

(Source: Adapted from Power & Logue, 2007)

1.3.6 Global distribution of CHIKV

CHIKV is believed to have originated from Central/East Africa where forest dwelling mosquitoes circulate the virus among non-human primates such as baboons and *Cercopithecus* monkeys (Kumarasamy et al., 2006). This sylvatic transmission cycle may be the key player in the occasional emergence and re-emergence of CHIKV infection (AbuBakar et al., 2007). The emergence of CHIKV in human-mosquito cycle in Africa may have started as early as the 18th century, and to date, CHIKV outbreaks have been identified around the world (Weaver & Lecuit, 2015). This global spread of CHIKV is probably caused

by immunocompromised human population in affected area, global trade, international travel and most notably, the adaptation of *A.albopictus* to CHIKV infection as they are expanding their territory around the globe (Murugan & Sathishkumar, 2016).

The first outbreak of CHIKV was reported in 1952 from Makonde plateaus, along the borders between Tanzania and Mozambique (Murugan & Sathishkumar, 2016). Subsequently, CHIKV was introduced into major parts of Africa and Southeast Asia throughout 1960s-1990s, most likely due to the lack of available diagnostics and the difficulty in differentiating chikungunya fever from dengue fever. In Asia, the first CHIK outbreak was reported in Bangkok, Thailand (1958) followed by various regions of India, including Kolkata (1963), Pondicherry and Vellore (1964), Visakhapatnam, Rajmundry and Kakinada (1965), Nagpur (1965), and Barsi (1973). Some minor outbreaks have been documented in Cambodia, Vietnam, Laos and Myanmar as well (Presti et al., 2014). In Africa, several epidemics occurred in different countries, including Democratic Republic of the Congo, Central African Republic, Malawi, Uganda, Zimbabwe, Burundi, Angola, Senegal, Guinea, South Africa and Nigeria.

In 2004, a large epidemic of CHIKV sustained by the circulation of the ECSA genotype dramatically re-emerged in Kenya, and spread to several islands of the Indian Ocean, including Comoros, Mayotte, Seychelles and most notably La Reunion Island in early 2005. Approximately 266,000 cases of CHIKV, nearly one-third of the population had been reported in La Reunion Island outbreak (Presti et al., 2014; Rezza, 2014). From the CHIKV outbreak in La Reunion Island, CHIKV was found to be susceptible to *A. albopictus* due to the specific mutation (E1-A226V) in E1 structural protein of CHIKV (Presti et al., 2016). The mutation presenting a substitution of alanine with valine in the position 226 of the E1 structural protein, enhances CHIKV infectivity for *A.albopictus* and facilitates in transmission of the CHIKV variant (Tsetsarkin et al., 2007). This viral variant was selected

during the course of the epidemic and became predominant in affected area where *A.albopictus* is heavily urbanized compared to *A.aegypti*, such as La Reunion Island and India (Rezsa, 2014).

In 2006, this epidemic strain has caused overwhelming epidemics in islands of Indian Ocean and Indian subcontinent, in which more than 1.4 million of cases have been reported in 13 states of India (Jaffar-Bandjee et al., 2010). Thereafter, CHIKV has spread from India to Southeast Asia followed by Italy, Europe. In 2007, first epidemic of CHIKV was reported in Italy, a temperate region in Europe, probably brought in by viremic travellers from CHIKV endemic regions of India (Staples et al., 2009).

In 2009, CHIKV transmission re-emerged in La Reunion Island, after which, sporadic CHIKV outbreaks were identified in Oceania, Eastern, Western, South eastern and Southern Asia, Central and Western Africa, Indian Ocean islands and Europe until 2012 (Galán-Huerta et al., 2015). With an increase in global travel, CHIKV has been imported into the western hemisphere in late 2013. After the initial discovery of CHIKV in Caribbean Island, CHIKV has spread to all countries in Central America, most of the countries in South America, Mexico and United States. The geographical transmission of CHIKV and distribution of different lineages are shown in Figure 1.9.

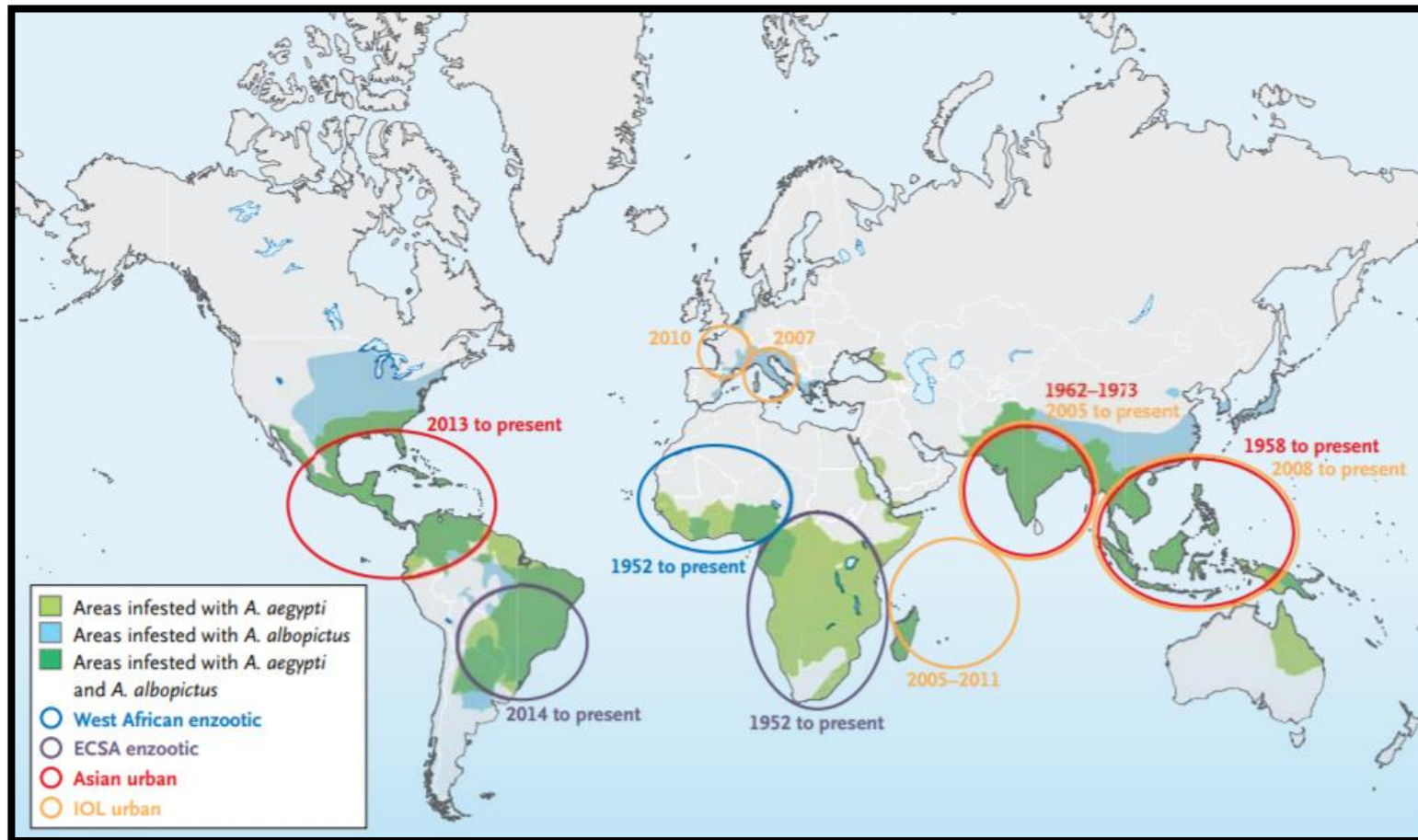


Figure 1.9: Geographical distribution of CHIKV and the vectors, *Ae.aegypti* and *Ae. Albopictus*.
(Source: Weaver & Lecuit, 2015)

1.3.7 Epidemiology of CHIKV in Malaysia

Like most of the developing countries, CHIKV outbreaks have been identified in Malaysia as well. It has been proposed that the transmission of CHIKV in Malaysia may have been introduced by migrant workers from countries where CHIKV is endemic as the development of Malaysia is heavily dependent on these workers (Lam et al., 2001). The first CHIKV outbreak in Malaysia occurred from December 1998 to February 1999 in Port Klang, Selangor when 51 persons were infected (AbuBakar et al., 2007). According to Lam et al. (2001), this affected area was characterized by poor sanitation and unsatisfactory waste management.

After a lapse of 7 years, CHIKV outbreak re-emerged in a fishing village located at Bagan Panchor, Perak in March 2006, affecting more than 200 persons with no deaths reported. Phylogenetic analysis revealed that this outbreak was closely related to the 1998 outbreak and both were attributed to the Asian genotype (Sam et al., 2009). Although this outbreak concurred with the peak of the 2005/2006 outbreak on La Reunion Island, India and islands of Indian Ocean, the authors suggested that the outbreak could have originated from an endemic CHIKV cycle not previously identified in Malaysia. Besides this, the apparent absence of CHIKV within these 7 years was probably due to an undetectable level of CHIKV transmissions among human or misdiagnosis of CHIK fever as dengue fever (AbuBakar et al., 2007).

In December 2006, CHIKV of ECSA genotype imported from India was re-introduced in Kinta district, Perak (AbuBakar et al., 2007). This outbreak was controlled successfully due to an early detection and establishment of intensive vector control measures (Zainah et al., 2010).

In April 2008, the largest CHIKV outbreak caused by circulation of E1-A226V mutation in ECSA strain occurred in Tangkak, Johor after two years of lapse (Zainah et al.,

2010; Ap et al., 2011). It then spread to other states in Peninsular Malaysia such as Negeri Sembilan and Melaka in late June and early July. Thereafter, the outbreak had spread to 14 out of 15 states and federal territories with a total of 2692 cases investigated by end of 2008 (Sam et al., 2009; Apandi et al., 2010). Most of the infected cases were identified in Kuala Lumpur, Malacca, Johor and Selangor, while fewer cases were identified in Kelantan, Sabah and Sarawak. However, in 2009, a shift was observed when these three states became the most affected areas in the country with the number of CHIKV cases increasing to 4430 cases in 2009 (Apandi et al., 2009).

Several factors have accounted for the rapid transmission of CHIKV from Johor to other states of Malaysia: a) Modification of ecosystem due to economic development in Malaysia; b) Abundance of vectors (*A.aegypti* and *A.albopictus*); c) The presence of a large immunocompromised population in Malaysia who do not have immunity against CHIKV; d) Failure of vector control measures implementation in CHIKV affected area due to the late detection of CHIKV occurrence (Zainah et al., 2010).

1.4 CHIKV vaccines development

Vaccines have been widely used as one of the most effective means to protect humans from the risk of infection by stimulating body's immune system and developing immunity to the infectious disease. At present, there is no licensed vaccine for CHIKV, although more than 15 vaccine candidates based on a range of platforms (inactivated, live attenuated, live vectored, chimeric, virus-like particle, subunit protein, consensus-DNA based) are currently in preclinical development and several in collaboration with industry. Table 1.2 summarizes the representative vaccine candidates based on a range of platform.

In the 1960s, the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) developed the first CHIKV vaccine prototype, a formalin-inactivated CHIKV vaccine by exposing the African green monkey tissue culture-derived

CHIKV (strain 15561, isolated from Thailand in 1964 outbreak) to formaldehyde (Harrison et al., 1971). This vaccine was safe when administered to new born mice and did not show any pathological or histological changes when injected into the central nervous system of rhesus macaques (Erasmus et al., 2016). However, the main concern of inactivated virus vaccine is high manufacturing costs as high level containment (BSL3) is required to reduce the risks associated with handling large quantities of CHIKV prior to inactivation (Rezza, 2015). Thus, this vaccine candidate may not be ideal for markets in developing countries.

Scientists from Walter Reed Army Institute of Research (WRAIR) propagated the seed of CHIKV strain 15561 in green monkey kidney and created CHIKV strain 15562. The 11th passage of strain 15562 was sent to USAMRIID and a live-attenuated vaccine seed virus, namely 181/clone 25 (hereafter 181/25) was developed after serial passages in the human lung fibroblast MRC-5 cells (Levitt et al., 1986). CHIKV 181/25 was shown to protect mice against lethal challenge and protect rhesus macaques from viremia after challenge. The live-attenuated vaccine seed virus was then manufactured in The Salk Institute-Government Service Division (TSI-GSD) and named as TSI-GSD-218 (Hoke et al., 2012). This vaccine candidate was demonstrated to be safe and immunogenic in a phase I trial. In the phase II trial, 59 volunteers received a single subcutaneous dose of the TSI-GSD-218 vaccine, 95 % of them elicited neutralizing antibodies and 85 % remained seropositive 1 year after immunization, however, transient arthralgia was experienced by 8 % of the volunteers. Thus, USAMRIID decided to halt further development of this attenuated vaccine strain in 1998, although it showed promising results in phase 1 and 2 clinical trials (Edelman et al., 2000). Thereafter, Indian Immunologicals Ltd (IIL), a leading vaccine manufacturer, received vaccine materials from WRAIR and reinitiated the vaccine development. However, Gorchakov et al. (2012) suggested that the attenuated 181/25 strain is attenuated by two

point mutations, it can be reactogenicity and possible reversion to virulence. Thus, this vaccine development remains questionable due to safety concerns.

In addition to the USAMRIID vaccine, novel alternative live-attenuated virus vaccine candidates against CHIKV have been developed and tested, with promising results in animal models. Initially, chimeric alphavirus vaccine was developed by Wang et al. (2008), using the backbones of attenuated Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and Sindbis virus (SIV), with the ORF encoded for structural polyprotein replaced by CHIKV structural polyproteins genes to produce the chimeras. These chimeric vaccine strains were found to stimulate strong humoral immunity and did not trigger reactogenicity in immunized adult mice. Another vaccine construct generated by Plante et al. (2011) contains an encephalomyocarditis virus internal ribosome entry site (IRES) between the CHIKV non-structural and structural genes of La Reunion CHIKV strain. The CHIKV/IRES vaccine is considered safer and stable compared to traditional attenuating single-point mutations, which may cause the risk of mutation reversion and pathogenicity reversion in vivo. Besides that, the insertion of IRES sequence down-regulates the expression of CHIKV structural genes, preventing the virus from infecting mosquitoes due to inefficient translation of the proteins from this IRES element.

There are always safety concerns associated with live-attenuated or inactivated virus vaccines, including under-attenuation and risk of reversion to virulence related to live-attenuated vaccines (naturally attenuated or chimeric) and incomplete inactivation of inactivated virus vaccine. To address these issues, scientists developed a consensus-DNA based vaccine. This vaccine was found to improve safety profile as compared to live-attenuated or inactivated virus vaccine. Furthermore, consensus-DNA based vaccine is able to induce cross-reactive immune responses against the three distinct groups of CHIKV, and

thus protect large populations from multiple strains of the virus. Promising results were obtained using consensus-DNA based vaccine candidates, which were found to be protective in mice and to induce neutralizing antibodies in nonhuman primates (Thiboutot et al., 2010).

In recent times, many vaccine candidates were developed using CHIKV structural proteins delivered by recombinant viral or plasmid vectors or prepared as subunit/VLPs. Subunit protein vaccines are constructed based on a limited number of viral proteins (Kumar et al., 2012). However, the vaccine is often less immunogenic due to absence of other viral and cellular components, most probably because a proportion of this protein is presenting a misfolded conformation relative to native protein (Roy & Noad, 2009). Consequently, the vaccine usually requires co-administration of adjuvants, frequent administrations and higher doses of the immunogen in order to achieve same level protection as inactivated virus vaccine (Josefsberg & Buckland, 2012).

In contrast to the subunit protein vaccine, a specific class of subunit vaccine called virus-like particles (VLPs) has emerged as a new attractive option to combat CHIKV infection. VLPs are generally formed by the self-assembly of envelope and/or capsid proteins. They mimic the native virus surface architecture and protein conformations, which are readily recognized by the immune system and induce protective antibody responses in the absence of adjuvants. VLPs have therefore shown dramatic effectiveness as candidate vaccines (Grgacic & Anderson, 2006).

Another approach that has been used to construct vaccine against CHIKV infection is the use of vaccine vectors such as live-attenuated measles virus (MV). This strain has demonstrated to elicit robust humoral and cellular immune response, yielding protective immunity against other arthropod-borne diseases, such as DENV and West Nile virus in mice and monkeys. MV-CHIK vaccines are constructed by inserting the CHIKV structural genes into live-attenuated measles virus vector's genome to generate a virus that initiates

CHIKV structural proteins expression upon infection. However, the concern of MV-based vaccines is the immunity yielded by previous MV infection or vaccination might disturb the protective efficacy and immunogenicity of a recombinant MV-based vaccine (Schwameis et al., 2016).

Thus far, there are two experimental vaccine candidates have completed phase 1 clinical trials and advanced to phase 2 clinical trials. The first candidate is a VLP vaccine, namely VRC-CHIKVLP059-00-VP (ClinicalTrials.gov: NCT02562482). This VLP vaccine comprised VLPs expressed from VRC293 mammalian cells, which transfected with bacmid encoding complete structural protein genes (C-E3-E2-6K-E1), derived from CHIKV strain 37997 (West African lineage). Another vaccine candidate which has been reported to reach phase 2 clinical trials is a live virus-vectored vaccine, namely MV-CHIK vaccine. MV-CHIKV has been tested in three Phase II trials conducted simultaneously at three different locations (ClinicalTrials.gov: NCT02861586, NCT03101111, NCT03028441) (Goyal et al., 2018). This is a vectored recombinant measles virus (MV) expressing complete structural proteins (C-E3-E2-6K-E1) derived from a La Reunion CHIKV. According to Schwameis et al. (2016), both vaccine candidates are unlikely to induce CHIK-like adverse events including arthralgia and chronic rheumatism due to the lack of live and replicating CHIKV. Therefore, there is no complaints of side effect likes arthralgia after vaccinated with VRC-CHIKVLP059-00-VP, underlining the vaccine is well tolerated and considered safe for immunosuppressed population.

Table 1.2: Vaccine candidates against CHIKV infection

Vaccine Type	Platform/Strategy	CHIKV Antigen	Developer	References
Inactivated virus	Formalin-treated	Whole virus (strain Thailand/15561)	USAMRIID	Harrison et al., 1971
		Whole virus (strain 181/ 25)	Indian Immunological	Levitt et al., 1986; Weaver et al., 2012
		Whole virus (strain DRDE06)	DRDE India	Tiwari et al., 2009
		Whole virus (strain CHK03/06)	Bharat Biotech	Ella et al., 2014
Live-attenuated virus	TSI-GSD-218 (181/25)	Full-length of strain Thailand/15562	USAMRIID/TSI-GSD	Levitt et al., 1986
	CHIKV/IRES	Full-length with IRES sequence	Tadeka/UTMB	Plante et al., 2011
	TM17-2	Full-length with truncation mutation in <i>trans</i> -membrane domain	Arbovax/NCSU	Piper et al., 2013
	CHIKV-Δ5nsP3	Full-length with deletion in nsP3	Valneva/ Karolinska Inst.	Hallengård et al., 2014
	CHIKV-Δ6K	Full-length with lacking 6K gene		
Chimeric alphavirus	Various genetic backbones (VEEV, EEEV, SIV)	Full-length chimera	UTMB/UAB	Wang et al., 2008
Live virus-vectored	MV-CHIKV	Measles virus expressing CE3E26KE1	Themis Bio GmbH/ Inst. Pasteur	Brandler et al., 2013
	VSVΔG-CHIKV	Vesicular stomatitis virus expressing E3E26KE1	Profectus/Yale/UTM B	Chattopadhyay et al., 2013
	MVA-CHIKV	Modified vaccinia Ankara virus expressing CE3E26KE1	Karolinska Inst./ CSIC Madrid	García-Arriaza et al., 2014
		Modified vaccinia Ankara virus expressing E3E2	Univ. Wisconsin/ Takeda	Weger-Lucarelli et al., 2014
	CAdVax-CHIKV	Adenovirus expressing CE3E26KE1	GenPhar Inc.	Wang et al., 2011

Table 1.2 continued

VLP	VRC-CHKVLP059-00-VP	CE3E26KE1 (strain 37997)	NIAID	Akahata et al., 2010
	VLP-CHIKV-S27	CE3E26KE1 (strain S27)	TI Pharma/ Wageningen Univ.	Metz et al., 2013
Subunits proteins	rCHIK-E1/E2-DRDE06	E1E2 (strain CHK-DRDE06)	DRDE India	Khan et al., 2012
	rE2-CHIK	E2	NIV, India	Kumar et al., 2012
Consensus DNA	p181/25-7CHIKViDNA	Full-length of strain 181/clone 25 with 2 attenuating mutations in E2 under pCMV promoter	Medigen	Tretyakova et al., 2014

1.5 Baculovirus-insect cells expression system

To date, baculovirus-insect cell expression system is widely used in producing recombinant proteins. It has been used to express heterologous genes from various sources, including fungi, plants, bacteria and viruses in insect cells.

1.5.1 Baculoviruses

Baculoviruses are generally a diverse group of insect-specific viruses, with sizes about 250-300 nm in length and 30-60 nm in diameter. The Baculovirus genome consists of a circular supercoiled double-stranded DNA (dsDNA) that is 80-180 kb in size, packaged in rod-shaped nucleocapsid and enclosed within an envelope to form a virion (Contreras-Gómez et al., 2014). The name “baculovirus” is derived from the Latin word “baculum”, which reflects to the rod shape of a baculovirus (Rohrmann, 2013).

Baculoviruses are characterized by their very narrow host specificity, predominantly infect insect species of the Lepidoptera order. They can also enter mammalian cells, but unable to infect mammalian cells. Thus, various baculoviruses have been developed as environmentally friendly biocontrol agents of insect in agriculture and forestry (Ikeda et al., 2015).

Based on the morphology of occlusion bodies, baculoviruses are distinguished by the nucleopolyhedrovirus (NPV) and granulovirus (GV). The occlusion body (OB) of NPV contains multiple virions and the NPVs are further divided into single or multiple nucleopolyhedrovirus (SNPV or MNPV), depending on whether the nucleocapsids are packaged singly or multiple into one infectious virion (Contreras-Gómez et al., 2014). *Autographa California multiple nucleopolyhedrovirus* (AcMNPV) is one of the first to be widely studied and used for the purposes of recombinant protein production. Figure 1.10 shows the classification of nucleopolyhedrovirus (NPV) and granulovirus (GV).

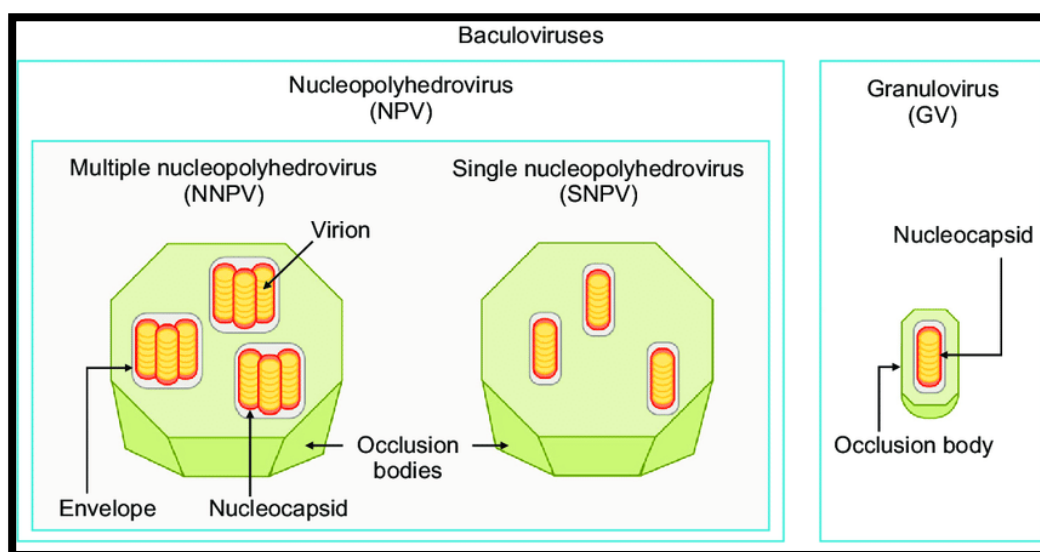


Figure 1.10: Classification of Nucleopolyherovirus (NPV) and Granulovirus (GV). Baculoviruses are divided into two genera (NPV and GV) which are characterized by morphology of occlusion bodies. NPV has multiple virions in polyhedral form of occlusion bodies, whereas GV has single virion in granule form of occlusion body. NPV strains are further divided into multiple nucleopolyhedrovirus (MNPV) and single nucleopolyhedrovirus (SNPV), depending on whether single or multiple nucleocapsids are present in each virion.

(Source: Lee et al., 2017)

NPV has a biphasic infection cycle, during which the two genotypically identical but phenotypically distinct forms of baculovirus viruses are produced: Budded virus (BV) and Occlusion bodies-derived virus (ODV) through a number of distinct and sequential steps (Ikeda et al., 2015). BV is generated after the primary infection via budding through plasma membrane of the infected cell and provides transmission of infection from cell to cell. On the other hand, ODV, a particle occluded in a crystalline protein matrix, called occlusion body (OB), initiates primary infection in the midgut epithelium of the ingesting insect and provides the transmission of infection from larvae to larvae (Contreras-Gómez et al., 2014). The replication cycle of the baculovirus is shown in Figure 1.11.

The polyhedron (pH) and p10 proteins, structural components of OB, are generated in the very late stage of infection. These two proteins are non-essential for recombination protein expression in insect cell cultures. Therefore, the pH and p10 promoters can be used

to drive recombinant protein expression. This finding results in the establishment of the baculovirus-insect cells expression system.

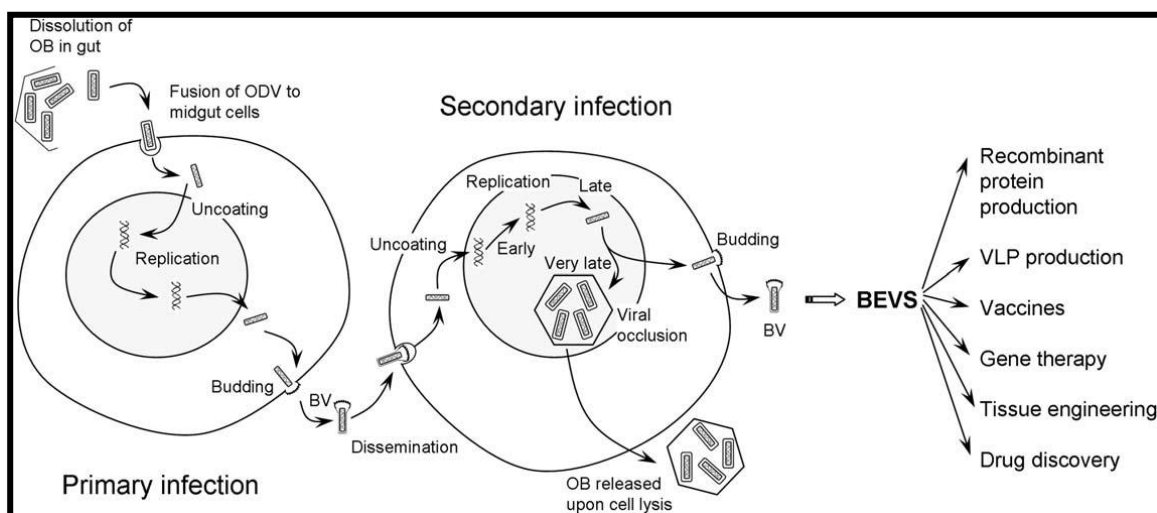


Figure 1.11: The replication cycle of a nucleopolyhedrovirus (NPV). Ingestion of foods contaminated with occlusion bodies containing ODVs by lepidopteran larvae results in primary infection of lepidopteran insects. The ingested OBs are disassembled in the alkaline environment of midgut lumen and liberating numerous ODVs for primary infection initiation. The primary infection occurs when the liberated ODV fuses with the insect midgut epithelial cells. The virus then replicated in the midgut cells and leads to the production of BVs. The BVs are liberated from the cell and initiate the secondary infection by infecting other tissue. The secondary infection gives rise to the generation of occlusion bodies which are then liberated from the cells via cell lysis and the death of the insect, to continue horizontal transmission of the infection. Genes polyhedron and P10 are responsible for BVs production, thus, these genes can be replaced by foreign genes to drive the expression of the target proteins. This also avoids the production of OBs in insect cells in culture.

(Source: Contreras-Gómez et al., 2014)

1.5.2 Bac-to-bac® baculovirus expression system

In this study, a bac-to-bac® baculovirus expression system (Invitrogen, California, USA) was used to express target CHIKV recombinant proteins. In principle, bacmid shuttle vector of this expression system is recombined with a gene expression cassette via site-specific transposition to generate a recombinant expression bacmid in *E.coli* cells. The recombinant bacmid is then transfected into insect cells (e.g. *Sf9* cells) with the aid of Cellfectin® II

reagent and results in the production of recombinant baculoviruses, which can express target recombinant proteins in the insect cells (Invitrogen, 2015).

The first component of the system is pFastBacTM vector into which the target genes will be cloned to generate a recombinant donor plasmid. There are three types of pFastBacTM vectors in this system, including pFastBacTM 1, pFastBacTM HT and pFastBacTM Dual vectors. In this study, pFastBacTM Dual vector was used to clone the target genes. The pFastBacTM Dual vector contains an expression cassette flanked by left and right arms of transposon Tn7 (Tn7L and Tn7R). Tn7L and Tn7R form a mini Tn7 that allows site-specific transposition of the target genes into the baculovirus genome. Within the expression cassette, there are two AcMNPV promoters, called polyhedron (P_H) and p10 promoters, two multiple cloning sites (MCS), Herpes Simplex Virus (HSV) thymidine kinase (tk) polyadenylation signal, SV40 polyadenylation signal and a gentamicin resistance gene. Beyond the expression cassette, pFastBacTM Dual vector also contains an ampicillin resistance gene, a pUC origin and a f1 origin. The map and features of pFastBacTM Dual vector is shown in Figure 1.12. The function of each feature is summarized in Table 1.3 (Invitrogen, 2015).

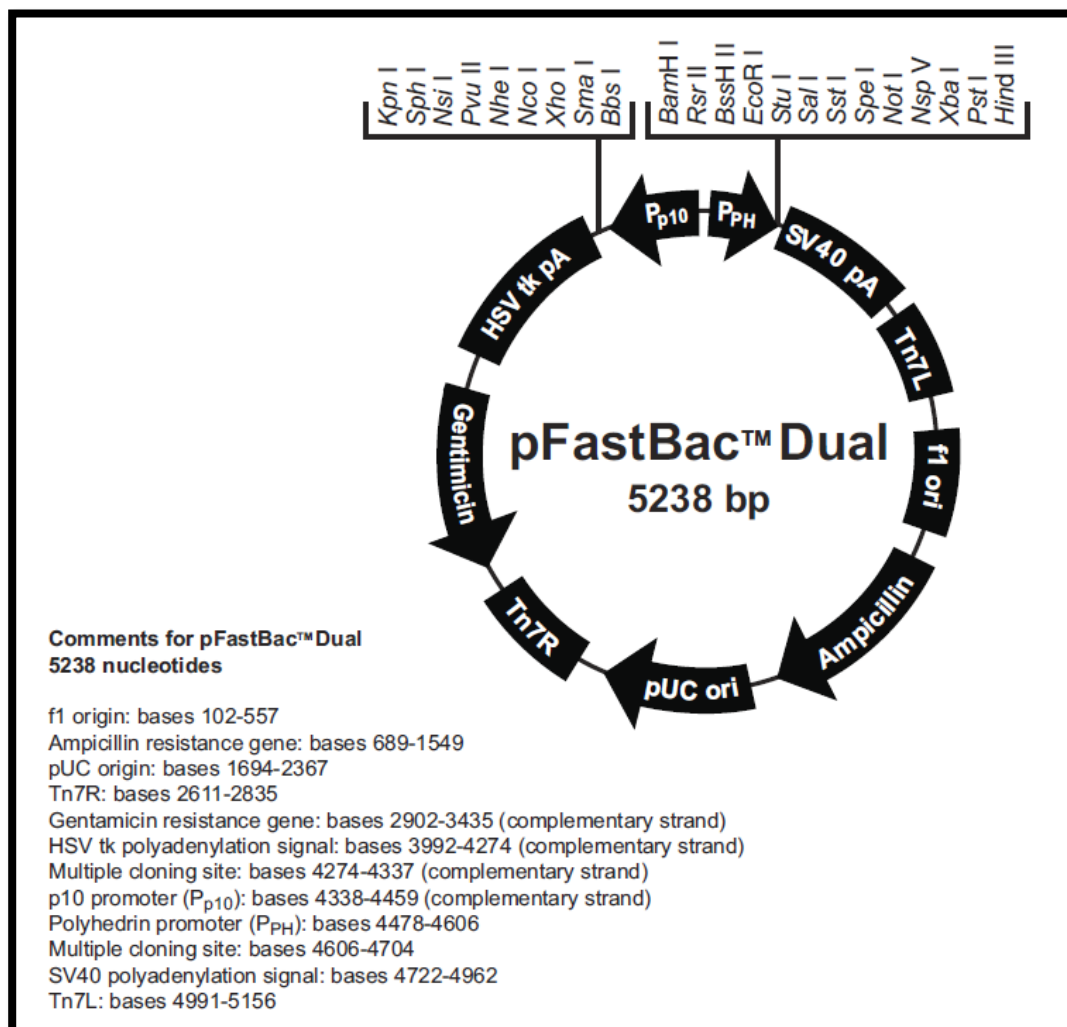


Figure 1.12: Map of the pFastBac™ Dual vector
(Source: Invitrogen, 2015)

Table 1.3: The function of each feature in pFastBac™ Dual vector

Feature	Function
Polyhedrin promoter (P_{PH})	Allows efficient, high-level expression of recombinant protein in insect cells
Multiple cloning site (P_{PH})	Allows restriction enzyme-mediated cloning of gene of interest
SV40 polyadenylation signal	Permits efficient transcription, termination and polyadenylation of mRNA
Tn7L and Tn7R	Mini Tn7 elements that permits site-specific transposition of the gene of interest into the baculovirus genome
f1 origin	Allow rescue of single-stranded DNA
Ampicillin resistance gene	Allows selection of the plasmid in <i>E.coli</i>
pUC origin	Permits high-copy replication and maintenance in <i>E.coli</i>
Gentamicin resistance gene	Permits selection of the recombinant bacmid in DH10Bac™ <i>E.coli</i>
Herpes Simplex Virus (HSV) thymidine kinase (tk) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
Multiple cloning site (P_{p10})	Allows restriction enzyme-mediated cloning of your gene of interest
P10 promoter (P_{p10})	Allows efficient, high-level expression of recombinant protein in insect cells

(Source: Adapted from Invitrogen, 2015)

The second component of this system is DH10Bac™ *E.coli* strain, which is used as host for the pFastBac™ Dual vector. Within the DH10Bac™ *E.coli* cell, there is a baculovirus shuttle vector (bacmid) with a mini-*att*Tn7 target site and a helper plasmid. When the pFastBac™ Dual recombinant plasmid is transformed into DH10Bac™ cells, transposition occurs between the mini-Tn7 element on the pFastBac™ Dual recombinant

plasmid and the mini-*att*Tn7 target site on the bacmid to produce a recombinant bacmid, which can then be used to transfect the insect cells. The transposition is occurred in the presence of transposition protein, which is supplied by the helper plasmid. Transposition makes it possible to distinguish between the parental and recombinant bacmid-containing DH10Bac™ cells as the attachment of mini-Tn7 element to mini-*att*Tn7 target site will interrupt LacZ gene, which encodes a protein that will result in blue colony formation in the bacteria. Therefore, in the parental cells, no transposition occurs and no interruption of the LacZ gene results in blue colonies formation. On the other hand, incorporation of the target gene in the bacmid of recombinant bacmid-containing cells result in the interruption of LacZ gene, therefore, no color formation. The high molecular weight of recombinant bacmid DNA is isolated and then transfected into the insect cells to generate recombinant baculovirus that can be used for preliminary expression (Invitrogen, 2015). Figure 1.13 shows the schematic representation of the bac-to-bac® baculovirus expression system.

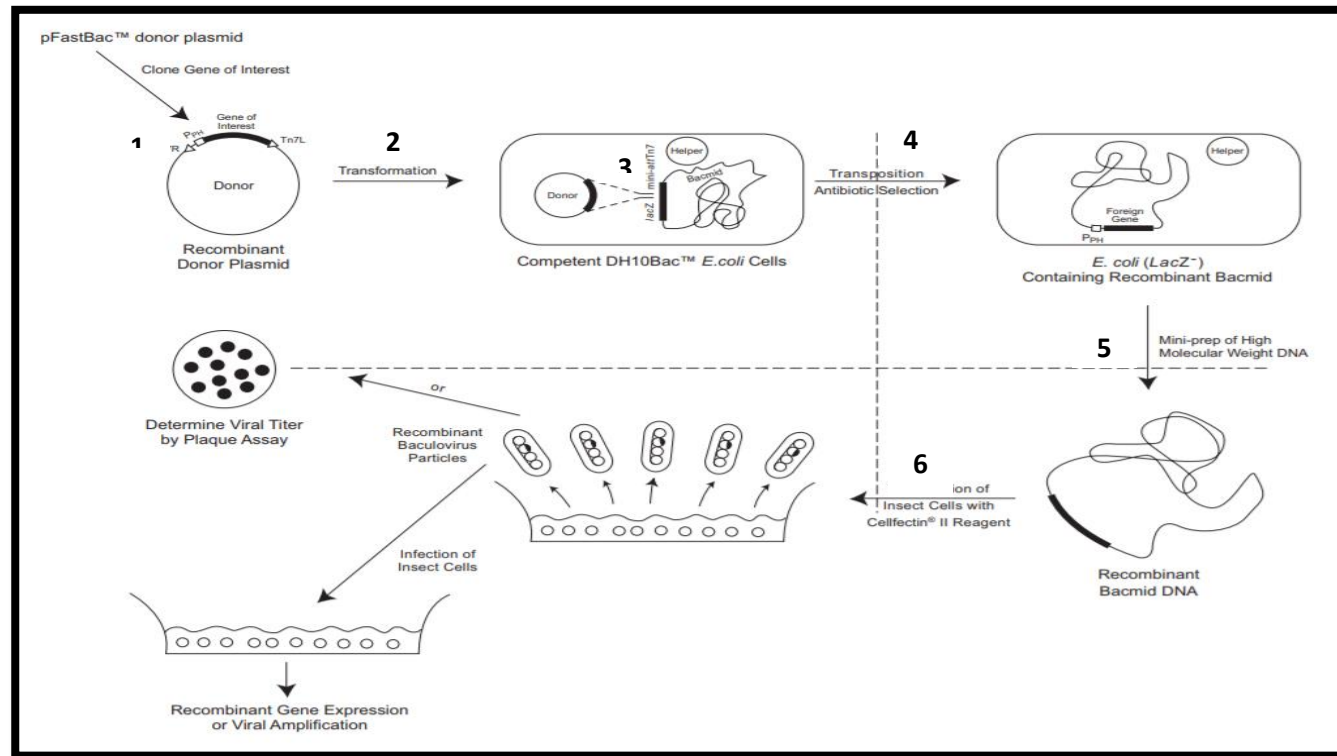


Figure 1.13: Overview of the bac-to-bac® baculovirus expression system (Invitrogen, Carlsifornia, USA). (1) The gene of interest is cloned into the pFastBac™ vector. (2) Transformation of the recombinant donor plasmid into competent DH10Bac™ *E. coli* cells which contain the bacmid with a mini-Tn7 target site and a helper plasmid. (3) Site-specific transposition occurs between the mini-Tn7 element on the recombinant donor plasmid and mini-Tn7 target site on parent bacmid in DH10Bac™ *E. coli* to form recombinant bacmid. (4) The absence of lacZ-alpha complementation factor in the recombinant bacterial cell is identified with blue-white screening. White colonies indicate recombinant bacterial cells. (5) The high molecular weight of recombinant bacmid DNA is isolated from selected colonies. (6) The insect cells are cultured and transfected with the recombinant bacmid DNA to generate recombinant baculovirus particles.

(Source: Adapted from Invitrogen, 2015)

1.6 Statement of research problem

Re-emergence of CHIKV has resulted in the unexpected rise in epidemics of debilitating febrile arthralgia in the past decade. Although various strategies have been adopted to combat CHIKV infection, the transmission of CHIKV continues to increase globally. In Malaysia, modification of ecosystem due to economic development, abundance of vectors (*A.aegypti* and *A.albopictus*), presence of a large immunocompromised population who do not have immunity against CHIKV and failure of vector control measures implementation in CHIKV affected area due to the late detection of CHIKV occurrence were accounted for the rapid transmission of CHIKV among different states. Additional concerns have arisen when there are relatively high incidence of severe disease manifestations and occasional fatalities among the immunocompromised population. Ineffective management of CHIKV outbreaks can cause impaired quality of life, loss in productivity, negative economic impact on tourism, and hindrance in economic growth. The most effective measure to reduce the CHIKV burden is through the use of a vaccine. However, there is no licensed human vaccine currently available for CHIKV. VLPs are a safe and highly effective class of subunit vaccine that mimic the native virus surface architecture and protein conformations, which are readily recognized by the immune system and induce protective antibody responses in the absence of adjuvant. Recombinant CHIKV structural proteins will be expressed from the target region of structural polygene (E3-E2-6K-EctoE1) and the expressed products are antigenically reactive against the CHIKV positive sera.

1.7 General objective

Accordingly, the general objective of this study was to construct recombinant CHIKV structural proteins.

1.8 Specific objectives

- 1.8.1 To construct recombinant structural proteins from a CHIKV isolated from Sarawak outbreak of 2010.
- 1.8.2 To express the recombinant proteins in an insect cells-baculovirus expression system.
- 1.8.3 To test the antigenicity of the recombinant construct against human CHIKV positive sera

CHAPTER 2

MATERIALS AND METHODS

2.1 Propagation of CHIKV

2.1.1 Cell line

A cloned insect cell line derived from *Aedes albopictus* mosquito, C6/36, was used to propagate Chikungunya virus (CHIKV) in this study. C6/36 cells were provided by the Institute of Health and Community Medicine (IHCM), UNIMAS, Sarawak.

2.1.2 Maintenance of C6/36 cell culture

Leibovitz's L-15 complete medium (L-15 medium) (Gibco, New York, USA) supplemented with 10 % heat inactivated Fetal Bovine Serum (FBS) (Gibco, New York, USA) (Appendix A.1.1 & A.1.2) was used to culture C6/36 cells. The medium was prepared according to manufacturer's instruction. One percent of Penicillin G and 1 % of Streptomycin were added to the media to prevent the microbial contamination of cell culture.

C6/36 cells were propagated in a 25 cm² disposable culture flask (TPP AG, Trasadingen, Switzerland) and passaged continually. To do so, 4 ml of old medium was first discarded and around 1 ml of medium was left inside the flask. The flask was gently tapped to disrupt the confluent cell monolayer and allow the cells to detach. The 1 ml of cell suspension was gently mixed by pipetting, split and then transferred into two new culture flasks containing 4.5 ml of fresh L-15 medium supplemented with 10 % FBS and Tryptose Phosphate Broth (TPB) each flask. The cells were then incubated in a static incubator (WTC Binder, Tuttlingen, Germany) set at 30 °C.

2.1.3 Source of CHIKV

A CHIKV isolate with a given code S233, provided by IHCM was used throughout this study. CHIKV S233 was isolated from patient serum sample collected from Sibu during a 2009 Chikungunya outbreak in Sarawak.

2.1.4 CHIKV infection in C6/36 cells

A 25 cm² flask of C6/36 cells with 70-80 % confluency was prepared a day before. Before infection, 4 ml of the old medium was discarded and around 1 ml of medium was left inside the flask. Following this, 200 µl of CHIKV was added onto the cell monolayer and the flask was rocked on the rocker (Edmund Bühler GmbH & Co., Bodelshausen, Germany) for about 15 minutes. Subsequently, 4 ml of fresh L-15 medium with 1 % heat inactivated FBS (Appendix A.1.1 & A.1.3) was added into the flask and incubated at 30 °C. A mock infection was prepared in another flask as the same step above except 100 µl of L-15 medium was added instead of CHIKV.

The infected cells were observed daily for cytopathic effect (CPE) under an Olympus IMT-2 phase contrast microscopy (Olympus Co., Tokyo, Japan). The cell images were taken at 20X magnification using Nikon A1 Confocal Laser Microscope (Nikon Inc., Melville, NY, USA).

The CHIKV-infected culture was harvested when more than 90 % CPE observed. The CHIKV-infected cell culture supernatant was transferred into a 15 ml sterile centrifuge tube and centrifuged (5810R, Eppendorf, Hamburg, Germany) at 244 × g, 4 °C for 10 mins. The clarified supernatant was aliquoted into microcentrifuge tube and subjected to RNA extraction.

2.2 Validation of CHIKV isolate

2.2.1 Viral RNA extraction

CHIKV isolate as obtained in the section above was subjected to viral RNA extraction using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the instructions provided by the manufacturer.

First, 200 µl of binding buffer [6 M guanidine-HCl, 10 mM Tris-HCl, 20 % Triton[®] X-100 (w/v), pH 4.4 (25 °C)] was added into 1.5 ml microcentrifuge tube containing 200 µl of virus culture (S233, Passage 4). Fifty-five microliters of premixed working solution containing 4 µg/µl of Poly (A) and 20 µg/µl of Proteinase K was added to the mixture and vortexed to mix briefly. The mixture was incubated in a Wisebath[®] fuzzy control system water bath (Daihan Scientific, Seoul, Korea) set at 72 °C for 10 minutes to lyse the virus. After incubation, 100 µl of isopropanol (Merck, Darmstadt, Germany) was added to the mixture and gently mixed by pipetting. A filter tube was inserted into a disposable collection tube to form a High Pure filter tube assembly. The sample was then pipetted to the upper reservoir of the filter tube assembly and centrifuged for 1 minute. In this step, the viral nucleic acids were bound to the glass fibre fleece on the filter of the upper reservoir. The collection tube containing flow-through was discarded and replaced by a new collection tube.

Subsequently, 500 µl of inhibitor removal buffer [5 M guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6 (25 °C)] was added to the upper reservoir of filter tube and centrifuged for 1 minute. The flow-through was discarded and 450 µl of wash buffer [20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (25 °C)] was added to the upper reservoir of the filter tube and centrifuged for 1 minute to remove enzymes, salts and other impurities. The collection tube was discarded and replaced by a new collection tube. The filter tube was washed again with 450 µl of wash buffer and centrifuged for 1 minute. Flow-through was discarded and extra centrifugation step was performed to ensure the removal of any residual wash buffer.

After two washing steps, the collection tube was discarded and the filter tube was assembled with a microcentrifuge tube. The entire filter tube assembly was dried in an oven (Binder WTC, Tuttlingen, Germany) set at 80 °C for 5 minutes to completely remove all residual alcohol from the washing steps. The nucleic acid was then eluted in 100 µl of elution buffer [nuclease-free, sterile, double distilled water (ddH₂O)] and centrifuged for 2 minutes. The eluate was used directly in gene amplification.

All centrifugation steps in viral RNA extraction were performed at 13,400 rpm (rotation per minute) or 12,045 × g on the bench-top minicentrifuge (Eppendorf, Hamburg, Germany).

2.2.2 Reverse-transcription polymerase chain reaction (RT-PCR)

The molecular detection of CHIKV was done through RT-PCR using in-house designed primers as listed in Table 2.1. The primers were designed to specifically target the CHIKV E1 region.

Reverse transcription (RT) was performed in a 10 µl reaction volume using CHIKV RNA (prepared in Section 2.2.1) as a template. In RT step, RNA was converted to the more stable form of complementary DNA (cDNA) molecule. First, 5.5 µl of RNA was incubated with 1 µl of 10 µM reverse primer at 70 °C for 10 minutes to denature RNA secondary structures. Meanwhile, a master mix containing 2 µl of 5X RT reaction buffer [250 mM Tris-HCl (pH 8.3 at 25 °C), 250 mM KCl, 20 mM MgCl₂, and 50 mM DTT] (MBI Fermentas, Vilnius, Lithuania), 0.5 µl of 10 mM dNTPs mix (MBI Fermentas, Vilnius, Lithuania) and 1 µl of 100 units Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) was prepared.

After 10 minutes incubation, the reaction mix was immediately incubated on ice for 2 minutes to prevent the reformation of secondary structures. A total volume of 3.5 µl master

mix was then added to the reaction mix and incubated at 37 °C for 1 hour followed by inactivation of enzymes at 70 °C for 10 minutes.

The cDNA molecule synthesized in RT step was used as a template for the following PCR step in a single 50 µl PCR reaction containing 5 µl of cDNA, 5 µl of 10X Taq buffer with (NH₄)₂SO₄ [750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM (NH₄)₂SO₄, 0.1 % Tween 20] (MBI Fermentas, Vilnius, Lithuania), 3µl of 1.5 mM MgCl₂ (MBI Fermentas, Vilnius, Lithuania), 1 µl of 2.5 units recombinant Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 1.5 µl of 10 mM dNTPs Mix, 2 µl of each 10 µM reverse and forward primers, and 30.5 µl of sterile UHQ water.

The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 20 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 minute. The final extension was at 72 °C for 5 minutes. All PCR reactions throughout this study were performed in a Nexus Gradient thermocycler (Eppendorf, Hamburg, Germany).

2.2.3 Agarose gel electrophoresis

The amplicons obtained from RT-PCR were analyzed on a 2 % agarose (w/v) gel (Amresco, Solon, OH, USA) prepared in 1X Tris-borate EDTA (TBE) buffer [89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.8] (Vivantis, Oceanside, CA, USA) by gel electrophoresis using EasyCast™ Mini Electrophoresis System (Owl, Portsmouth, NH, USA) following the instructions provided by the manufacturer.

To prepare 2 % agarose gel, 2 g of agarose was completely dissolved in 100 ml of 1X TBE by heating in a microwave, followed by the addition of 0.05 µg/ml of ethidium bromide (EtBr, 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide) solution as staining agent, which fluoresces under UV-light. The gel was mixed well and poured into

casting tray with gel comb placed in the notch. In this step, any bubbles were removed with a pipette tip and the gel was allowed to set.

Before loading samples to their respective wells, 6X loading buffer were added to the samples and vortexed to mix briefly. Molecular markers (GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) or Lambda DNA/HindIII+EcoRI (Thermo Fisher Scientific Inc., Waltham, MA, USA) were loaded alongside samples, onto the gel for the purpose of amplicon size determination. The gel electrophoresis was carried out in a horizontal tank containing 1X TBE and was run at a constant voltage of 180V. The gel was then visualized under the electronic UV-transilluminator (Ultra Lum Inc., Carson, California, USA) and the gel picture was taken with a gel documentation system. The expected sizes of amplicons were shown in table 2.1.

Table 2.1: In-house designed primers for molecular detection of CHIKV through RT-PCR

Primer name	Primer sequences (5'-3')	Expected size (bp)	Genome position ¹
Chik-E1/PCR/F	CTGGCAGCCCTGATTGTTCT	685	9886-9905
Chik-E1/SEQ/R2	CCAAAGGGCGGGTAGTCCA		10553-10571
Chik-E1/SEQ/F1	TCTGCGACGCTGAAAATAC	743	10277-10295
Chik-E1/SEQ/R1	CAGCTTCCCGAATAGTGAC		11002-11020
Chik-E1/SEQ/F2	TAAGTATTGGCTAAAAGAAC	688	10713-10732
Chik-E1/PCR/R	GGTTGCGTAGCCCTTTGAT		11383-11401

¹ Genome position given with reference to the complete sequence of CHIKV S233 (unpublished) provided by IHCM.

2.2.4 Purification of DNA fragments from agarose gel

Prior to DNA sequencing, the amplicons as prepared in Section 2.2.3 were purified by using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to manufacturer's instruction. This extraction kit allows the removal of impurities from the

sample and ensures up to 80 % recovery of 70 bp to 10 kb DNA fragments size range through a fast bind-wash-elute procedure.

The amplicons of interest were first excised from the agarose gel using sterile disposable blades (B. Braun, Melsungen, Germany). In binding step, 450 µl of QG buffer [5.5 M guanidine thiocyanate, 20 mM Tris-HCl, pH 6.6] was added to 4 slices of excised gels and the mixture was incubated at 50 °C for 10 minutes. After 10 minutes incubation, 100 µl of isopropanol was added to the mixture to enhance the yield of DNA. The mixture was transferred into a QIAquick spin column and centrifuged for 1 minute to allow the DNA binds to the silica membrane. The flow-through was discarded and 500 µl of QG buffer was added to the spin column again, then centrifuged for 1 minute to ensure complete removal of agarose gel traces in the column.

In washing step, 750 µl of PE buffer [10 mM Tris-HCl, pH 7.5, 80 % ethanol] was added to the spin column and incubated at room temperature for 2 minutes followed by centrifugation for 1 minute. The purpose of this washing step is to remove unwanted primers and impurities, such as salts, enzymes, unincorporated nucleotides, agarose, dyes and EtBr which are escaped from membrane binding and flow through the column. The flow-through was discarded and additional centrifugation step was performed to remove any residual PE buffers which may interfere with subsequent enzymatic reactions. After extra centrifugation, the spin column was assembled with a microcentrifuge tube and dried at 80 °C for 5 minutes to completely remove the residual ethanol.

In the elution step, the amplicons were eluted by adding sterile UHQ water to the centre of QIAquick membrane, followed by incubation at room temperature for 2 minutes and centrifugation for 2 minutes. The purified amplicons were used as template in sequencing.

All centrifugation steps in this section were performed at $12,045 \times g$ on the bench-top minicentrifuge.

2.2.5 DNA sequencing and analysis

DNA sequencing throughout this study was performed using an automated DNA Sequencer, 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Prior to DNA sequencing, sequencing PCR followed by sequencing clean-up were performed.

2.2.5.1 DNA concentration measurements

Before sequencing PCR, the concentration and purity of purified amplicons as prepared in Section 2.2.4 were determined using NanoDrop Spectrophotometer[®] ND-1000 v3.5 (NanoDrop Technologies Inc., Wilmington, USA). The concentration of purified amplicons was calculated in ng/ μ l based on absorbance at 260 nm, while the purity was measured by ratio of sample absorbance at 260 and 280 (A_{260}/A_{280} ratio). Good quality DNA will have an A_{260}/A_{280} ratio of 1.8 to 2.0. On the other hand, if the ratio is appreciably lower or higher in either case, it may be because of the presence of proteins, phenol, salts or other contaminants that absorb strongly at or near 280 nm.

2.2.5.2 Sequencing PCR

The DNA template was first prepared in a final volume of 10 μ l at 90-110 ng concentration. A total volume of 10 μ l of sequencing PCR master mix containing 4.5 μ l of premixed reaction from BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, California, USA), 1 μ l of 3.2 μ M/ μ l sequencing primer and sterile UHQ water were added to the 10 μ l DNA template to give a final volume of 20 μ l.

The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 60 °C for 2 minutes.

2.2.5.3 Sequencing PCR clean up

The sequencing PCR products clean-up was performed using BigDye® Sequencing Clean Up Kit (McLab, California, USA) and DynaMag™-96 Side Magnet (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each component in BigDye® Sequencing Clean Up Kit has been optimized for eliminating salts and unincorporated dye terminators from DNA sequencing reaction mixtures.

Sequencing clean-up was performed on a 96-well plate. BigDye® Cleaning Bead (BCB) was swirled before used to resuspend the beads. Then, 20 µl of BCB was added to 20 µl of sequencing PCR products as obtained in Section 2.2.5.2 and mixed by pipetting. The mixture was incubated at room temperature for 5 minutes. After 5 minutes incubation, 84 µl of 85 % absolute ethanol (Scharlab SL, Barcelona, Spain) was added to the mixture and gently mixed. Again, the mixture was incubated at room temperature for 5 minutes. The 96-well plate was then placed onto the 96-well magnetic plate (DynaMag™-96 Side Magnet) for 5 minutes until the solution was clear. While the plate was on the magnetic plate, the supernatant was aspirated out from the well without disturbing the beads and discarded.

In washing step, 100 µl of 85 % absolute ethanol was added to each well and incubated at room temperature for 5 minutes. The washing step was repeated twice and the plate was remained on the magnetic plate throughout the process. The supernatant was then aspirated out from the well as much as possible in order to remove ethanol and other contaminant residues. The plate was then taken off from the magnetic plate and dried in the oven set at 80 °C for 5 minutes to ensure completely removal of ethanol. The samples were cooled down at room temperature for few minutes before elution.

In elution step, 15 µl of elution buffer was added onto each sample and mixed thoroughly by pipetting until the mixture were fully resuspended. The plate was left at room temperature for 5 minutes, then placed on magnetic plate for another 5 minutes until the

solution was clear. A total volume of 10 µl of supernatant was transferred onto new wells without disturbing the beads and briefly spun (MPC-P25, ALLSHENG, Hangzhou, China). Following this, the supernatant was heated at 95 °C for 2 minutes to denature secondary structures prior to analysis by 3130 Genetic Analyzer.

2.2.5.4 Sequence data analysis

The sequence data generated from 3130 Genetic Analyzer were analyzed using LaserGene software suite (DNASar Inc., Madison, WI, USA) which included programs such as SeqMan that was used to trim and assemble sequence data; MegAlign that offered both DNA and protein sequence alignment using a range of pairwise and multiple alignment methods; and EditSeq which was used for manually editing sequence details and provided functions such as reverse complement, invert, translate, back-translate and ORF identification.

Before aligned the generated sequences to the reference genome, the raw forward and reverse sequences were first trimmed to remove low quality bases and then assembled into a single contig. Following this, the contig sequence was aligned to a reference genome.

The reference genome used throughout this study was an unpublished CHIKV S233 DNA sequence provided by IHCM, which was generated using Sanger Sequencing method. The contig sequence was also compared to the sequences deposited in the public database GenBank at the website using basic local alignment search tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.3 Cloning and expression in bac-to-bac[®] BEVS

Bac-to-bac[®] baculovirus expression system (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used to express CHIKV structural proteins (E3-E2-6K-EctoE1) in this study. The cloning and expression strategies used in this study were summarized in flowchart as shown in Figure 2.3. The schematic representation of CHIKV target region was shown in

Figure 2.1 and the schematic representation of insert DNA (2,874 bp) was shown in Figure 2.2.

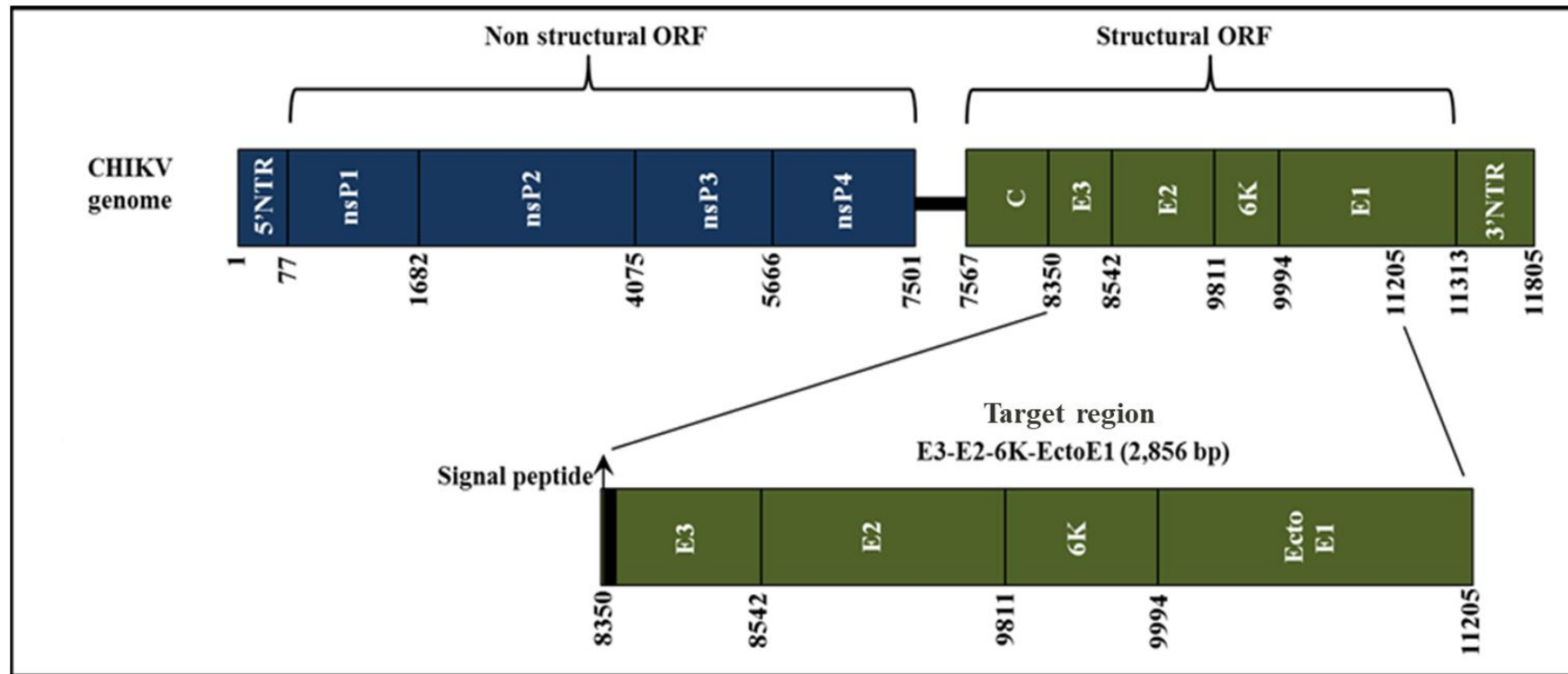


Figure 2.1: Schematic representation of the CHIKV genome and target region to be cloned into pFastBac™ Dual donor plasmid. The gene region (2,856 bp) encoding CHIKV structural polyproteins (E3-E2-6K-ectodomain of E1) was selected as the target region for recombinant structural proteins expression in this study.

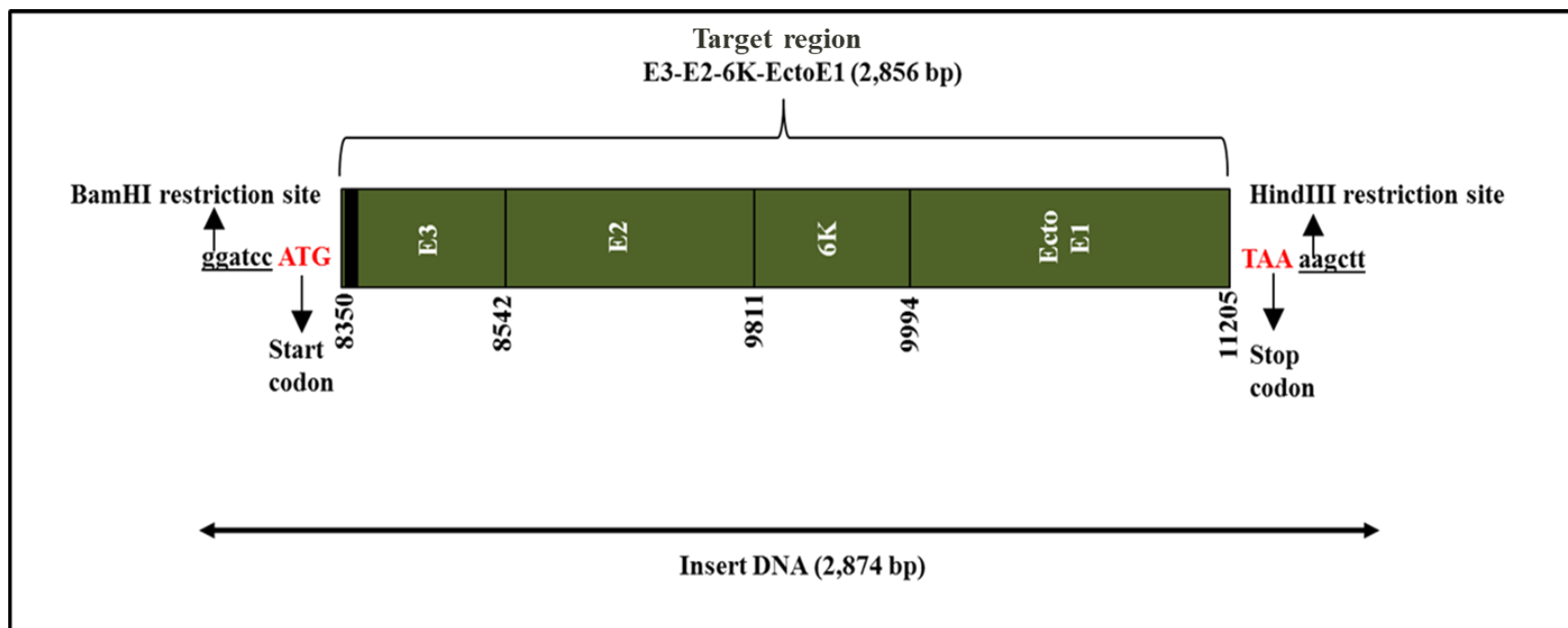


Figure 2.2: Schematic representation of insert DNA (2,874 bp) that are cloned into pFastBacTM Dual donor plasmid. The BamHI and HindIII restriction sites together with start and stop codon are indicated.

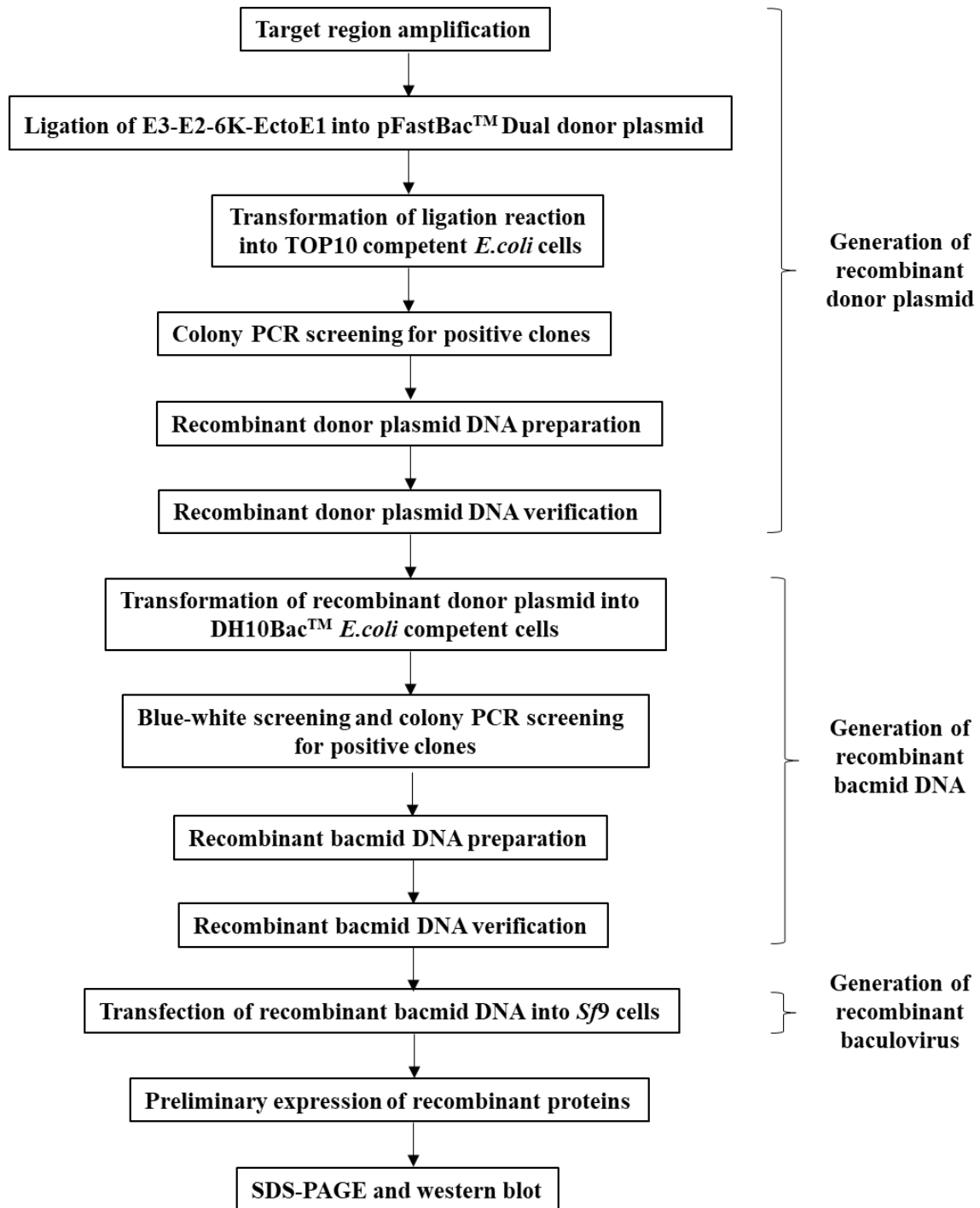


Figure 2.3: Flowchart of cloning and expression strategies in this study.

2.3.1 Amplification of target region (E3-E2-6K-EctoE1)

The procedures of target region (E3-E2-6K-EctoE1) amplification is summarized in a schematic representation as shown in Figure 2.4. High fidelity Pfx50™ DNA polymerase (Invitrogen, California, USA) was used in the amplification because it offers better fidelity than Taq DNA polymerase which couples low mis-incorporation rates with proofreading activity. The use of high fidelity DNA polymerase in the PCR is essential for reducing the introduction of amplification errors in amplicons to be cloned.

2.3.1.1 Primers design

Primers specifically targeting the E3-E2-6K-EctoE1 region were designed using the PrimerSelect program in the LaserGene software suite and a web-based application, Primaclade (<http://primaclade.org/cgi-bin/primaclade.cgi>).

The CHIKV S233 sequence available at the IHCM was used as a template to design all the primers used in the following steps. The primers used in the E3-E2-6K-EctoE1 amplification were listed in Table 2.2. For the primers flanked the 5' and 3' ends of E3-E2-6K-EctoE1, an ATG start codon was included in forward primer for the initiation of translation and a TAA stop codon was included in reverse primer for termination of translation. BamHI and HindIII restriction sites were also included in the primer right after the start and stop codons respectively for the purpose of ligation. The presence of BamHI and HindIII unique restriction sites in the E3-E2-6K-EctoE1 sequence was confirmed with MapDraw program in the LaserGene software suite.

All the primers used in this study were ordered and synthesized at First BASE Laboratories (Selangor, Malaysia).

Table 2.2: Primer sets used in PCR amplification of fragment E3-E2-6K-EctoE1

Primer name	Primer Sequence (5'-3')	¹ Genome position	Expected size (bp)	Function
CHIKV/BamHI/F8350 CHIKV/HindIII/R11205	² <i>gaga</i> ³ <i>ggatcc</i> ⁴ <i>atg</i> AGTCTTGCCATCCCAGTT ² <i>gaga</i> ³ <i>aagctt</i> ⁴ <i>tta</i> AGCGGAGATGTCCTGGAC	8350-8367 11188-11205	2874 bp	To amplify target gene region (E3-E2-6K-EctoE1)
CHIKV/PCRF1/7958 CHIKV/PCRR1/8924	GGGACAAAGTAATGAAACCAGC GGGTGCGTACATGAATGACTAA	7958-7979 8903-8924	968 bp	To generate individual amplicons which are overlapping to each other
CHIKV/PCRF2/8781 CHIKV/PCRR2/9745	GGCGGGGCTATTTGTAAGA GTTCATACGGTGTGATGCATCT	8781-8799 9724-9745	965 bp	
CHIKV/PCRF3/9551 CHIKV/PCRR3/10522	AGTATTGGCCGCAGTTATCTACA TTTTGTTGTCGAAAGGTGTCC	9551-9573 10502-10522	972 bp	
CHIKV/F9886 CHIKV/R11205	CTGGCAGCCCTGATTGTTCTAT AGCGGAGATGTCCTGGACCCCGAGGGT GGTATGTGACGCCGGGTAGTTGACTAT GTGGTCCTTCGGGGGG	9886-9907 11136-11205	1320 bp	

¹ Genome position given with reference to the complete sequence of CHIKV S233 provided by IHCM

² Buffer sequences were included in forward and reverse flanking primers.

³ BamHI and HindIII restriction sites were included in forward and reverse flanking primers respectively.

⁴ Start and stop codons were included in forward and reverse flanking primers respectively.

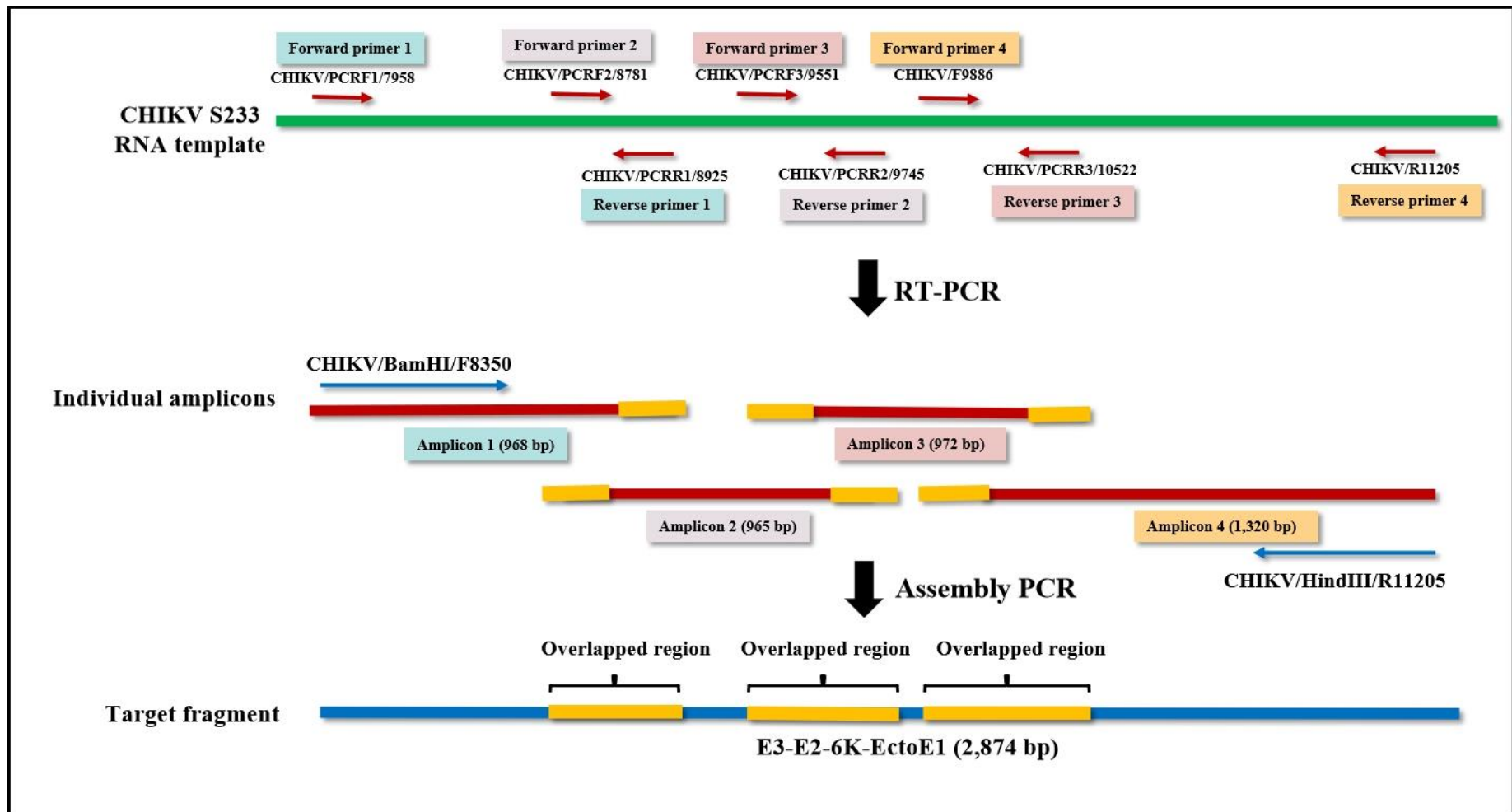


Figure 2.4: Schematic representation of E3-E2-6K-EctoE1 fragment amplification.

2.3.1.2 Generation of individual amplicons by RT-PCR

RT step of CHIKV S233 RNA as prepared in Section 2.2.1 was performed as the same step described in Section 2.2.2 except the reverse primers used for the synthesis of cDNA molecule were different (as shown in Table 2.2).

In PCR step, a master mix containing 5 µl of 10X Pfx50™ buffer [20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50 % (v/v) glycerol], 1.0 µl of 10 mM dNTP Mix, 1.0 µl of 5U/µl of Pfx50™ DNA polymerase, 2 µl of each 10 µM of forward primer and reverse primer (as listed in Table 2.2) and appropriate amount of sterile UHQ water was added to the cDNA template to give a final volume of 50 µl.

The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 68 °C for 2 minutes 15 seconds and a final extension at 68 °C for 10 minutes.

The amplicons were then analyzed on a 1.5 % agarose (w/v) gel in 1X TBE buffer. All the individual amplicons with expected sizes were excised from agarose gel and purified as described in Section 2.2.4.

2.3.1.3 Assembly PCR

An assembly PCR with some modification was performed to assemble the target fragment (E3-E2-6K-EctoE1) from equimolar of individual amplicons as obtained in Section 2.3.1.2 with specific primers.

Multiple reactions of 50 µl assembly PCR mix were prepared so as to enhance the yield of the products. Each reaction contained 5 µl of 10X Pfx50™ buffer [20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 1mM DTT, stabilizers, 50 % (v/v) glycerol], 1.5 µl of 10 mM dNTP Mix, 1 µl of 5U/µl of Pfx50™ DNA polymerase, 5 µl of each 20 µM

forward and reverse specific primers (listed in Table 2.2), 200 ng of each individual amplicons and appropriate volume of sterile UHQ water.

The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 68 °C for 4 minutes 30 seconds and a final extension at 68 °C for 10 minutes.

Five microliters of each PCR reaction were aspirated out from each tube and resolved on 1 % agarose gel in 1X Tris-acetate-EDTA (TAE) buffer [40 mM Tris, 19mM Acetic acid, 1 mM EDTA, pH 8.0] (First BASE Laboratories Sdn. Bhd., Selangor, Malaysia) to confirm the amplification of target fragment. Following this, the leftover of the PCR reactions were pooled in one tube and subjected to PCR purification.

2.3.1.4 PCR purification

PCR purification was performed using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The purification kit is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. DNA fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns and a bench-top minicentrifuge.

First, 5 volumes of PB buffer was added to 1 volume of the PCR reaction and mixed thoroughly by pipetting. The mixture was applied to a QIAquick spin column and centrifuged for 1 minute to allow the DNA binds to the silica membrane. The flow-through was discarded.

In washing step, 750 µl of PE buffer [10 mM Tris-HCl, pH 7.5, 80 % ethanol] was added to the QIAquick spin column and left stand at room temperature for 2 minutes followed by centrifugation for 1 minute. The flow-through was discarded, and additional

centrifugation was performed to remove any PE Buffer residues, which may interfere with subsequent enzymatic reactions. The spin column was dried at 80 °C for 5 minutes to completely remove the ethanol residues.

In elution step, the assembled DNA fragments were eluted by adding 70 µl of sterile UHQ water to the centre of QIAquick membrane, incubated at room temperature for 8 minutes and centrifuged for 4 minutes. The concentration and purity of purified PCR product was determined using NanoDrop Spectrophotometer® ND-1000 v3.5.

All centrifugation steps in PCR purification were performed at $12,045 \times g$ on the bench-top minicentrifuge.

2.3.2 Generation of recombinant donor plasmid (pFBD-E3E26KEctoE1)

2.3.2.1 BamHI-HindIII double digestion

A molar ratio of 1:20 pFastBac™ Dual donor plasmid (vector) to purified PCR products (insert) as prepared in Section 2.3.1.4 were double digested simultaneously in two separate tubes with both BamHI (MBI Fermentas, Vilnius, Lithuania) and HindIII (MBI Fermentas, Vilnius, Lithuania) restriction enzymes.

For the digestion of vector, a total volume of 100 µl digestion reaction containing 10 µl of 10X tango buffer [33mM tris-acetate (pH 7.9, 37 °C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA] (MBI Fermentas, Vilnius, Lithuania), 3 µl of 10 U/µl BamHI, 3 µl of 10 U/µl HindIII, 0.5 µg/µl pFastBac™ Dual donor plasmid and appropriate amount of sterile UHQ water was prepared. On the other hand, the digestion reaction of insert was prepared in another tube as the same step above except purified PCR products was added instead of pFastBac™ Dual donor plasmid.

Both reactions were digested at 37 °C for 4 hours followed by heat inactivation at 80 °C for 20 minutes. After that, 3 µl of digested vector was resolved on 0.7 % agarose gel in 1X TAE buffer to confirm the complete digestion of the plasmid DNA.

Prior to ligation, both digested vector and insert were subjected to ethanol precipitation for the clean-up purpose. They were first pooled in one microcentrifuge tube, flicked and centrifuged at $12,045 \times g$ for 1 minute. Subsequently, 400 μ l of ice cold absolute ethanol was added to the mixture and mixed by inverting the tube for few times. The mixture was incubated at room temperature for 5 minutes, and then centrifuged at $12,000 \times g$ (MIKRO 200R, Hettich Zentrifugen, Tuttlingen, Germany), 4 °C for 10 minutes. The supernatant was discarded and additional 1 minute centrifugation was performed. The leftover supernatant was aspirated out and discarded. The pellet was washed with 70 % ethanol and centrifuged briefly. The supernatant was removed by aspiration and the pellet was dried at 80 °C for 5 minutes to completely remove the ethanol residues. Subsequently, the pellet was resuspended in 20 μ l of sterile UHQ water by pipetting until the pellet was fully dissolved. The nucleic acid was then incubated at room temperature for 5 minutes and centrifuged at $12,045 \times g$ for 1 minute on the bench-top minicentrifuge.

2.3.2.2 Ligation

A total volume of 10 μ l ligation reaction was set up by mixing 8 μ l of the purified digested products mixture as prepared in Section 2.3.2.1 with 1 μ l of 10x T4 DNA ligase buffer [400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP (pH 7.8 at 25 °C)] (Thermo Fisher Scientific Inc, Waltham, MA, USA) and 1 μ l of 5 U/ μ l T4 DNA ligase (Thermo Fisher Scientific Inc, Waltham, MA, USA). The ligation reaction was incubated at 16 °C for 16 hours.

2.3.2.3 Transformation of ligation reaction into TOP10 *E.coli* cell

A vial of One Shot™ TOP10 chemically competent cells was first thawed on ice. Following this, 5 μ l of the ligation reaction was added directly onto the thawed competent cells and mixed by gently stirring with a pipette tip. The competent cell/DNA mixture was then incubated on ice for 30 minutes. Subsequently, each transformation tube was heat-

shocked by placing the bottom of the tube into a 42 °C water bath for 45 seconds without shaking. In this step, a sudden increase in temperature creates pores in the plasma membrane of the bacteria and allows plasmid DNA to enter the bacterial cell through the pores. The transformation tube was then immediately placed back on ice for 2 minutes to allow the cell membrane to self-heal.

Next, 250 µl of pre-warmed SOC-medium [2 % Tryptone, 0.5 % Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose] was added to the cells and incubated horizontally in a 37 °C Innova 4000 Incubator Shaker (New Brunswick Scientific, New Jersey, USA) set at 225 rpm for 1 hour. After 1 hour shaking, the transformation mix was plated onto Luria Bertani (LB) agar containing 150 µg/ml of ampicillin and incubated in a 37 °C of static incubator (JISICO, Seoul, Korea) for 18 hours.

2.3.2.4 Colony PCR screening

Ampicillin-resistant transformants were selected and subjected to colony PCR to identify the clones that harbour recombinant donor plasmid. Internal primers CHIKV/F8857 and CHIKV/R9272 were used to amplify internal region of the insert DNA (as listed in Table 2.3). During colony PCR reaction, the colony is disrupted by high temperature to release nucleic acid, which is then used as template for PCR reaction.

Single colony was picked from the plate using sterile toothpick and resuspended in a total volume of 50 µl of PCR master mix containing 5 µl of 10X Taq Buffer with $(\text{NH}_4)_2\text{SO}_4$ [750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 % Tween 20], 3 µl of 25 mM MgCl_2 , 0.5 µl of 5 U/L Taq DNA polymerase, 1 µl of 10 mM dNTP Mix, 2 µl of each 10 µM forward and reverse primers (CHIKV/F8857 and CHIKV/R9272) and 36.5 µl of sterile UHQ water.

PCR was performed using the cycling profile: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for

1 minute and extension at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes.

The amplicons were resolved on 2 % agarose (w/v) gel in 1X TBE buffer.

Table 2.3: Primers used in colony PCR

Primer name	Sequences (5'-3')	¹ Genome Positions	Expected size (bp)
CHIKV/F8857	CCAAAAGGGGAAACTCTGACG	8857-8877	413
CHIKV/R9272	CGCGGGACCAGAGGGGAGTTAT	9272-9251	

¹ Genome position given with reference to the complete sequence of CHIKV strain S233 (unpublished) provided by IHCM

2.3.2.5 Plasmid DNA isolation

Positive clones as detected in Section 2.3.2.4 were subjected to plasmid DNA isolation. Plasmid DNA was isolated from transformed bacterial cells using Miniprep ExpressTM Matrix kit (MP Biomedicals, California, USA) according to the manufacturer's instructions with some modifications. The plasmid DNA isolation procedure is based on three basic steps, including growth of bacterial cells and amplification of the plasmid, harvesting and alkaline lysis of the bacterial cells, and purification of the plasmid DNA using Miniprep ExpressTM matrix.

First, single colony was picked and inoculated into 5 ml of LB medium containing 150 µg/ml ampicillin. The culture was incubated overnight in an incubator shaker set at 37 °C, 225 rpm. To harvest the bacterial cells, 1.5 ml of overnight culture was transferred to a microcentrifuge tube and centrifuged at 12,045 × g for 5 minutes. The supernatant was discarded by aspiration without disturbing the pellet. Next, the pellet was completely resuspended in 200 µl of ice-cold Solution I [50 mM Glucose, 25 mM Tris.Cl (pH 8), 10 mM EDTA (pH 8)]. To lyse the bacterial cells, 400 µl of Solution II [0.2 M NaOH, 1 % SDS] was added to the mixture and gently mixed by inverting the tube for few times. In this step, bacterial cell membrane is disrupted by sodium dodecyl sulphate (SDS) and the

compact super coiled plasmid DNA molecules are released into the solution. Meanwhile, the genomic, plasmid DNA and cellular proteins are denatured by high concentration of NaOH. Next, 300 μ l of Solution III [5 M potassium acetate, glacial acetic acid, ddH₂O] was added to the mixture and the tube was shaken vigorously until white precipitates are observed. In this step, the mixture is neutralized with potassium acetate and allowed the plasmid DNA to re-anneal rapidly. The white precipitates are formed once the chromosomal DNA and bacterial proteins are trapped in a complex formed between the potassium and SDS. The white precipitates were removed by centrifugation at $12,045 \times g$ for 10 minutes. After centrifugation, 800 μ l of clarified supernatant was transferred to a new tube.

Subsequently, 600 μ l of Miniprep ExpressTM matrix was added to the clarified supernatant. The contents were mixed by inverting the tube for 5 times followed by placed on the tube rotator (LUCKHAM CM100, Cambridgeshire, UK) for 15 minutes to allow plasmid DNA bound to the matrix. After rotation, the content was centrifuged at $12,045 \times g$ for 1 minute and the supernatant was discarded. In washing step, 600 μ l of 80 % ethanol was added to the matrix/DNA complexes and resuspended by gently stirring with a pipette tip. The contents were then centrifuged at $12,045 \times g$ for 1 minute and the supernatant was discarded. Additional centrifugation was performed and the remainder of ethanol was removed by aspiration. The matrix/DNA complexes were dried at 80 °C for 5 minutes to completely remove the ethanol residues, followed by resuspension in 50 μ l of sterile UHQ water by gently stirring with a pipette tip until uniform suspension was formed. The suspension was centrifuged at $12,045 \times g$ for 1 minute and the clarified supernatant containing plasmid DNA was transferred to a new tube.

2.3.2.6 Verification of recombinant donor plasmid DNA

The recombinant donor plasmid DNA (pFBD-E3E26KEctoE1) was verified by restriction digestion analysis followed by DNA sequencing.

In restriction digestion, the plasmid DNA was digested with unique restriction enzymes into specific sized pieces. A total volume of 50 µl of digestion reaction containing 5 µl of 10X tango buffer [33mM Tris-acetate (pH 7.9, 37 °C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA], 5 µl of 10 U/µl BamHI, 5 µl of 10 U/µl HindIII, 15 µl of plasmid (prepared in Section 2.3.2.5), and 25 µl of sterile UHQ water was prepared. The digestion reaction was incubated at 37 °C for 4 hours and heat inactivated at 80 °C for 20 minutes. The restriction digestion reactions were resolved on 0.7 % agarose gel prepared in 1X TAE buffer. The plasmid DNA with expected vector and insert sizes was then subjected to DNA sequencing as described in Section 2.2.5 using sequencing primers as listed in Table 2.4.

Table 2.4: Sequencing primers used in DNA sequencing of insert DNA

Primer name	Primer sequences (5'-3')	¹ Genome position
CHIKV-SEQF1-8431	GAAAAGGAACCGGAGGAAAC	8431-8450
CHIKV-SEQR1-8485	CAGGTCTCATGACGTTGTCCT	8485-8505
CHIKV-SEQF2-9245	GGCAGTATAACTCCCCTCTGG	9245-9265
CHIKV-SEQR2-9292	GGTCCCCAAGTTCAGCATT	9292-9310
CHIKV-SEQF3-9955	TTAGCCGTAATGAGCGTCG	9955-9973
CHIKV-SEQR3-10000	GTTCGTACGCGCTCACAGT	10000-10018
CHIKV-SEQF4-10747	CAGCACACAGCACCATTG	10747-10765
CHIKV-SEQR4-10797	CGCTCTTACCGGGTTTGTT	10797-10815

¹ Genome position given with reference to the complete sequence of CHIKV strain S233 (unpublished) provided by IHCM

2.3.3 Gene correction of missense mutations in mutant clones

DNA sequence analysis revealed that mutations were found in the positive clones as obtained in Section 2.3.2. It is necessary to correct the mutations because a single nucleotide change can result in a codon that codes for a different amino acid and affect the protein structure and function. The procedures of gene correction were summarized in the schematic diagram as shown in Figure 2.5.

In this section, clone 32 with 2 mutated nucleotides (9,086C>T and 9,880A>G) was used as a template for primers design. Two sets of nested primers were designed to amplify the region spanning the mutated nucleotides. *AleI* and *PshAI* unique restriction sites that flanked the region containing mutated nucleotides were digested to allow the ligation of digested clone 32 and nested amplified amplicon to occur.

2.3.3.1 Nested PCR

Nested PCR involved the use of two primer sets as listed in Table 2.5 and two successive PCR reactions. The outer primer set (CHIKV/F8857 and CHIKV/R10268) binds a little further away from the target sequence (*AleI*-*PshAI* region) in the first PCR reaction. The amplicons resulting from the first PCR reaction are used as template for a second PCR reaction using inner primers (CHIKV/F8975 and CHIKV/R10099) which bind nearer to the target sequence. By using this assay, the sensitivity and specificity of the amplification were enhanced. In term of sensitivity, the product of the first reaction is easily re-amplified in the second reaction, while in term of specificity, the inner fragment is only amplified if a specific product from the first PCR reaction is synthesized. The nested PCR strategy was summarized in Figure 2.6.

First, CHIKV RNA was freshly extracted and reverse transcription was performed to generate cDNA. In the first stage of nested PCR, a total volume of 45 µl PCR master mix

containing 5 µl of 10X Pfx50™ buffer [20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50 % (v/v) glycerol], 1.5 µl of 10 mM dNTPs Mix, 0.5 µl of 5U/µl of Pfx50™ DNA polymerase, 2 µl of 10 µM of each primer (CHIKV/F8857 and CHIKV/R10268) and 34 µl of sterile UHQ water was added to 5 µl of cDNA template.

Cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute and extension at 68 °C for 3 minutes and a final extension at 68 °C for 10 minutes. Following this, 25 µl of amplicon was resolved on a 1.0 % agarose (w/v) gel in 1X TBE buffer to confirm the success amplification. The remainder of the amplicon was then diluted with sterile UHQ in a 1:10 dilution and used as template in second stage of amplification.

In the second stage of the nested PCR, 5 µl of diluted amplicons was added to 45 µl of PCR master mix containing 5 µl of 10X Pfx50™ buffer [20 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.1 mM EDTA, 1mM DTT, stabilizers, 50 % (v/v) glycerol], 1.5 µl 10 mM dNTPs Mix, 0.5 µl of 5U/µl of Pfx50™ DNA polymerase, 2 µl of each 10 µM forward and reverse primer (CHIKV/F8975 and CHIKV/R10099) and 34 µl of sterile UHQ water.

The PCR cycling conditions were same as first stage nested PCR. To enhance the yield of the amplicons, multiple reactions of amplicons were pooled in one microcentrifuge tube. The pooled amplicons were subjected to PCR purification as described in Section 2.3.1.4 and eluted in 100 µl of sterile UHQ water. The concentration and purity of the purified amplicons were determined before proceed to restriction digestion.

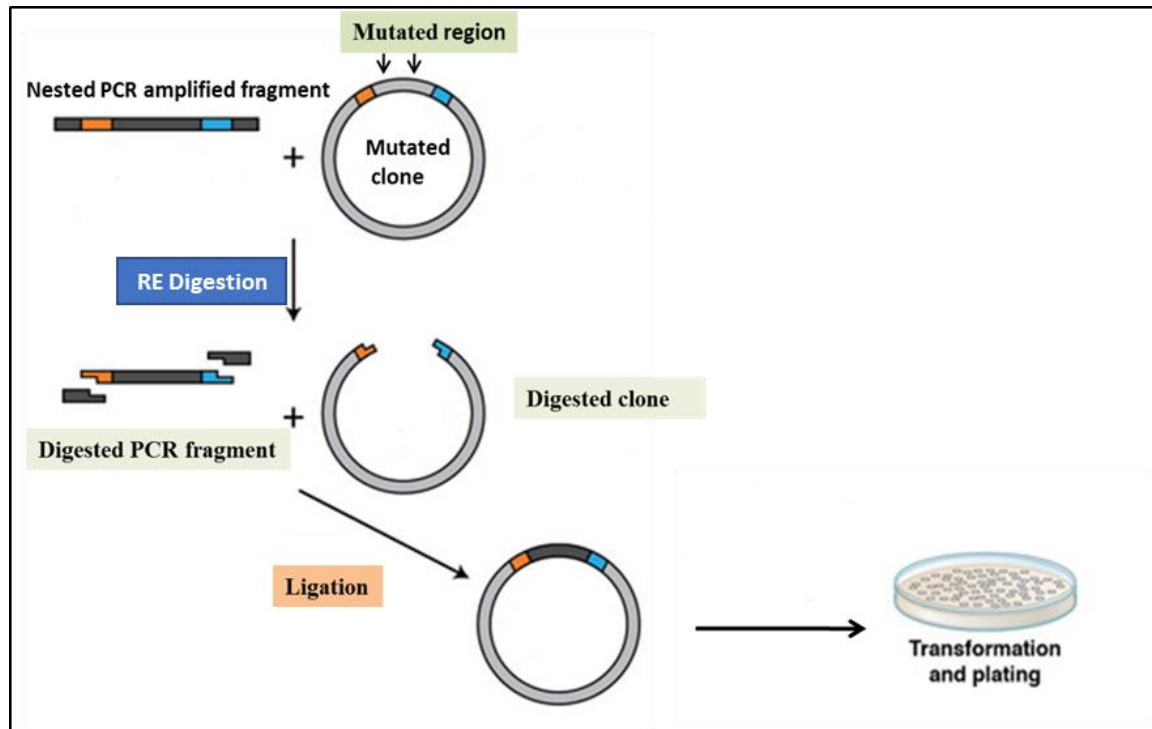


Figure 2.5: A schematic diagram of gene correction procedures. The region containing mutation sites was removed via restriction digestion and replaced by the corrected one via ligation.

Table 2.5: Primer sets used in nested PCR

Nested PCR	Primer name	Nucleotide Sequence (5'-3')	¹Genome position	Expected size (bp)	Function
First PCR	CHIKV/F8857	CCAAAAGGGAAAACCTCTGACG	8857-8877	1412	Binds to sequences outside the target DNA
	CHIKV/R10268	GCGCCGCCCCACATAAA	10252-10268		
Second PCR	CHIKV/F8975	CGCAGCACGGTAAAGAGC	8975-8992	1125	Amplify target DNA within the products of the first reaction
	CHIKV/R10099	TATTGGAGACGGAACACTACTGTCAG	10079-10099		

¹Genome position given with reference to the complete sequence of CHIKV strain S233 (unpublished) provided by IHCM

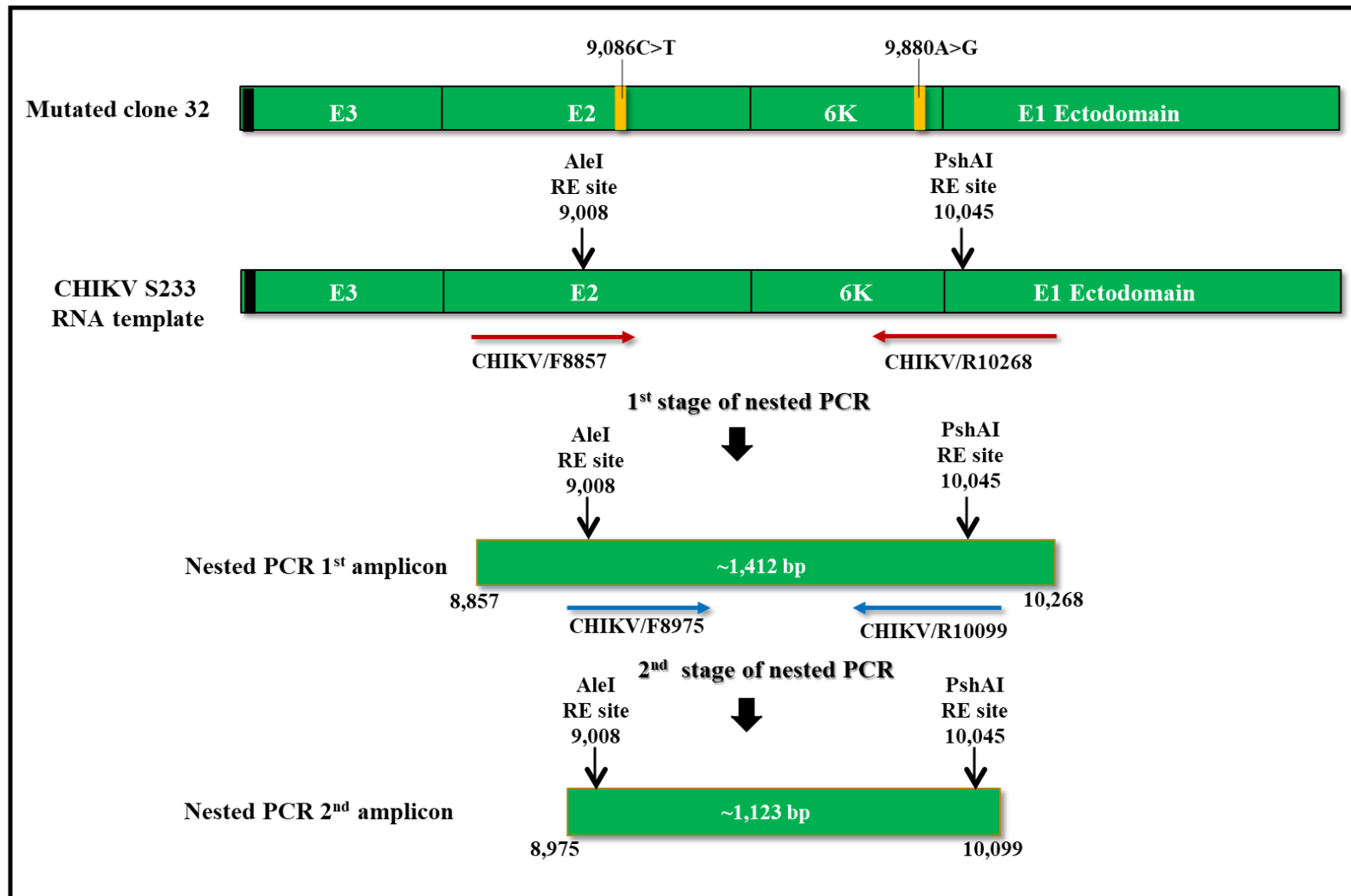


Figure 2.6: Schematic diagram of nested PCR. The outer primer set is indicated as red arrows and the inner primer set is indicated as blue arrows. The mutated nucleotides are indicated as yellow strips.

2.3.3.2 AleI-PshAI double digestion

A total volume of 100 µl digestion reaction containing 10 µl of CutSmart[®] buffer [50 mM potassium acetate, 20 mM Tris-acetate (pH 7.9 at 25 °C), 10 mM magnesium acetate, 100 µg/ml BSA], 5 µl of 10 U/µl AleI (NEB, Massachusetts, USA), 5 µl of 10 U/µl PshAI (NEB, Massachusetts, USA), 60 µl of 190 ng/µl purified amplicons as prepared in Section 2.3.3.1 and 15 µl of sterile UHQ water was prepared.

Meanwhile, another tube of digestion reaction containing 10 µl of CutSmart[®] buffer [50 mM potassium acetate, 20 mM Tris-acetate (pH 7.9 at 25 °C), 10 mM magnesium acetate, 100 µg/ml BSA], 5 µl of 10 U/µl AleI (NEB, Massachusetts, USA), 5 µl of 10 U/µl PshAI (NEB, Massachusetts, USA), 40 µl of 80 ng/µl mutated recombinant plasmid (clone 32) and 40 µl of sterile UHQ was prepared.

Both digestion reactions were subjected to double digestion simultaneously at 37 °C for 18 hours followed by heat inactivation at 80 °C for 20 minutes. Both tubes of entire digested products were loaded in 1.5 % agarose (w/v) gel prepared in 1X TBE buffer. The expected bands were excised and pooled into one microcentrifuge tube in a ratio of 2 volume digested amplicons (1.1 kb) to 1 volume digested mutated recombinant plasmid (6.8 kb). The pooled bands were subjected to gel extraction.

2.3.3.3 Ligation

A 10 µl of ligation reaction was set up by mixing 8 µl of the gel-extracted products as prepared in Section 2.3.3.2 with 1 µl of 10X T4 DNA ligase buffer [400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP (pH 7.8 at 25 °C)] and 1 µl of 5 U/µl T4 DNA ligase. The reaction was incubated at 16 °C for 16 hours. The ligation reaction was used directly in transformation.

2.3.3.4 Transformation

The ligation reaction mixture was transformed into One Shot™ TOP10 Chemically Competent *E. coli* as described in Section 2.3.2.3.

2.3.3.5 Colony PCR screening

Positive clones were identified by performing colony PCR as described in Section 2.3.2.4.

2.3.3.6 Verification of recombinant donor plasmid

Plasmid DNA isolated from positive clones were verified by restriction digestion and DNA sequencing as described in Section 2.3.2.6.

2.3.3.7 Glycerol stock preparation

The bacterial culture containing the desired recombinant donor plasmids was streaked on LB agar plates containing 150 µg/ml ampicillin and incubated overnight at 37 °C. A single colony was picked using sterile toothpick and inoculated into 3 ml LB broth containing 150 µg/ml ampicillin, incubated in a 37 °C incubator shaker set at 225 rpm for 18 hours until the culture reaches stationary phase. To prepare seed culture, 280 µl of overnight culture was added to 2.52 ml of LB broth containing 150 µg/ml ampicillin in a ratio of seed suspension to LB broth 1:10 followed by incubation for 1-2 hours. Subsequently, 300 µl of autoclaved 50 % glycerol was added to 700 µl of transformed TOP10 *E.coli* cell cultures in a 2-ml screw top tube and mixed by gently pipetting. The glycerol stocks were then stored at -80 °C.

2.3.4 Generation of recombinant bacmid DNA

2.3.4.1 Transformation of recombinant donor plasmid DNA into DH10Bac™ *E.coli* cells

For each transformation, one vial of MAX Efficiency® DH10Bac™ competent cells was thawed on ice. Next, 1 ng the recombinant donor plasmid construct (pFBD-E3-E2-6K-

EctoE1) as prepared in Section 2.3.3 was added directly onto thawed competent cells and mixed by gently stirring with a pipette tip. At the same time, control transformation reactions were performed using 1 ng of pFastBacTM Dual-Gus/CAT control plasmid and 1 ng of pUC 19 control. The competent cell/DNA mixtures were incubated on ice for 30 minutes. Subsequently, each transformation tube was heat-shocked by placing the bottom of the tube into a 42 °C water bath for 45 seconds without shaking. After heat shocked, the transformation tubes were immediately chilled on ice for 2 minutes. Next, 900 µl of preheated SOC-medium [2 % Tryptone, 0.5 % Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose] was added to the cells and incubated horizontally in a 37 °C shaking incubator set at 225 rpm for 4 hours. For pUC 19 transformation, the tube was only incubated for 1 hour.

For each pFastBacTM donor plasmid transformation, 10-fold serial dilutions of the cells (10^{-1} , 10^{-2} , and 10^{-3}) were prepared with S.O.C medium. One hundred microliters of each dilution were spread on DH10BacTM LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. For the pUC 19 transformation, the cells were diluted with S.O.C medium in a ratio of 1:100 and 100 µl of the diluted transformed cells were spread on LB agar plate containing 150 µg/ml ampicillin. All the plates were incubated in a 37 °C incubator for 48 hours.

After 48 hours incubation, white colonies were picked and re-streaked on DH10Bac LB agar plate to verify the phenotype. To avoid selecting false positives, the largest and the most isolated white colonies were chosen.

2.3.4.2 Colony PCR screening

Colony PCR was used as a screening tool to determine if the transposition event had occurred. Positive clones were identified by performing colony PCR as same as described in Section 2.3.2.4 except the primers used were different. The primers are listed in Table 2.6.

Table 2.6: Primers used in colony PCR screening of bacmid DNA

Primer	Sequences (5'-3')	¹ Genome Positions	Expected size (bp)
CHIKV/F9018 CHIKV/R10937	CACCGCCGCAACTACCGAGGAG ACGCCCCCAAAGTCTGAGGAATG	9018-9040 10937-10960	1920

¹ Genome position given with reference to the complete sequence of CHIKV strain S233 (unpublished) provided by IHCM

2.3.4.3 Recombinant bacmid DNA isolation

Recombinant bacmid DNA was purified from DH10Bac™ *E.coli* using PureLink™ HiPure Plasmid DNA Miniprep Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the instructions provided by manufacturer. The kit is based on anion-exchange chromatography, which utilizes a patented resin composed of small particles with a uniform pore size to efficiently purify plasmid DNA to a level equivalent to two passes through cesium chloride (CsCl) gradient.

First, a single white colony was inoculated into 3 ml of DH10Bac™ LB broth. The bacterial culture was incubated overnight in a 37 °C shaking incubator set at 225 rpm.

To harvest the bacterial cells, 3 ml of overnight culture was centrifuged at $9,000 \times g$ for 15 minutes followed by removing the supernatant completely without disturbing the cell pellet. The cell pellet was resuspended in 400 µl of resuspension buffer [50 mM Tris-HCl, pH 8.0] containing RNase A [20 mg/ml in 10 mM Sodium acetate, pH 5.2] until homogenous. To lyse the bacterial cells, 400 µl of lysis buffer [0.2 M NaOH, 1 % SDS] was added to the mixture and mixed gently by inverting the capped tube for 5 times. The mixture was incubated at room temperature for 5 minutes. Following this, 400 µl of precipitation buffer [3.1 M potassium acetate, pH 5.5] was added and mixed immediately by inverting the capped tube until the mixture is homogenous. The mixture was centrifuged at $15,320 \times g$ at room temperature for 10 minutes.

After centrifugation, the supernatant was loaded onto the equilibrated column and the solution was drained by gravity flow. In this step, the negatively charged phosphate on the backbone of the DNA interact with the positive charges on the surface of the resin in the column. The column was washed twice with 2.5 ml of wash buffer [0.1 M Sodium acetate, pH 5.0, 825 mM NaCl]. The solution in the column was drained by gravity flow after each wash and the flow-through was discarded. In this step, bacmid DNA remains bound to the resin while RNA, proteins, carbohydrates and other impurities are washed away with wash buffer.

To elute the bacmid DNA, a sterile microcentrifuge tube was placed under the column. Nine hundred microliters of elution buffer [100 mM Tris-HCl, pH 8.5, 1.25 M NaCl] was added to the column and drained by gravity flow. The purified bacmid DNA was collected in the elution tube and 630 μ l isopropanol was added to the purified DNA, mixed gently by inverting the tube for few times. The tube was incubated on ice for 10 minutes. The mixture was centrifuged at $15,320 \times g$ at 4 °C for 20 minutes. The supernatant was carefully removed and discarded. The DNA pellet was resuspended in 1 ml of 70 % ethanol, followed by centrifugation at $15,320 \times g$ at 4 °C for 5 minutes. The supernatant was removed and the DNA pellet was air-dried for 10 minutes. After drying, the DNA pellet was resuspended in 40 μ l TE buffer [10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA] by pipetting only 2 times to avoid shearing the DNA. The tube was then incubated on ice for at least 10 minutes to dissolve the pellet.

2.3.4.4 Glycerol stock preparation

The bacterial culture containing the desired recombinant bacmid was streaked on DH10Bac™ LB agar plates. The plate was incubated overnight at 37 °C. A single colony was then isolated from the agar plate and inoculated into 3 ml DH10Bac™ LB broth. The bacterial culture was incubated in a 37 °C shaking incubator set at 225 rpm for 18 hours until the culture reaches stationary phase.

To prepare seed culture, 300 µl of overnight culture was added to 2.7 ml of DH10Bac™ LB broth in a ratio of seed suspension to LB broth 1:10 followed by incubating for 1-2 hours. Three hundred microliters of autoclaved 50 % glycerol was added to 700 µl of seed culture in 2 ml screw-cap tube and gently mixed. The glycerol stock was then stored at -80 °C.

2.3.5 Generation of recombinant baculovirus (AcMNPV-E3E26KEctoE1)

2.3.5.1 Maintenance of Sf9 cells

Sf9 cells were originally derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. They are highly susceptible to infection with *Autographa Californica* multiple nuclear polyhedrosis virus (AcMNPV), and capable of mammalian cell-like post-translational modifications. Thus, *Sf9* cells are widely used in baculovirus expression system.

The *Sf9* cells (Invitrogen, Carlsbad, CA) were maintained in a 25 cm² flask with 5 ml of Grace's Insect Medium (Gibco, NY, USA) supplemented with 10 % FBS (GIBCO, Grand Island, NY, USA) (Appendix A.1.4 & A.1.5). To subculture monolayer cultures, 3.0 ml of old medium were aspirated and discarded from an 80 % to 90 % confluent monolayer. A sterile tissue cell scraper (ThermoFisher Scientific, Switzerland) was used for gentle detachment of the adherent cells. The cells were resuspended by pipetting the suspension using serological pipette. After that, the cell suspensions were aspirated and split into two

sterile 25 cm² flask. To each of the flask, 4.0 ml of fresh media was added to top up the volume. The cells were incubated at 27 °C in an incubator.

2.3.5.2 Adaptation of *Sf9* cells into serum-free medium

In order to switch *Sf9* cells from serum-supplemented to serum-free medium (SFM), it is necessary to gradually adapt the cells to grow in serum-free conditions because it may be too stressful for the cells if the cells change directly to 100 % serum-free condition. Sequential adaptation of *Sf9* cells (Figure 2.7) was performed by following the manufacturer's instruction.

To adapt *Sf9* cells in 25 % SFM, a 25 cm² flask of *Sf9* cells (as prepared in Section 2.3.5.1) with a minimum density of 5x10⁵ cells/ml were first grown in a mixed media containing 75 % of GIM supplemented with 10 % FBS and 25 % of Sf-900™ II SFM (Gibco, NY, USA). The cells were incubated at 27 °C in a static incubator until the cells reached confluency. Following this, the cells were adapted from 25 % to 50 % SFM. To do so, the cells were first detached from the flask using sterile scraper and resuspended by pipetting the suspension. Approximately 2 ml of cell suspension was transferred to a new flask that contain 3 ml of mixed media comprised of 50 % of GIM supplemented with 10 % FBS and 50 % of SFM. The cells were allowed to reach confluency at 27 °C, and further adapted in the same manners from 50 % to 75 % SFM, followed by 75 % to 100 % SFM.

Passage 1
75% serum-supplemented medium : 25% SFM
Passage 2
50% serum-supplemented medium : 50% SFM
Passage 3
25% serum-supplemented medium : 75% SFM
Passage 4
100% SFM

Figure 2.7: Sequential adaptation of *Sf9* cells from serum-supplemented to SFM.
(Source: Gibco manual)

2.3.5.3 Transfection of *Sf9* cells

Recombinant bacmid DNA as prepared in Section 2.3.4.3 was transfected into *Sf9* cells in the presence of Cellfectin® II reagent (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to the manufacturer's instruction. Cellfectin® II reagent is a proprietary cationic lipid formulation that provides the highest transfection efficiencies and protein expression levels on *Sf9* cell lines.

Serum-free adapted *Sf9* cells that were in the log phase ($1.5\text{--}2.5 \times 10^6$ cells/ml) with greater than 95 % viability were seeded at 8×10^5 cells per well in 2 ml of unsupplemented GIM (Gibco, NY, USA) (Appendix A.1.6) using a 6-well plate. The content of the plate was mixed by sliding the plate back and forth, side to side to distribute the cells evenly throughout the well. The cells were allowed to settle down at room temperature in the hood for 1 hour. For each transfection sample, two complexes were prepared as follows:
Solution A: 8 µl of Cellfectin® II reagent was diluted in 100 µl unsupplemented GIM and vortexed briefly to mix.

Solution B: 1-2 µg of bacmid DNA was diluted in 100 µl of unsupplemented GIM and mixed gently.

Solution A and B were mixed (total volume ~210 µl) in one tube and incubated at room temperature for 30 minutes. Mixtures with bacmid control (pFastBacTM Dual-Gus/CAT) and without bacmid (Cellfectin[®] reagent only) were also prepared as transfection positive and negative control respectively.

The transfection mixtures (~210 µl) were added dropwise onto the cells, then the mixture was gently mixed by sliding the plate from side to side to distribute the mixture evenly. The cells were then incubated at 27 °C for 5 hours. After 5 hours, the transfection mixtures were removed from the wells and replaced with 2 ml of GIM supplemented with 10 % FBS. The cells were incubated at 27 °C and observed daily until the signs of viral infection appear. The cell images were taken using Nikon A1 Confocal Laser Microscope.

2.3.5.4 Isolation of P1 viral stock

When the transfected cells show signs of late stage infection, the baculovirus-infected culture supernatant was harvested by centrifugation. The entire medium containing baculovirus was collected from each well (~2 ml) and transferred to 15 ml sterile centrifuge tube. The tubes were centrifuged at 500 × g (5810R, Eppendorf, Hamburg, Germany) for 5 minutes to remove the cells and large debris. The clarified supernatants were transferred into a new sterile tube and this was the P1 (passage 1) viral stock. The transfection positive and negative controls were also harvested as described above. The tubes were wrapped with aluminium foil to protect from light and kept at 4 °C for future use.

2.3.6 Preliminary expression of recombinant proteins

A 25 cm² flask of 6.6×10^6 cells/ml serum free-adapted Sf9 cells was prepared a day before. Before infection, 4 ml of old medium was discarded before adding 500 µl of the P1 viral stock as prepared in the Section 2.3.5.4. The flask was rocked on the rocker for about

30 minutes. After 30 minutes, 4 ml of fresh Sf-900TM II SFM was added into the flask and incubated in an Innova 4000 incubator shaker set at 27 °C. Gus/CAT-expressing baculovirus (AcMNPV-Gus/CAT) and a mock infection were also prepared using transfection positive control and negative control respectively. The cells were inspected daily for the signs of viral infection.

An approximately 100 µl of the culture medium containing AcMNPV-E3E26KEctoE1 were collected daily until the late stage of viral infection was demonstrated. At the late stage of viral infection, the AcMNPV-E3E26KEctoE1 was harvested as described in Section 2.3.5.4, together with AcMNPV-Gus/CAT and mock infection. The clarified supernatant was transferred into a sterile tube and this was the P2 (passage 2) viral stock.

To prepare cell lysate, 1 ml of Hypotonic Douncing Buffer [10 mM HEPES, 1.5 mM MgCl₂, 5 mM KCl] was added to the cell monolayer, and rocked on the rocker for about 30 minutes. After 30 minutes, the entire medium containing the cell lysate were transferred into the microcentrifuge tube and spin at 5,000 × g for 20 minutes at room temperature.

The tubes containing the supernatants and the cell lysates were wrapped with aluminium foil. The daily collected supernatants together with the harvested supernatants and cell lysates were analyzed by SDS-PAGE and western blot.

2.3.7 Antigenic testing of recombinant proteins

2.3.7.1 SDS-PAGE and western blot

The supernatants and cell lysates as prepared in Section 2.3.6 were analyzed by performing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. In SDS-PAGE, the protein contents of harvested samples were first incubated with SDS, which denatures the proteins and masks the proteins with negative charge, allowing them to be separated according to their molecular weight by PAGE. The proteins

were then transferred from gel to a nitrocellulose membrane, in which the target proteins can be probed using specific primary antibodies coupled with secondary antibody.

In this study, the clarified supernatant and cell lysates resuspended in 4X reducing protein sample buffer (Appendix A.2.3 & A.2.4) were resolved on 12-15 % resolving gel overlaid with 5 % stacking gel. Twelve percent resolving gel was first prepared by mixing 3.4 ml of UHQ water, 4 ml of 30 % acrylamide/bis-acrylamide (Bio-Rad Laboratories Inc., Hercules, CA), 2.5 ml of resolving buffer [1.5 M Tris-HCl, pH 8.8] (Bio-Rad Laboratories Inc., Hercules, CA), 100 μ l of 10 % SDS (Appendix A.2.1), 100 μ l of 10 % APS (Appendix A.2.2) and 4 μ l of TEMED (Bio-Rad Laboratories Inc., Hercules, CA). The resolving gel mixture was transferred into the gel caster system. UHQ water was added on the top of the gel in order to level the gel surface and avoid formation of bubbles. After that, 5 % stacking gel was prepared by mixing 3.4 ml of UHQ water, 1 ml of 30 % acrylamide/bis-acrylamide, 1.5 ml of stacking buffer [0.5 M Tris-HCl, pH 6.8] (Bio-Rad Laboratories Inc., Hercules, CA), 50 μ l of 10 % SDS, 50 μ l of 10 % APS (Appendix), and 5 μ l of TEMED. The stacking gel mixture was transferred on the top of the resolving gel after the UHQ water was discarded. Wells for the sample application were formed by a comb that was inserted into the space before polymerization occur.

For sample preparation, the clarified supernatants and cell lysates were dissolved in 4X reducing protein sample buffer and heated for 10 minutes prior to centrifugation at $12,045 \times g$ for 10 minutes. The samples were then loaded into the wells and electrophoresis was performed vertically at a constant voltage of 150 V in 1X running buffer [25 mM Tris, 192 mM Glycine, 0.1 % SDS, pH 8.3] in a MiniProtean III Cell (Bio-rad, Hercules, CA, USA). The proteins on the gel were either stained with Coomassie brilliant blue (CBB) G250 (Appendix A.2.5) (Bio-rad, Hercules, CA, USA) or electro transferred onto

nitrocellulose membrane (ADVANTEC, California, USA) in a mini Trans-Blot® module (Bio-rad, Hercules, CA, USA).

To transfer the proteins, the electrophoresis transfer cassette was first prepared in the following order: cathode (-) → sponge → filter paper → gel → nitrocellulose membrane → filter paper → sponge → anode (+). The whole cassette was placed into a chamber containing 1X Towbin buffer [25 mM Tris, 192 mM Glycine, 20 % v/v methanol] (Appendix A.2.6) and run at 200 mA for 1 hour. After 1 hour, the membrane was placed into a container containing 1XPBS with skimmed milk and rocked for 30 minutes for blocking purpose. The membrane was then washed with RO water, then probed with patient serum at dilution of 1:200 overnight. After overnight incubation on the rocker, the blot was probed with polyclonal rabbit anti-human IgG-HRP (Dako, Glostrup, Denmark) at 1 in 2000 dilution, followed by 2 hours incubation on the rocker at room temperature. In between each of the incubation, the blot was washed 3 times with 1X PBS at 10 minutes intervals. Reactive bands were detected when the blot was incubated in chromogenic substrate containing 4-chloro-1-naphthol in methanol/H₂O₂ substrate for 30 minutes at room temperature.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Validation of CHIKV isolate used in this study

C6/36 cells were infected with CHIKV strain S233 and incubated at 30 °C in a static incubator as described in Section 2.1.4. More than 90 % CPE was observed in CHIKV-infected C6/36 cells (Figure 3.1B) on day 8 post-infection. No CPE was observed in mock-infected C6/36 cells (Figure 3.1A). The result clearly demonstrates that C6/36 cells were successfully infected by CHIKV as CHIKV induced CPE in C6/36 cells which culminate in cell death via apoptosis on day 8-post infection was observed.

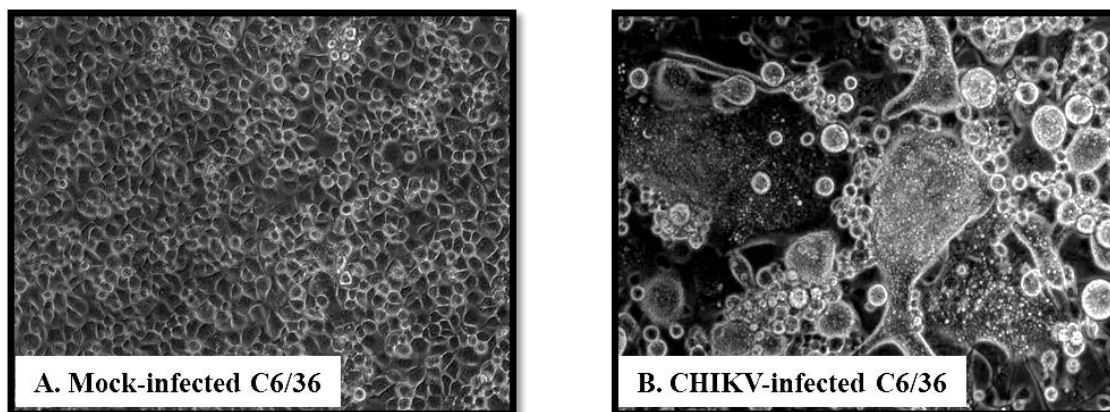


Figure 3.1: CPE of C6/36 cells infected with CHIKV A) mock-infected C6/36 and B) CHIKV-infected C6/36 on day 8 post-infection. Images were taken at the 20X magnification.

The presence of the CHIKV genome in the CHIKV-infected culture supernatant was detected by RT-PCR as described in Section 2.2.2. The primers used were specifically designed to target three different portions of the CHIKV E1 structural gene region which are 685 bp, 743 bp and 688 bp respectively in length. Figure 3.2 shows the electrophoresis result of the generated amplicons analysed on 2 % agarose gel in 1X TBE. In this figure,

detection of CHIKV in the CHIKV-infected culture was confirmed, in which approximately 685 bp, 743 bp and 688 bp respectively of the expected sized amplicons were detected when analyzed by electrophoresis. The CHIKV isolate was then sequenced verified via DNA sequencing. The sequences of all three amplicons were found to be of the expected CHIKV genome sequence when queried using BLAST.

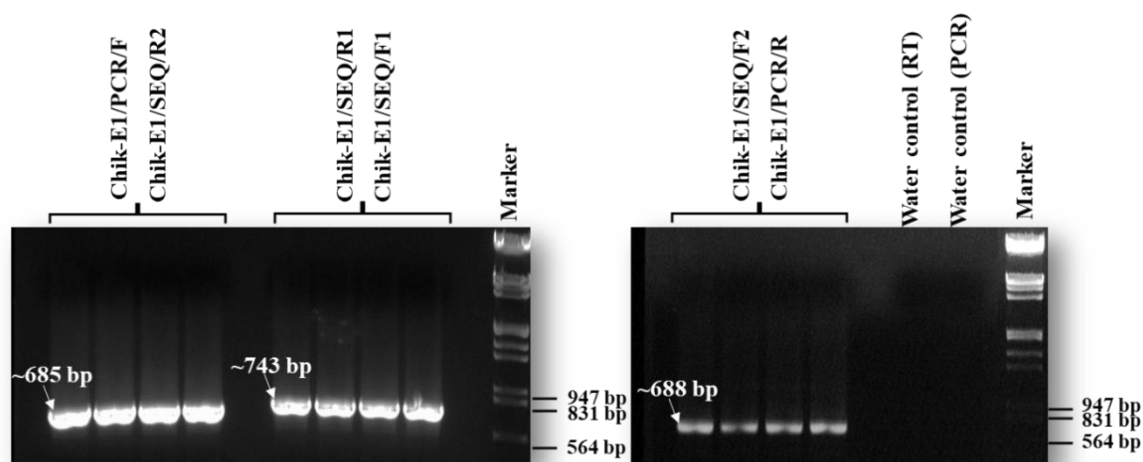


Figure 3.2: Agarose gel analysis of amplicons from RT-PCR. The amplicons with expected size are indicated by the arrows. The names of primers used are indicated on the top of gel lane. UHQ water was used as water control in RT step and PCR step. Lambda DNA/HindIII+EcoRI markers are indicated as marker on the right side of each gel image.

3.2 Amplification of target gene region (E3-E2-6K-EctoE1)

Several different strategies were attempted to amplify the target gene region (E3-E2-6K-EctoE1) from the verified CHIKV template. Initially a set of specific PCR primers were specifically designed to amplify the whole target region (E3-E2-6K-EctoE1) in a single RT-PCR, however, an approximately 350 bp of amplicon (result not shown) were detected lower than 2,874 bp of expected PCR. The 350 bp band was sequenced by DNA sequencing and the sequence was aligned to the CHIKV S233 sequence that was previously sequenced by others at the institute. From the alignment result (not shown), the forward primer was found to anneal to the wrong location and extended by polymerase to generate a short fragment of

amplicon. Optimization of the amplifications such as optimization of primer design, primer concentration and PCR conditions had been made. However, similar PCR products with nearly the same size were still detected.

The target gene region (E3-E2-6K-EctoE1) was only successfully amplified using the strategy as schematically presented in Figure 2.4. Using this method, the expected 2,874 bp of target target region was successfully assembled from four individual overlapping amplicons. Four separate overlapping amplicons were first generated from the a CHIKV S233 template through RT-PCR using designed 4 separate primer sets as listed in Table 2.2 and resolved on 1.5 % agarose gel prepared in 1X TBE. Figure 3.3 shows successful amplification of the individual amplicons from RT-PCR which resulted in the expected 968 bp, 965 bp, 972 bp, and 1,320 bp amplicons respectively. Non specific bands with sizes lower than the expected products were also observed in the same gel, this could be due to non-specific binding or primer dimer formation.

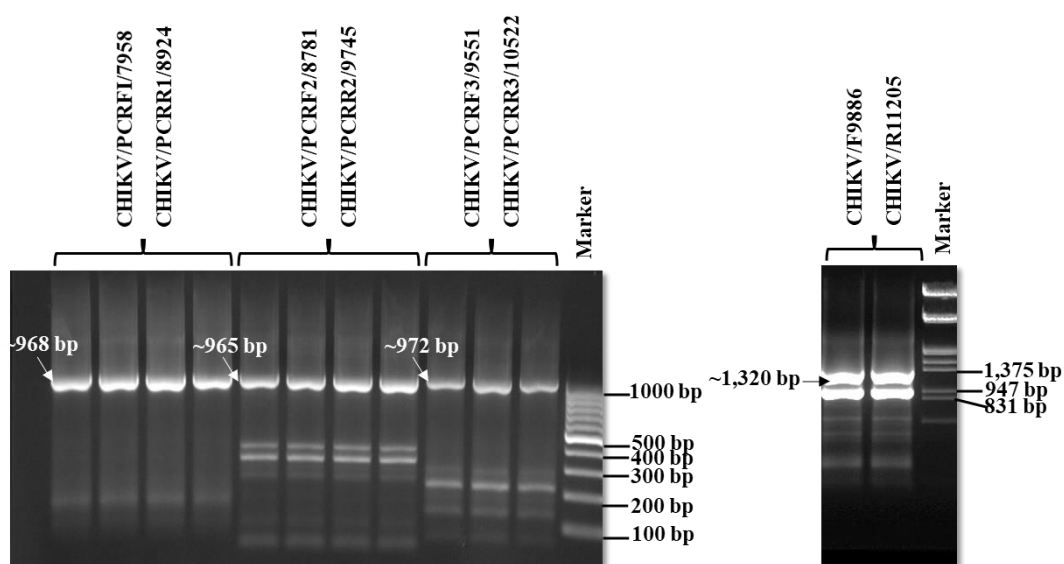


Figure 3.3: Agarose gel analysis of individual amplicons amplified via RT-PCR. The expected amplicons are indicated by the arrows. The name of primers used are indicated at the top of gel lane. GeneRuler 100 bp DNA ladder and Lambda DNA/HindIII+EcoRI marker are indicated as marker on the right side of each gel image.

Subsequently, a pool of equimolar templates containing the four gel-purified overlapping amplicons as obtained in Figure 3.3 were assembled and amplified using a pair of primers (CHIKV/BamHI/F8350 and CHIKV/HindIII/R11205) flanking the target region as listed in Table 2.2. Figure 3.4 demonstrates that the expected amplicons were successfully generated through assembly PCR and resolved on 1 % agarose gel in 1X Tris-acetate-EDTA (TAE) buffer. From the gel image, two potential bands with expected size nearly 2,874 bp were observed. The water or negative control included in the gel was clear, this indicates no contamination occurs in the amplification. The two bands which are indicated by the arrows were migrated very close to each other between 2,027 bp and 3,530 bp marker. It was very difficult to differentiate which one is the 2,874 bp target band. Besides that, the yield of the amplicons in the gel was too low to be extracted and used in subsequent step. To overcome these problems, multiple PCR reactions were prepared, then pooled in one microcentrifuge tube. The pooled PCR reactions were then subjected to PCR purification prior to cloning step. This strategy was efficient in increasing the yield of the amplicons and also ensuring the target amplicons were cloned into pFastBacTM Dual donor plasmid in the subsequent cloning step.

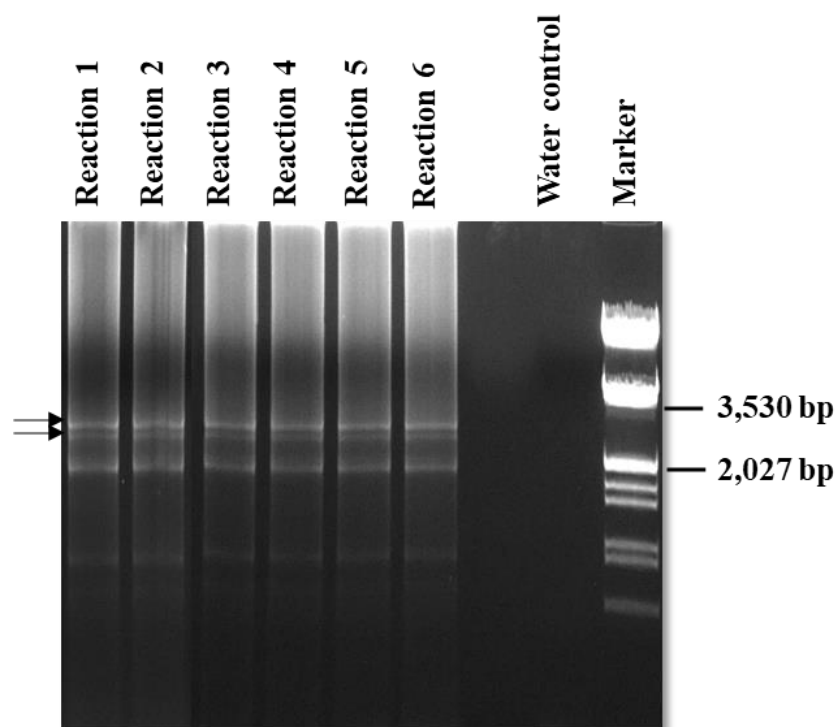


Figure 3.4: Agarose gel analysis of amplicons generated through assembly PCR. The potential expected amplicons are indicated by the arrows. UHQ water was used as a water control. Lambda DNA/HindIII+EcoRI is indicated as marker on the right side of the gel image.

In order to ligate the insert DNA (purified amplicons as obtained in Figure 3.4) into pFastBacTM Dual donor plasmid, they were double digested with restriction enzymes BamHI and HindIII as described in Section 2.3.3.1. Figure 3.5 demonstrates the complete digestion or linearization of pFBD donor plasmid on 7 % agarose gel in 1X TAE buffer. From the gel image, the pFastBacTM Dual donor plasmid was confirmed to be successfully linearized as an approximately 5,145 bp of single band, a 93 bp of short fragment which can be found between the restriction sites of BamHI-HindIII was digested out from the pFBD donor plasmid. However, the undigested pFBD donor plasmid (first lane) was separated into two major bands, which corresponded to the nature form of supercoiled plasmid (more intense band with lower product size) and open-circular plasmid (less intense band with higher product size).

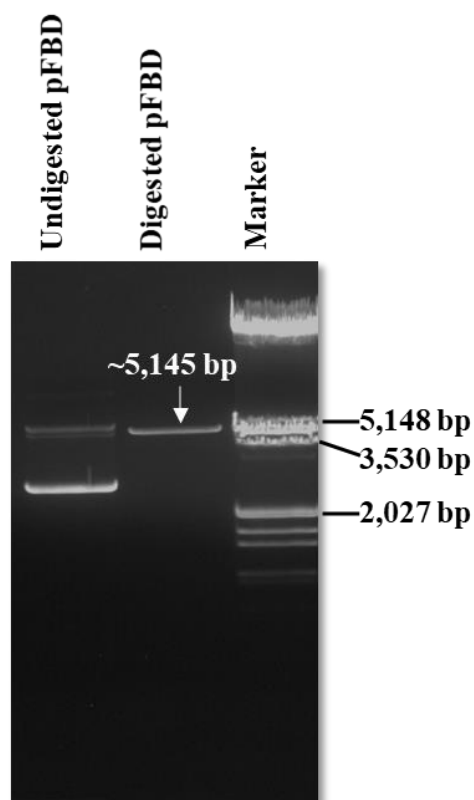


Figure 3.5: Agarose gel analysis of BamHI-HindIII double digested pFastBacTM Dual donor plasmid (pFBD). The linearized pFBD is indicated by the arrow. Undigested pFastBacTM Dual donor plasmid was used as a control. Lambda DNA/HindIII+EcoRI is indicated as marker on the right side of the gel image.

Prior to ligation, the digested products (pFBD donor plasmid and insert) were first pooled in one tube and subjected to PCR purification in order to get rid of contaminating salts from the solution and concentrate the digested products. The ligation of pFBD donor plasmid and insert were performed as described in Section 2.3.2.2. In this step, both the digested donor plasmid backbone and the insert were mixed in a single tube to allow the compatible overhangs generated by restriction digestion to stick to one another and form a complete, circular plasmid. Following this, the ligated plasmid was transformed into TOP 10 *E.coli* cells to form recombinant pFBD donor plasmid (pFBD-E3E26KEctoE1).

3.3 Clone verification

To identify the clones that harbour the recombinant donor plasmid, ampicillin-resistant colonies were selected and confirmed via colony PCR using the forward insert primer (CHIKV/PCRF5/11086) and reverse vector primer (pFBD/Sv40Tn7L/4849R) as listed in Table 2.3 to check if the insert is in correct orientation.

Ampicillin-resistant transformants were selected and subjected to colony PCR in order to identify the clones that harbour recombinant donor plasmid. Figure 3.6 shows the amplicons generated through colony PCR on 2 % agarose gel in 1X TBE buffer. Thirty-two colonies were picked and only 2 colonies were successfully amplified. From the gel image in Figure 3.6, clones 30 and 32 show intense bands with expected size approximately 413 bp, indicating that these two clones contain insert DNA of interest. Water control was included as a negative control and the lane was clear, this represents no contamination in the amplification. These two clones were then subjected to plasmid DNA isolation and further sequencing confirmation of the entire insert.

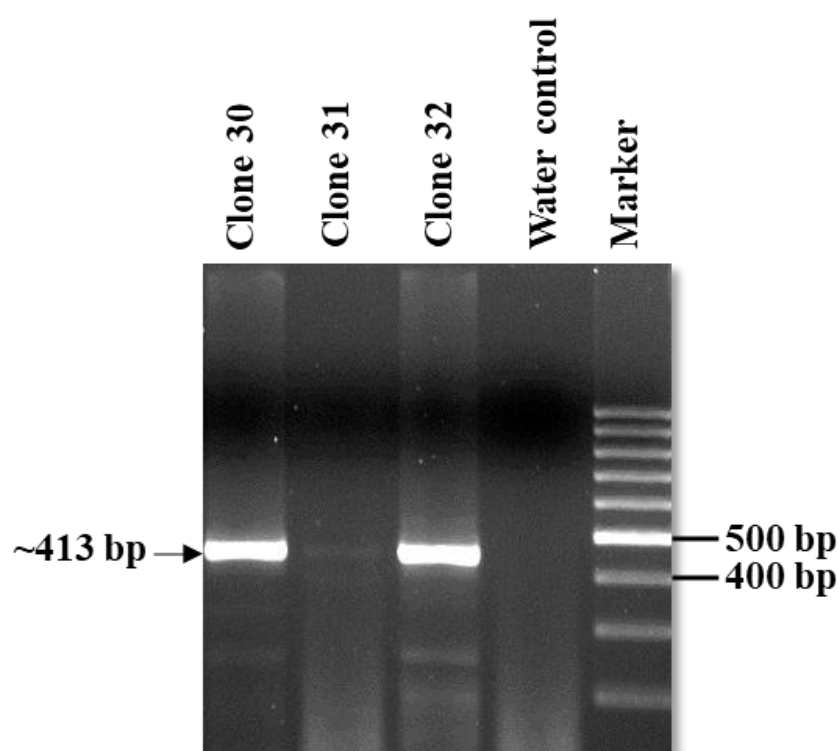


Figure 3.6: Agarose gel electrophoresis of colony PCR amplification of selected clones. Amplicons with expected size are indicated by the arrow. UHQ water was used as water control. GeneRuler 100 bp DNA ladder is indicated as marker on the right side of the gel image.

Figure 3.7 shows restriction digestion analysis of clones 30 and 32 on 0.7 % agarose gel in 1X TAE buffer. Based on the gel image result in Figure 3.7, as compared to the undigested clone, in which two bands that represent supercoiled and open-circular plasmids were observed, the plasmid of clones 30 and 32 were correctly digested by BamHI and HindIII restriction enzymes, in which approximately 5.1 kb and 2.8 kb of the products respective to the linearized backbone of pFastBacTM Dual and insert DNA (E3-E2-6K-EctoE1) were observed. This indicates that the BamHI and HindIII restriction enzymes works efficiently.

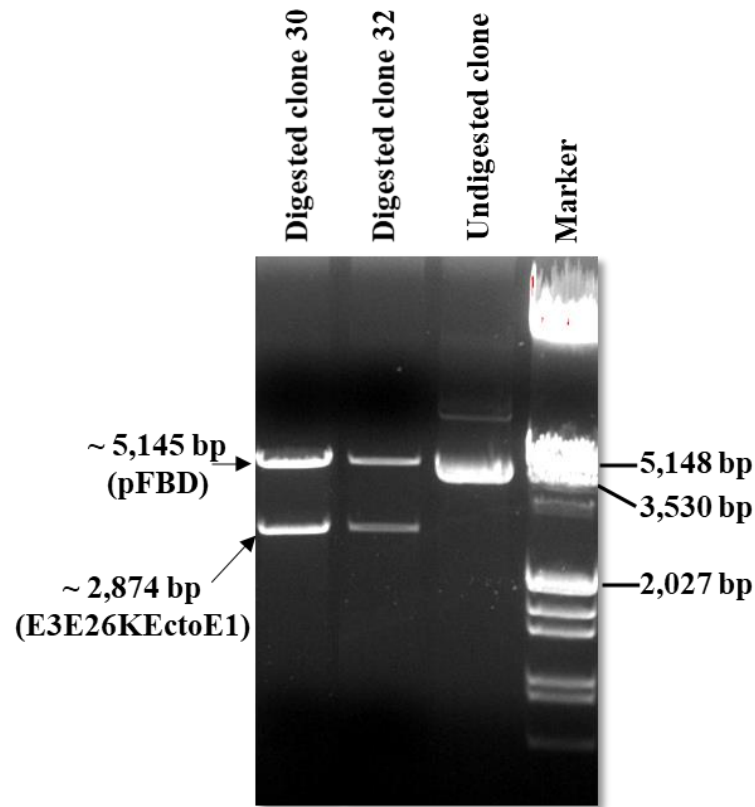


Figure 3.7: Agarose gel electrophoresis of double digestion of positive clones (clone 30 and 32). The desired bands with size approximately 5.1 kb and 2.8 kb, respective to the backbone of pFastBac[™] Dual donor plasmid and the insert DNA (E3-E2-6K-EctoE1) are indicated by the arrows. Lambda DNA/HindIII+EcoRI is indicated as marker on the right side of the gel image.

The plasmid DNA of the two clones were sequenced verified using sequencing primers as listed in Table 2.4. In this step, the entire insert DNA were sequenced and analyzed. The sequences generated were then aligned to the reference sequence of CHIKV S233. The sequence analysis revealed the successful recombination of insert DNA (E3-E2-6K-EctoE1) with donor plasmid (pFBD donor plasmid). However, missense mutations and silent mutations were found in the clones. Missense mutations altered the amino acid sequences and led to a different phenotype, while silent mutations did not. Table 3.1 shows the nucleotide substitution and amino acid substitution in clones 30 and 32 in comparison with the reference sequence. The nucleotide substitution which resulted in silent mutation were ignored and not included in the table as it did not alter the amino acid sequence.

Table 3.1: Nucleotide and amino acid variations identified in clone sequence in comparison to the reference sequence

Clone	Gene region	Nucleotide variations	Type of mutations	Amino acid variations
30	E2	¹ g.8567A>G	Missense mutation	² p.Y9C
	E1	³ g.10051_10052insC	Frameshift mutation	⁴ p.N20TfsX384
32	E2	g.9086C>T	Missense mutation	p.S182L
	6K	g.9880A>G	Missense mutation	p.I24V

*Nucleotide positions are relative to the complete genome sequence of CHIKV strain S233 (unpublished) provided by IHCM

*Amino acid positions are relative to the deduced amino acid sequences of structural proteins in CHIKV strain S233 provided by IHCM

- ¹ g.8567A>G denotes nucleotide A at position 8567 is substituted by G. Alphabet g. denoted genomic sequence.
- ² p.Y9C denotes amino acid Y (Tyrosine) at amino acid position 9 is changed to C (Cysteine)
- ³ g.10051_10052insC denotes a nucleotide C is inserted between nucleotides 10051 and 10052
- ⁴ p.N20TfsX384 denotes amino acid asparagine (N), at amino acid positions 20 changed to amino acid threonine (T), the length of the frame shift (fs) is 384 amino acid, including the premature stop codons (X)

In clone 30, a missense mutation g.8567A>G resulted in amino acid substitution of Tyrosine (Y) to Cysteine (C) at amino acid position 9 of E2 protein; frameshift mutation of g.10051_10052insC resulted in an amino acid asparagine (N) at amino acid position 20 of protein E1 changed to threonine (T), followed by a shift of reading frame downstream of the mutation and creation of premature stop codon in the sequence. This could lead to production of a protein which is abnormally short. In clone 32, two missense mutations (g.9086C>T and g.9880A>G) resulted in amino acid substitution of Serine (S) to Leucine (L) at amino acid position 182 of E2 protein and substitution of Isoleucine (I) to Valine (V) at amino acid position 24 of 6K protein respectively.

It was very important to correct the mutated amino acids as a change of amino acid from one to another do affect the protein structure and function. The gene correction was carried out as described in Section 2.3.3, in which clone 32 was chosen as a template for

gene correction because clone 32 has two unique restriction sites (AleI and PshAI) which flank the mutated sites (from position 9,086 to 9,880) and this region is much more shorter as compared to clone 30 (from position 8567 to 10052), therefore, it was easier to generate a new fragment from the original virus isolate, CHIKV S233 and replaced the mutated region in clone 32 by restriction digestion.

3.4 Gene correction in mutant clones

Nested PCR was performed to generate a new DNA fragment from the CHIKV S233 virus isolate and this resulted DNA fragment was used to replace the mutated region in clone 32. The strategies used were schematically represented in Figure 2.5 and 2.6. Instead of RT-PCR, nested PCR is preferable to be used to generate the target fragment which is 1,123 bp in length as the sensitivity and specificity of DNA amplification is significantly better than RT-PCR.

Figure 3.8 shows the electrophoresis results of nested PCR first amplicon, second amplicon and a pool of purified amplicons on 1.0 % agarose gel in 1X TAE. From the gel image in Figure 3.8 A, a band with expected size, approximately 1,412 bp was observed, indicating the amplicon generated through first stage of nested PCR was successfully synthesized by using the nested outer primers CHIKV/F8857 and CHIKV/R10268 as listed in Table 2.5. From the gel image in Figure 3.8 B, a band with expected size, approximately 1,123 bp was observed, indicating the second amplicon which is internal to the first amplicon was successfully generated via second stage of nested PCR using the nested inner primers CHIKV/F8977 and CHIKV/R10099 as listed in Table 2.5. Water controls included in both stages of nested PCR were clear and this indicates no contamination during amplification. In order to increase the yield of amplicons, a total of 8 amplification reactions were prepared and pooled in a single tube prior to PCR purification. The pooled and purified amplicon was

resolved on the 1.0 % agarose gel in 1X TAE as shown in Figure 3.8 C, in which a higher yield band was observed.

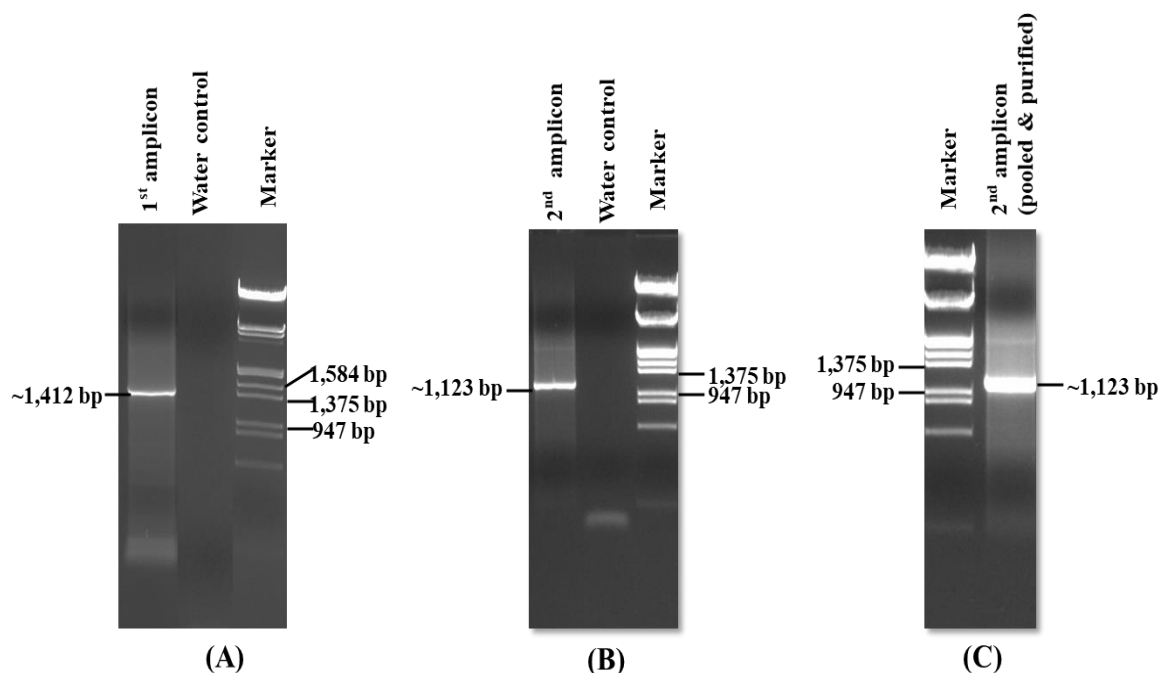


Figure 3.8: Agarose gel electrophoresis of amplicons generated through nested PCR. UHQ water was used as water control. Lambda DNA/HindIII+EcoRI is indicated as marker on the right side of the gel image.

Following this, both insert DNA (purified amplicons as shown in Figure 3.8 C) and plasmid of clone 32 were subjected to *AleI*-*PshAI* double digestion. Based on the result, the digested products were at correct size and released approximately 1.0 kb and 6.9 kb of insert and plasmid backbone (result not shown). Both insert and plasmid backbone obtained were gel purified and ligated in a single tube as described in Section 2.3.3.3. Following this, the ligated construct was transformed into TOP 10 *E.coli* cells.

Twenty colonies were screened with primers (CHIKV/F8914 and CHIKV/R9272) and the amplicons were resolved on 2 % agarose gel in 1X TBE. Based on the gel image in Figure 3.9, 19 out of 20 colonies were found to be positive by colony PCR screening, in

which the amplicons with expected size, approximately 359 bp were observed. The plasmid of clone 32 was used as positive control in this colony PCR assay as this clone contains insert DNA with correct size of the target region (E3-E2-6K-EctoE1). Following colony PCR screening, the 8 plasmids of positive clones with intense band were chosen and confirmed by restriction digestion using enzymes BamHI and HindIII. Figure 3.10 demonstrates the restriction digestion analysis of the selected 8 clones on 0.7 % agarose gel in 1X TAE buffer. Based on the result in Figure 3.10, 2 out of 8 clones (clones 5 and 10) were digested correctly to release correct size of donor plasmid and insert DNA. The plasmid of clone 32 was included as positive control in this colony PCR assay as this clone has been confirmed to harbour the correct size of the insert DNA. Double digestion of clone 5 and 10 demonstrated identical pattern of the digested products as positive control, which are 5.1 kb and 2.8 kb respectively. Colony PCR and double digestion of clone 5 and 10 showed that the target region (E3-E2-6K-EctoE1) was successfully cloned into pFBD donor plasmid.

Following this, clone 5 and 10 were subjected to DNA sequencing. The entire insert DNA were sequenced using the primers as listed in Table 2.4. The alignment result revealed that mutated region in clone 32 was corrected through the replacement with newly synthesized AleI-PshAI fragment. In protein E2 of clones 5 and 10, the mutated amino acid L (Leucine) was successfully corrected to S (Serine), whereas in protein 6K of clones 5 and 10, the mutated amino acid V (Valine) was successfully corrected to I (Isoleucine) (Figure 3.11).

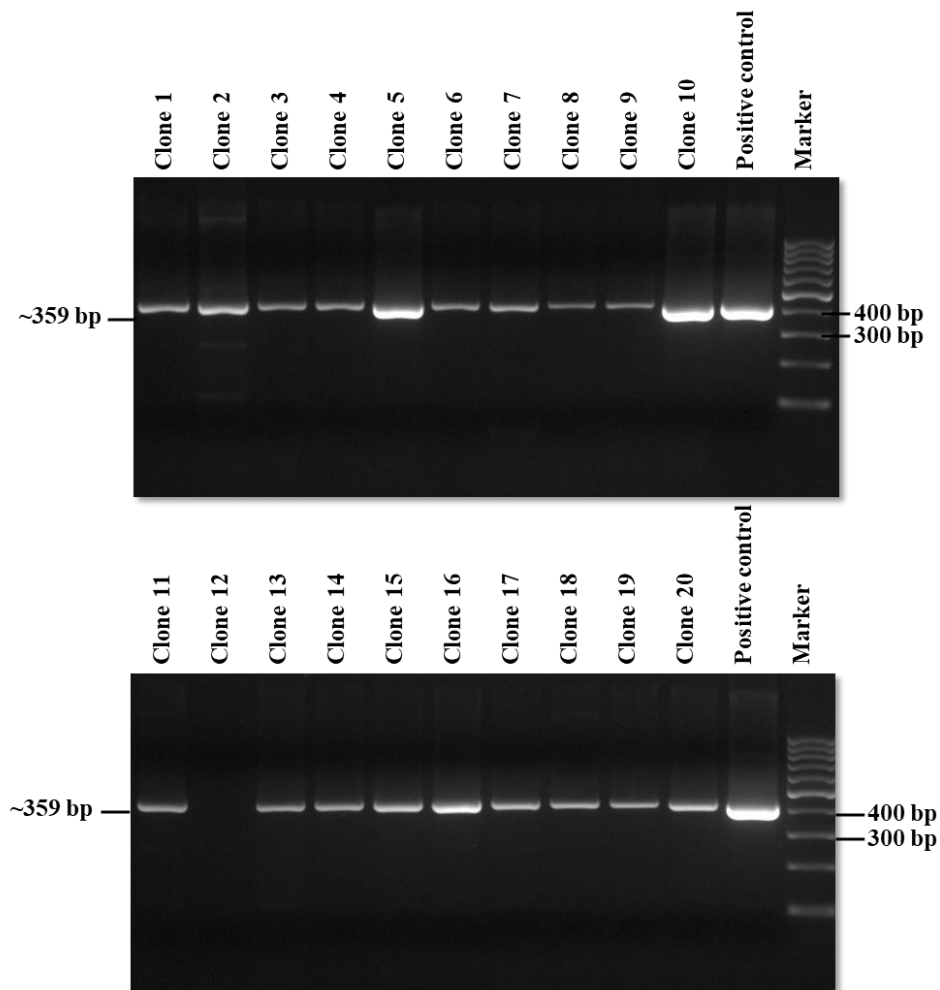


Figure 3.9: Agarose gel electrophoresis of colony PCR amplification of selected clones. Amplicons with expected size are indicated. Clone 32 was used as positive control. GeneRuler 100 bp DNA ladder are indicated as marker on the right side of each gel image.

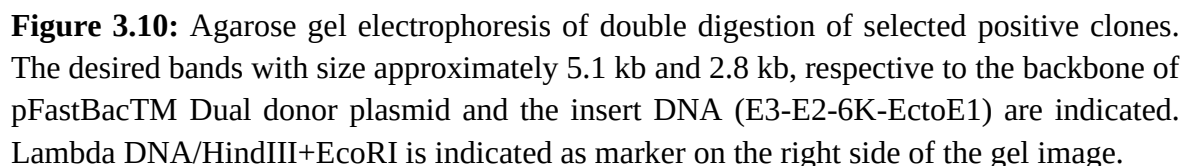


Figure 3.11: Alignment of clones 32, clones 5 and 10 sequences to the reference sequence of CHIKV S233 (unpublished). The mutated sites at amino acid position 182 in E2 protein and position 24 in 6K protein are highlighted in light blue.

3.5 Generation of recombinant bacmid

The verified recombinant donor plasmid (pFBD-E3E26KEctoE1) was transformed into DH10BacTM *E.coli* containing bacmid and helper plasmid as described in Section 2.3.4 to generate recombinant bacmid. After 48 hours incubation, the colonies containing recombinant bacmids were visible as large white colonies among the blue ones which harbouring the unaltered bacmids on DH10BacTM LB agar plate in the presence of X-gal and IPTG. The formation of white colonies was due to the disruption of the LacZ gene expression that caused by the occurrence of transposition event. Ten true white colonies were picked and re-streaked onto the DH10BacTM LB agar plate to ensure if they have true white phenotype. These 10 colonies were subjected to colony PCR screening only since the verification of high molecular weight recombinant bacmid DNA is not convenient to be performed through restriction digestion and DNA sequencing. Insert-specific primers (CHIKV/F9018 and CHIKV/R10937) were used in colony PCR assay to confirm the success transposition. Figure 3.12 shows the amplicons generated through colony PCR resolved on 1 % agarose gel in 1X TAE. Based on the gel image in Figure 3.12, all selected bacmid DNA shows positive results, in which 1,920 bp of amplicons were observed, indicating the successful transposition of recombinant donor plasmid (pFBD-E3E26KEctoE) into bacmid shuttle vector. Again, the plasmid of clone 32 was used as positive control in this assay, it demonstrates a positive result, indicates that the amplification is efficient. Following this, bacmid 10 were selected and used to transfect *sf9* cells as described in section 2.3.5.3.

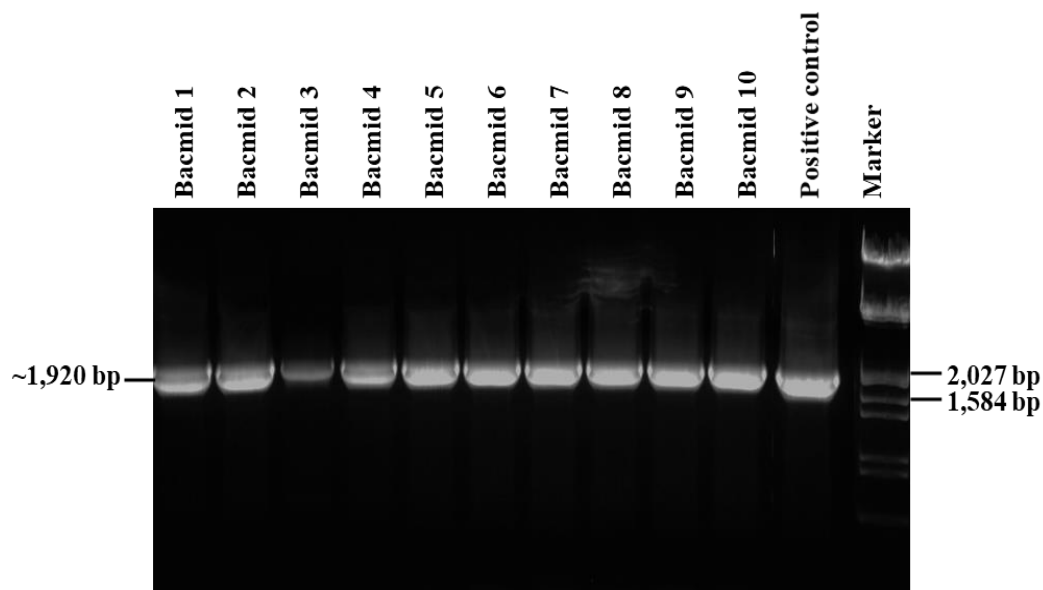


Figure 3.12: Agarose gel electrophoresis of colony PCR amplification of selected clones. Amplicons with expected size are indicated. Clone 32 was used as positive control. GeneRuler 100 bp DNA ladder are indicated as marker on the right side of each gel image.

3.6 Generation of recombinant baculovirus

The morphology of the *Sf9* cells was monitored daily. Early phase and late phase of bacmid transfection were observed on day 4-post transfection and day 7-post transfection respectively. At early phase of transfection, the cells diameter and nuclei size were increased compared to mock-transfected *Sf9* cells. At late phase of infection, the cells appeared to stop propagating and detached from the flask in comparison to the mock-transfected *Sf9* cells (Figure 3.13). These results have indicated that *Sf9* cells were successfully transfected with recombinant bacmid. Therefore, passage 1 (P1) of AcMNPV-E3E26KEctoE1, AcMNPV-Gus/CAT (derived from pFastBacTM Dual-Gus/CAT control) and mock-transfected cells were harvested on day 7-post transfection.

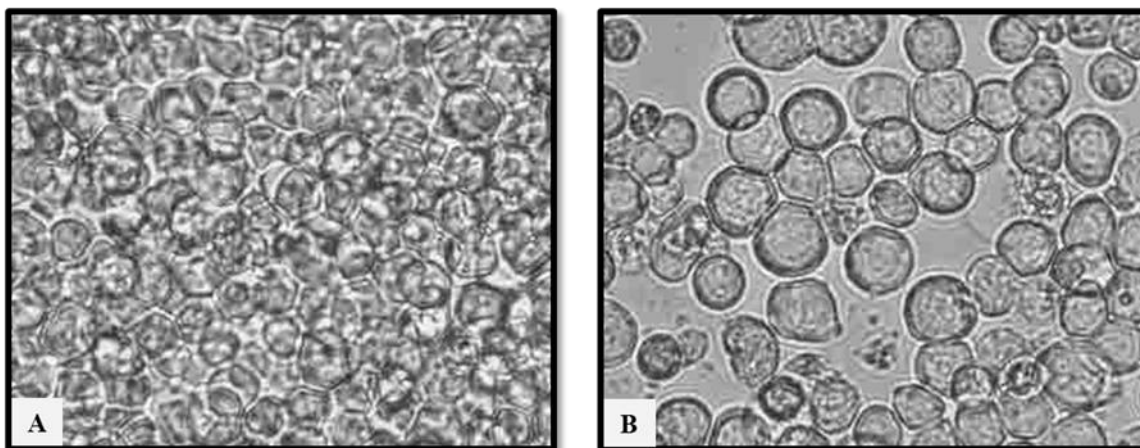


Figure 3.13: CPE of *Sf9* cells transfected with recombinant bacmid (A) Mock-transfected *Sf9* cells (B) *Sf9* cells transfected with recombinant bacmid on day 7 post-transfection. Images were taken at the 20X magnification.

3.7 Antigenicity of recombinant CHIKV structural proteins generated in *Sf9* cells

Preliminary expression was performed as described in Section 2.3.6. The expressed products were then analysed by SDS-PAGE and western blot assay against CHIKV positive and negative sera in order to test their antigenicity.

Passage 2 (P2) of baculoviruses were prepared by infecting the *Sf9* cells with P1 baculovirus stocks as obtained in Section 3.6. The culture medium was collected daily until the late phase of baculovirus infection was observed. The late phase of baculovirus infection was observed on day 4-post infection when the cells appeared to stop growing compared to the mock-infected cells. Therefore, P2 of recombinant baculoviruses (AcMNPV-E3E26KEctoE1 and AcMNPV-Gus/CAT) and mock-infected cells were harvested on day 4-post infection.

The daily collected P2 crude supernatants and cell lysates were first analysed by SDS-PAGE and stained with CBB to evaluate the expression level of the recombinant proteins. The recombinant proteins were separated on 12-15 % resolving gel and treated with reduced and heated condition. However, no bands at the expected product size were visible on the gel (data not shown). The CHIKV recombinant proteins were also transferred

onto nitrocellulose membrane and probed with CHIKV positive and negative sera to detect the expressed products. AcMNPV-Gus/CAT and mock infection were used as negative controls. Figure 3.14 shows the blot on the time course study of the CHIKV recombinant proteins expression. Two patient sera (CHIKV positive and CHIKV negative) as shown in Table 3.2 were used in the time course study. Based on Figure 3.14A, positive reactions can be observed as bands of about 120 kDa, 39 kDa and 38 kDa on the immunoblot when the CHIKV proteins (derived from day-4 collected supernatants and cell lysates) were probed with CHIKV positive serum. The recombinant proteins were also tested against CHIKV negative sera, and the corresponding bands were not observed on the immunoblot (as shown in Figure 3.14B). This result demonstrates the presence of the recombinant proteins derived from AcMNPV-E3E26KEctoE1 that showed a positive reaction with IgG antibodies in CHIKV positive sera. Hence, the day-4 collected supernatants and cell lysates were further used to confirm the antigenicity against other CHIKV positive and negative sera. In the day-4 (D4) supernatant, a band at approximately 72 kDa was observed on the immunoblot that was probed with CHIKV positive serum, however, this corresponding band was also observed in CHIKV negative serum as well as in control groups. The 72 kDa band is considered as non-specific due to the recognition and non-specific binding to other proteins in human sera. A band at approximately 66 kDa is also observed in the D4 supernatant when the immunoblot was probed with CHIKV negative serum. This band is considered as non-specific band as the corresponding band is not observed on the blot probed with CHIKV positive serum.

Table 3.2: Positive and negative serum used in the time course study

No.	Patient sera	Neutralization	PRNT ₅₀
1	Positive serum 1	Positive	1:2560
2	Negative serum 1	Negative	NA

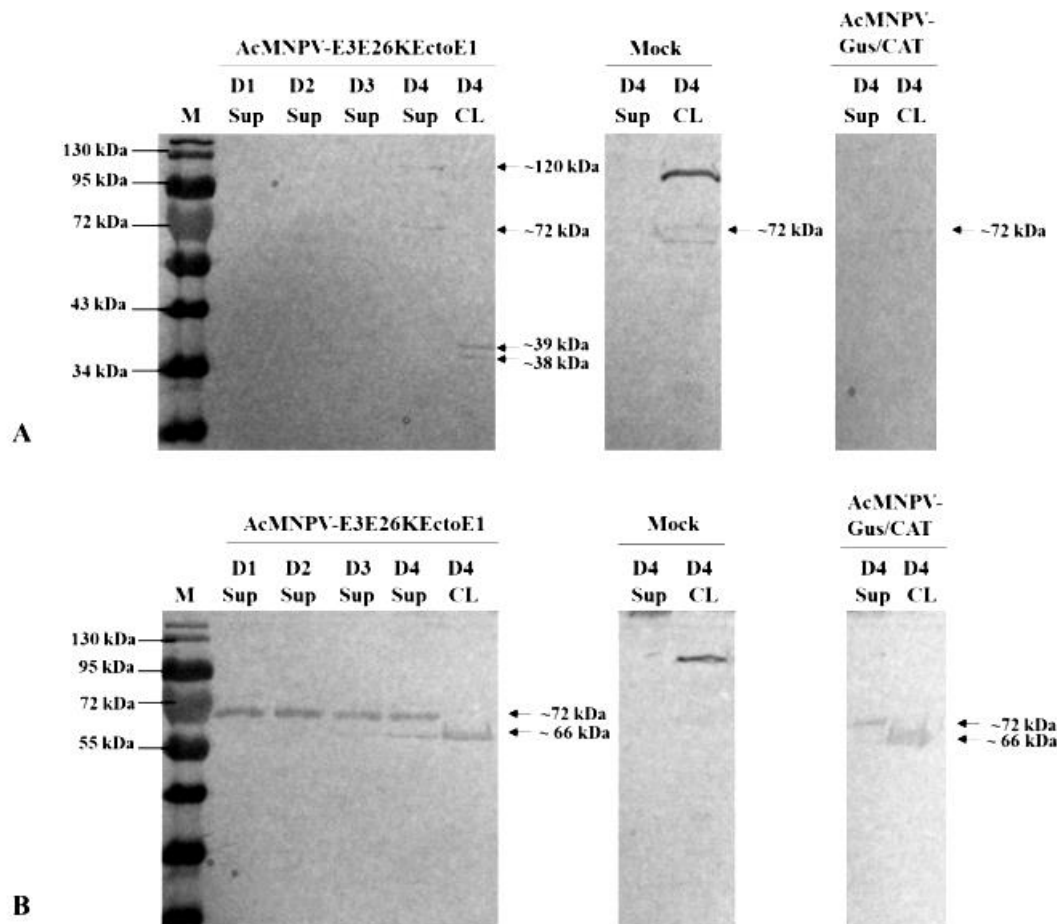


Figure 3.14: Western blot of the daily collected recombinant proteins tested against CHIKV positive and negative sera. A) Immunoblot probed with CHIKV positive serum B) Immunoblot probed with CHIKV negative serum. Fisher BioReagents™ EZ-Run™ prestained rec protein ladders are indicated as M on the left side of each image. Supernatants are indicated as Sup; Cell lysates are indicated as CL. The proteins were electrophoresed at reduced heated condition.

Table 3.3 shows panel of patient sera used to confirm the antigenicity of the day-4 post infected CHIKV recombinant proteins. Positive control used in these western blot assays was CHIKV-infected C6/36 cell lysate under non-reducing and non-heated conditions, positive results were observed on the blot probed with the positive patient serum, in which two bands were visible as indicated by red arrows (Figure 3.15) representing E1 and E2 proteins, but these bands were not observed on the blot probed with the negative patient serum, indicating specific reaction and confirming the validity of the immunoassay.

Again, bands of approximately 120 kDa, 39 kDa and 38 kDa were seen on the immunoblots (as shown in Figure 3.15) that were probed with the CHIKV positive sera. Meanwhile, the equivalent bands were not seen on the immunoblots that were probed with the CHIKV negative sera (as shown in Figure 3.16). In the immunoassay conducted with the mock-infected cell lysate (as shown in Figure 3.14, 3.15 and 3.16), a protein band of approximately 130 kDa, was constantly observed in the mock-infected cell lysate but the corresponding band was not observed with both recombinant baculovirus-infected cells (AcMNPV-E3E26KEctoE1 and AcMNPV-Gus/CAT) that probed were probed with the positive and negative serum. Apart from this, several discrete protein bands were also constantly observed in the mock-infected cell lysate only. The presence of these bands in mock-infected cells could be probably due to the presence of the minor protein components that have been expressed by the host cells, meanwhile, in the baculovirus-infected cells, the host cells protein expression is inhibited, this resulted in a different protein profile as compared to mock-infected cells.

Table 3.3: Positive and negative sera used to evaluate the antigenic reactivity of the recombinant CHIKV structural proteins

No.	Lab Number	Neutralization	PRNT ₅₀
1	Positive serum 2	Positive	1:2560
2	Positive serum 3	Positive	1:640
3	Negative serum 2	Negative	NA
4	Negative serum 3	Negative	NA

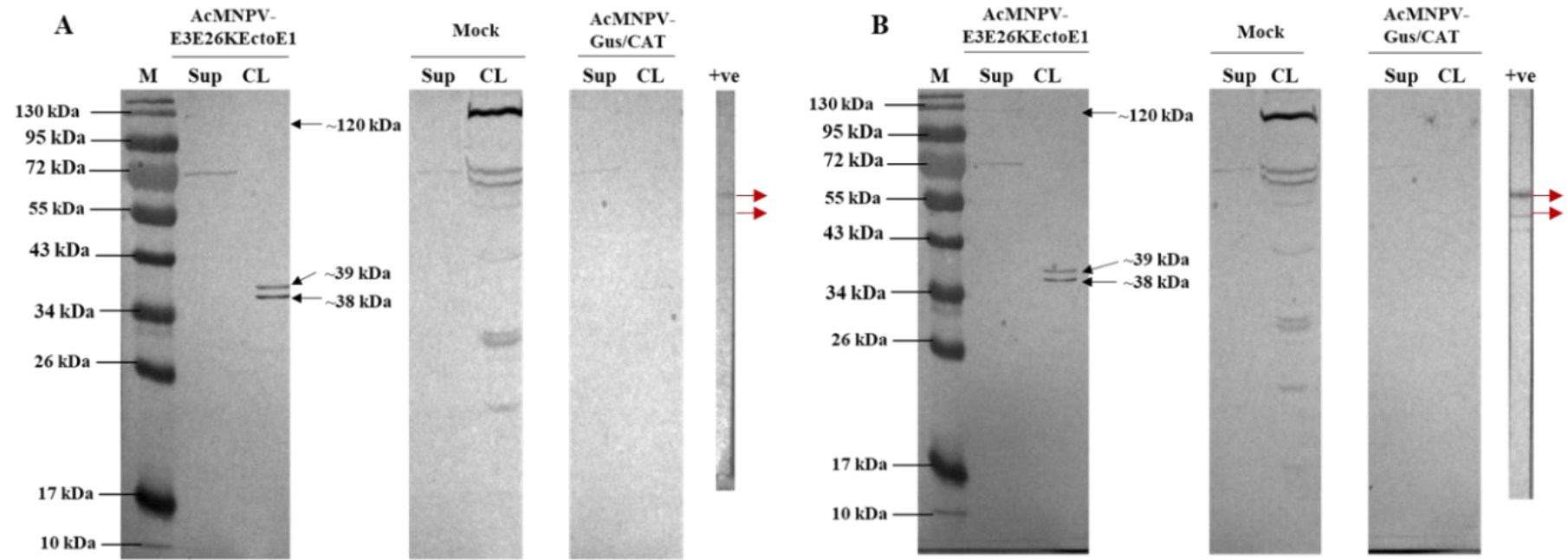


Figure 3.15: Western blot of day 4-post infected recombinant proteins tested against CHIKV positive sera. CHIKV structural proteins (E3-E2-6K-EctoE1) with size approximately 120 kDa, 39 kDa and 38 kDa are observed when A) Immunoblot probed with CHIKV positive serum 2 B) immunoblot probed with CHIKV positive serum 3. Fisher BioReagents™ EZ-Run™ prestained rec protein ladders are indicated as M on the left side of each image. Supernatants are indicated as Sup; Cell lysates are indicated as CL; Positive controls used are indicated as +ve and the expected bands are indicated by red arrows. The proteins were electrophoresed at reduced heated condition.

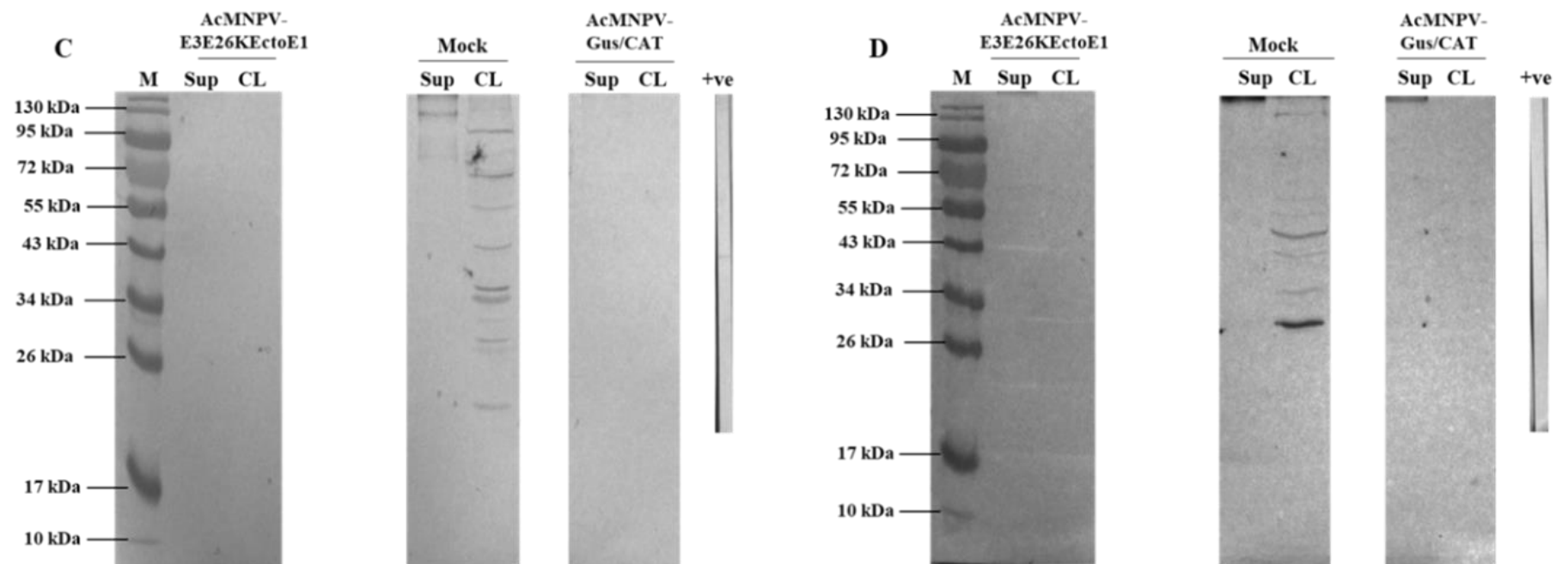


Figure 3.16: Western blot of day 4-post infected recombinant proteins tested against CHIKV negative sera. No CHIKV structural proteins are detected when A) Immunoblot probed with CHIKV negative serum 2 B) immunoblot probed with CHIKV negative serum 3. Fisher BioReagents™ EZ-Run™ prestained rec protein ladders are indicated as M on the left side of each image. Supernatants are indicated as Sup; Cell lysates are indicated as CL; Positive controls used are indicated as +ve. The proteins were electrophoresed at reduced heated condition.

3.8 Recombinant baculoviruses expressing CHIKV structural proteins

A 120 kDa CHIKV recombinant structural protein was successfully expressed from the CHIKV recombinant construct (E3-E2-6K-EctoE1) by Bac-to-Bac[®] BEVS. The western blot analysis revealed an approximately 120 kDa recombinant protein which was antigenic reactive when probed with IgG antibodies in CHIKV positive sera obtained from patients previously infected with CHIKV. The theoretical calculated protein size of the E3-E2-6K-EctoE1 structural proteins is about 115 kDa, and a band of the approximate size was observed on the immunoblot. It is very common when there is a slight discrepancy between the theoretical calculated protein size and the apparent protein size that observed on the immunoblot, this size discrepancy could be due to a number of possibilities: 1) chemical modifications of the protein, especially glycosylation and ubiquitination which causes drastically retarded gel mobility 2) phosphorylation modification that may cause subtle but obvious band shift on the gel 3) high content of acidic aa in the content (Guan et al., 2015). We also observed two unexpected protein bands of 38 and 39 kDa respectively, that were also found to be antigenically reactive to CHIKV positive sera. These results may possibly indicate incomplete or unassembled component proteins of the target protein (E3-E2-6K-EctoE1).

Several different expression systems have previously been used to express CHIK structural recombinant proteins and VLPs, such as yeast expression system (Saraswat et al., 2016), mammalian expression system (Akahata et al., 2010; Velez et al., 2012; Noranate et al., 2014) and baculovirus expression system (Metz et al., 2013; Wagner et al., 2014). In this study, the insect cell-baculovirus expression system (IC-BEVS) has been shown to express recombinant CHIKV structural proteins successfully. An advantage of using IC-BEVS when compared to other recombinant expression systems is that IC-BEVS has the capability to express large enveloped proteins (>50 kDa) in high yield and induce post-translational

modifications such as folding, oligomerization, phosphorylation, glycosylation, acylation, disulfide bond formation and proteolytic cleavage without disturbing the activity of original proteins (Makvandi et al., 2016). This expression system has been widely used to produce recombinant proteins for a wide variety of viruses and applied as a scalable industrial production platform for human vaccine such as the production of influenza vaccine (Flublok, Protein Sciences Corporation) and human papillomavirus (HPV) vaccine (Cervarix, GlaxoSmithKline), which are the licensed human vaccines that have been approved by the regulatory agency (Wagner et al., 2014).

Several different IC-BEVS strategies are available to produce a recombinant baculovirus containing the target gene such as BaculoDirect™ BEVS, *flashBAC*™ BEVS, BacMagic™ BEVS, BaculoGold™ BEVS and so on. These BEVS rely on either using site-specific transposition or homologous recombination approaches to obtain recombinant baculovirus. The issues such as cost, simplicity, and yield (Haines et al., 2006; Bleckmann et al., 2016) make the bac-to-bac® BEVS used in this study as a preferred strategy for VLP production.

3.9 Uses of recombinant CHIKV structural proteins

To date, re-emergence of CHIKV has become a global concern due to the unexpected rise of outbreaks throughout the world and the absence of any CHIKV licensed vaccine for human use. Currently, a CHIK VLP-based candidate, namely VRC-CHIKVLP059-00-VP (ClinicalTrials.gov: NCT02562482) was expressed from the whole structural polyprotein genes (C-E3-E2-6K-E1) of CHIKV strain 37997 using mammalian expression system and it has completed phase 1 clinical trials recently. This suggests that CHIK VLPs have the potential for use in development of chikungunya vaccines. In this study, the recombinant CHIKV construct (E3-E2-6K-EctoE1) is comprised of the CHIKV enveloped polyprotein only. This enveloped polyprotein is important to form the outer protein shell of the virus

particles and it has the potential to be used as a source of CHIK VLP. As compared to the VRC-CHIKVLP059-00-VP, which was expressed from the full-length structural polyprotein genes (C-E3-E2-6K-E1), the construct of this study did not include the capsid protein and E1 endodomain protein. Although this 120 kDa construct is antigenically reactive to the CHIKV IgG antibodies in human sera, it remains unknown if it forms VLPs. The BEVS used in this study is considered as the most versatile and powerful eukaryotic vector systems for the expression of recombinant proteins that retains the morphological structure of expressed proteins and therefore mimics the natural configuration of the native protein. Thus, the morphological similarity of this recombinant CHIKV structural protein to the native CHIKV particles can be further identified by using transmission electron microscope (TEM) to see if the structural proteins can fold correctly to mimic authentic conformation structure of the nature virus and form VLPs.

Besides VLPs, CHIKV recombinant protein expression technology as used in this study has the potential to offer reliable source of CHIKV antigens which can be used in the immunoassays to detect CHIKV infection in a more specific and rapid way. The conventional assays of CHIKV infection diagnosis include virus isolation, serological tests for the detection of viral specific antibodies or viral proteins, and RT-PCR based methods to detect viral genomes and gene products (Johnson et al., 2016). Among these assays, virus isolation is the most definitive test and is usually considered to be the gold standard. However, the process is time-consuming and the degree of success is dependent on a number of complicating factors. Meanwhile, RT-PCR test is expensive, time-consuming, labor-intensive and coupled with a high risk of contamination. IgM antibodies-containing serum can be detected in CHIKV positive patients as early as 3-5 days after the onset of fever, and generally last for 30-90 days, although detectable levels may remain up to 8 month post-infection. Early and specific detection is crucial in the management of patients infected with

CHIKV. According to Kumar et al. (2012), antigen and antibody based immunoassays are cost-effective and can be used as rapid screening test for early detection of large numbers of patients during epidemics. Apart from this, the CHIKV antigenic recombinant proteins can also be used as standards such as ELISA standards when used in conjunction with a matched antibody pair or as positive controls in Western blot.

The antigenic CHIKV proteins can also be potentially used to generate monoclonal antibodies (Mabs) which is also the most reliable option for the development of immunoassays like ELISA to detect IgM and IgG immunoglobulins in the sera of suspected patients. Mabs can be obtained by injecting lab animals such as mice, rabbit or goat with recombinant proteins or antigens (Leenaars & Hendriksen, 2005) such as the recombinant CHIKV structural proteins generated in this study.

CHAPTER 4

CONCLUSION

4.1 General conclusion

CHIKV has re-emerged as an important human pathogen which responsible for an unexpected rise in epidemics of debilitating febrile arthralgia in the past decades. Ineffective management of CHIKV outbreaks can gives a negative impact on economic growth. One of the way to reduce CHIKV burden is development of CHIKV vaccine. VLPs are one of the ideal vaccine candidates used to combat viral infection as this vaccine candidate mimic the native virus surface architecture and protein conformations, which are then readily to be recognized by the immune system and induce protective antibody responses in the absence of adjuvant. This study was aimed to construct the CHIKV structural protein which could be potentially used as a source of VLPs from a selected gene region (E3-E2-6K-EctoE1) by using IC-BEVS. As shown in Figure 3.14 and 3.15, three potential CHIKV proteins have been generated from IC-BEVS. These proteins were found to be antigenic reactive against CHIKV patient sera and also have the potential to be used in the development of the diagnostic tools for the detection of CHIKV infections such as ELISA, immunochromatographic assay or indirect immunofluorescent assay.

4.2 Limitation of the study

In this study, I have demonstrated that the CHIKV expressed structural protein is reactive to human sera of patients that were tested positive for chikungunya. However we are unable to determine at this point whether this structural protein could form a VLP and the antibodies generated against this protein would have neutralising properties which is an important determinant of a vaccine candidate. Given that, the VLP could prove useful as an antigenic reagent in the development of future diagnostic tools. Another limitation was the

inability to produce a high yield expression of the recombinant protein that is necessary for downstream processes and applications.

4.3 Recommendation and future research

Optimization of the expression conditions is necessary to improve the yields of the protein produced. For an example, it is recommended to increase the culture pH to 6.6-6.8 in order to generate detectable concentrations of the proteins (Wagner et al., 2014). Besides that, protein purification such as CsCl density gradient equilibrium centrifugation should be conducted to separate the VLPs from product- and process-related impurities (Noranate et al., 2014).

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APPENDIX

Media, Antibiotics, Reagents and Solutions

A.1 Media, antibiotics and buffers used in maintaining cells

A.1.1 Leibovitz's L-15 stock media - 1Litre

Leibovitz's L-15 powder (Gibco, New York, USA)	1 packet
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UHQ water	1 litre
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Leibovitz's L-15 powder was dissolved in UHQ water and the volume were adjusted to 1 litre. The media was sterilized by negative pressure through 0.22 µm cellulose acetate filter (Nalgene, NY, USA) followed by performing vacuum filtration with filtration unit (TPP, Trasadingen, Switzerland). The media was kept at 4 °C after the sterility was checked.

A.1.2 Leibovitz's L-15 complete media with 10 % FBS - 100 ml

Leibovitz's L-15 stock media	100 ml
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Fetal Bovine Serum	10 ml
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100X Streptomycin	1 ml
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100X Penicillin G	1 ml
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A.1.3 Leibovitz's L-15 complete media with 1 % FBS - 100 ml

Leibovitz's L-15 stock media	100 ml
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Fetal Bovine Serum	1 ml
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100X Streptomycin	1 ml
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100X Penicillin G	1 ml
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A.1.4 Grace's insect stock media – 1 Litre

Grace's Insect Medium powder (Gibco, New York, USA)	1 packet
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Sodium bicarbonate	0.35 g
Lactalbumin hydrolysate	3.33 g
Yeastolate	3.33 g
UHQ water	1 litre

The powders were dissolved in UHQ water and the volume were adjusted to 1 litre.

The media was sterilized by negative pressure through 0.22 µm cellulose acetate filter (Nalgene, NY, USA) followed by performing vacuum filtration with filtration unit (TPP, Trasadingen, Switzerland). The media was kept at 4 °C after the sterility was checked.

A.1.5 Complete TNM-FH Grace's insect medium supplemented with 10 % FBS - 100ml

Grace's Insect stock media	100 ml
Fetal Bovine Serum	10 ml
100X Streptomycin	1 ml
100X Penicillin G	1 ml

A.1.6 Unsupplemented Grace's insect stock medium – 1 Litre

Grace's Insect Medium powder (Gibco, New York, USA)	1 packet
UHQ water	1 litre

The powder was dissolved in UHQ water and the volume were adjusted to 1 litre.

The media was sterilized by negative pressure through 0.22 µm cellulose acetate filter (Nalgene, NY, USA) followed by performing vacuum filtration with filtration unit (TPP, Trasadingen, Switzerland). The media was kept at 4 °C after the sterility was checked.

A.2 Buffers and solutions used in SDS-PAGE and western blot

A.2.1 10 % SDS - 100 ml

Sodium dodecyl sulphate	10 g
RO water	100 ml

SDS powder was dissolved into RO water and adjusted the volume to 100 ml.

A.2.2 10 % APS - 10 ml

Ammonium persulphate	1 g
RO water	10 ml

Ammonium persulphate was dissolved into 10 ml of RO water and stored at 4 °C until used.

A.2.3 4X non-reducing protein sample buffer – 9.5 ml

0.5 M Tris-HCl, pH 6.8 (Stacking gel buffer)	1 g
Glycerol	2.5 ml
10 % SDS	2.0 ml
0.5 % bromophenol blue	0.2 ml
RO water	3.55 ml

The solution was kept at 4 °C until used.

A.2.4 4X reducing sample buffer

4X non reducing sample buffer (A.4.3)	950 µl
2-mercaptoethanol	50 µl

A.2.5 Coomassie brilliant blue (CBB)

Coomassie brilliant blue (G250)	1 g
Methanol	400 ml
Glacial acetic acid	100 ml

RO water	500 ml
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The coomassie brilliant blue powder was diluted with RO water before adding methanol and glacial acetic acid. The solution was then filtered through filter paper before used.

A.2.6 1X towbin buffer - 1 Litre

25 mM Tris	3.03 g
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192 mM Glycine	14.4 g
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20 % v/v Methanol	200 ml
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RO water	800 ml
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The powders were dissolved in 600 ml RO water, followed by 200 ml methanol. RO water was added to the mixture and adjusted the volume to 1 litre. The towbin buffer was kept at 4 °C.