DEVELOPMENT OF A PEPTIDE-BASED ANTIBODY ASSAY TO DETECT CHIKUNGUNYA VIRUS (CHIKV)

YEW SZE SHIN

UNIVERSITI SAINS MALAYSIA
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DEVELOPMENT OF A PEPTIDE-BASED ANTIBODY ASSAY TO DETECT CHIKUNGUNYA VIRUS (CHIKV)

by

YEW SZE SHIN

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
µg	microgram
µg	microgram
µl	microlitre
µM	micromolar
AI	Antigenic Index
Amp	ampere
A_p	antigenicity propensity
BEBV	Bebaru virus
BFV	Barmah Forest virus
bp	base pair
BSL-3	biosafety level 3
CAdVax	Complex Adenovirus vaccine
CD	circular dichroism
CHIKV	Chikungunya virus
c-terminal	carboxyl terminal

CV	column volumes
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EC_{50}	half maximal effective concentration
ECSA	East-Central South Africa
EEE	Eastern Equine Encephalitis virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FDA	Food and Drug Administration
FFU	Focus Forming Unit
g	gram
GETV	Getah virus
GuHCl	guanidine hydrochloride
H_2O	water

HI	hemagglutination inhibition assay
HIC	Hydrophobic Interaction Chromatography
hpi	hours post infection
HPLC	High Performance Liquid Chromatography
HRP	horseradish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
IIFT	indirect immunofluorescence test
IMAC	Immobilized metal ion affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
IRES	internal ribosome entry sequence
K_{av}	partition coefficient
kb	kilobase pair (s)
kDa	kilodalton (s)
L	litre
LB	Luria-Bertani
M	molar

MAC-ELISA	IgM antibody capture ELISA
MAYV	Mayoro virus
mg	milligram
MIDV	Middleburg virus
min	minute (s)
ml	millilitre
mM	millimolar
Mr	molecular weight
NDUV	Ndumu virus
ng	nanogram
NJ	Neighbor-joining
nM	nanomolar
NT	neutralisation test
N-terminal	amino terminal
NTR	non-translated region
^{o} C	degree Celsius
OD	optical density

ONN	O’Nyong-nyong
ORFs	open reading frames
PAb	polyclonal antibody
PCR	polymerase chain reaction
PDB	Protein Data Bank
pmol	picomolar
PMSF	phenylmethanesulfonylfluoride
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNAi	RNA interference
RRV	Ross River virus
RT	reverse transcription
RT-LAMP	RT- loop-mediated isothermal amplification
s	second
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDV	Sleeping Disease virus
SFV	Semliki Forest virus

SIN	Sindbis virus
siRNAs	small interfering RNAs
SPDV	Salmon Pancrease Disease virus
TATase	terminal adenylyltransferase
TBE	tris borate ethylenediaminetetraacetic acid
TFA	trifluoroacetic acid
TMB	tetramethylbenzidine
TROCV	Trocara virus
UTR	un-translated region
UV	ultraviolet
V	volt
VEE	Venezuelan Equine Encephalitis
VLP	virus-like-particle
WA	West Africa
WEE	Western Equine Encephalitis virus
WNV	West Nile virus
x g	g-force

PENGHASILAN UJIAN ANTIBODI BERASASKAN PEPTIDA UNTUK MENGESAN VIRUS CHIKUNGUNYA (CHIKV)

ABSTRAK

Virus Chikungunya (CHIKV) yang pertama kali telah dilaporkan pada tahun 1953 di Tanzania telah muncul semula selepas tahun 2006 dalam magnitud yang tinggi di negara tropika dan juga negara bukan tropika. Jangkitan CHIKV boleh menyebabkan deman panas dan artritis yang berpanjangan. Persamaan wujud dalam manifestasi klinikal dan taburan geografi dengan demam Denggi. Kit pengesanan yang boleh membezakan virus ini amat penting untuk mengelakkan kesilapan dalam diagnosis dan dapat menyediakan rawatan perubatan yang effektif kepada pesakit. Kaedah pengesanan yang sedia ada sebelum ini termasuk pengkulturan dan pengekstrakan virus yang prosesnya memakan masa, tindak balas berantai polymerase (PCR) yang memerlukan teknologi pada kadar yang mahal dan ujian serologi mensasarkan antibody badan yang kurang sensitif. Kajian ini dilaksanakan untuk membangunkan satu sistem pengesanan virus CHIKV yang mudah dan berkesan untuk pengesanan pada tahap akut dengan mensasarkan glikoprotein virus yang terdedah di permukaan. Gen yang mengekodkan glikoprotein E2 virus yang terletak pada permukaan virus dan mandatori untuk pengenalan reseptor, telah diklonkan dan diekspreskan dalam sistem *E. coli*. Walau bagaimanapun, protein rekombinan ini adalah tak larut. Pengoptimuman lanjut dalam keadaan ekspresi dan pertukaran vektor ekspresi tidak meningkatkan kelarutan. Oleh itu, pembinaan E2 tanpa bahagian hidrofobik pada terminus-C telah dilakukan. Namun, rangkuman protein yang masih juga tidak larut dihasilkan. Rangkuman protein yang tak larut ini telah dilarutkan dengan urea, selepas itu, dilipat semula dan ditulenkan. Akan tetapi, hasil dan ketulenan protein ini adalah tidak

memuaskan. Disebabkan penyediaan glikoprotein yang larut dan tulen adalah rumit, fragmen peptida yang pendek dan senang disintesiskan telah digunakan untuk menghasilkan antibodi poliklon anti-CHIKV yang monokhusus. Daripada menjalankan pemetaan urutan epitop melalui eksperimen makmal basah yang berulang-ulangan, penentu antigen yang berpotensi telah diramal mengguna analisis bioinformatik epitop selanjar dan tidak selanjar. Analisis epitop tidak selanjar yang membuat ramalan berdasarkan struktur protein 3D adalah lebih dibolehpercayai berbanding dengan analisis selanjar. Akhirnya, 8 peptida antigenik telah dikenalpasti melalui kaedah hibrid dan konsensus. Peptida yang anggaran ketulenannya 85% telah disintesis secara kimia untuk pengimunan arnab putih. Antiserum yang dihasilkan daripada arnab ditulenkan menggunakan kromatografi affiniti peptida untuk menyediakan antibodi poliklon yang spesifik ke atas lokasi protein yang tertentu. Saringan afiniti pengikatan dengan glikoprotein rekombinan CHIKV menunjukkan bahawa antibodi yang dihasilkan oleh 2 peptida berprestasi lebih baik berbanding yang lain. Antibodi-antibodi ini adalah berpotensi untuk dikembangan ke peringkat ujian pengesanan yang lebih sensitif dan spesifik yang boleh digunakan di lapangan.

DEVELOPMENT OF PEPTIDE-BASED ANTIBODY ASSAY TO DETECT CHIKUNGUNYA VIRUS (CHIKV)

ABSTRACT

Chikungunya virus (CHIKV), first reported in 1953 in Tanzania, had reemerged in high magnitude after 2006 in tropical countries as well as spread into non-tropical regions. Chikungunya virus infection can cause viral fever with a side effect of severe and prolonged arthritis. Due to its similar clinical manifestation and geographical distribution with Dengue fever, a rapid detection kit to differentiate them is important to prevent misdiagnosis and provide effective medical care to the patient. Existing detection methods include time-consuming virus culturing and isolation, expensive technology requiring polymerase chain reaction (PCR) and less sensitive antibody targeting serological tests. This study was carried out to develop a simple and effective virus detection assay at the acute stage by targeting the surface exposed viral glycoprotein. The gene encoding E2, which is the glycoprotein that positioned on the viral spike and mandatory in receptor recognition, was cloned and expressed in *E. coli* system. However, the recombinant protein was insoluble. Further optimization on expression conditions and change of expression vector was not helpful either. Therefore, truncated E2 protein without hydrophobic C-terminal was constructed. However, it was also produced insoluble inclusion body. The inclusion body of E2 protein was solubilized with urea, then refolded and purified. Nevertheless, the yield and purity of the protein was not satisfying. Due to difficulty in preparing soluble and pure glycoprotein, the short peptide which is easy to produce with high purity was employed to produce mono-specific anti-CHIKV polyclonal antibodies. Instead of mapping the epitope sequence through laborious wet lab experiments, the

potential antigenic determinants of the CHIKV glycoprotein was predicted using sequential and discontinuous epitope bioinformatics analysis. Discontinuous epitope analyses that make prediction based on 3D protein structure is more reliable compare to sequential analysis. Finally, 8 antigenic peptides were identified using hybrid and consensus methods. They were chemically synthesized with purity of at least 85% for immunization of white rabbits. Antisera from the rabbits were purified using peptide affinity chromatography to produce site specific polyclonal antibodies. Recombinant CHIKV glycoprotein binding affinity screening showed that antibodies against two E2 peptides (E2_133-147 and E2_309-323) performed better than the others. These antibodies can potentially be developed into a highly sensitive and specific detection assay that can be used in the field.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Chikungunya, meaning “that which bend up” in Makonde language, was first reported in 1953 in Tanzania. During the 1950s to 1980s, several outbreaks were reported in African and Asian tropical countries. After a few decades of silence, it reemerged in 2005-2007 in India and Indian Ocean islands by infecting millions of citizens. Chikungunya is transmitted by mosquitoes belonging to the genus *Aedes* (*Aedes aegypti* and *Aedes albopictus*). Patients experience intermittent high fever, rashes, headache, vomiting, muscle pain and joint pain during the infection for one to two weeks. Some even develop chronic arthritis for few months or permanently.

To date, no effective antiviral treatment or vaccine is available for Chikungunya. Furthermore, due to the migration of the people, mutation of the virus and global climate changes, the disease is no longer limited to known tropical areas. The global distribution map of the disease is expanding and the number of victims continues to increase.

Clinical manifestations of Chikungunya fever are very similar to Dengue fever. Moreover, these diseases occur in most of the endemic areas concurrently. Due to earlier discovery of Dengue and lack of knowledge on Chikungunya, many cases might be mistakenly diagnosed solely by clinical observations. Precise detection of the virus is important for a physician to provide suitable treatment and execute efficient clinical management.

Chikungunya virus (CHIKV) belongs to the Alphavirus genus of the Togaviridae family. Clinical identification of CHIKV includes virus isolation, molecular amplification by polymerase chain reaction (PCR) and serological anti-Chikungunya antibody detection. The virus isolation is very time-consuming and requires skillful lab technicians and highly secured biosafety level three (BSL3) laboratories. Majority of the detection kits available in the market are targeting the presence of either the viral genome or anti-virus antibodies. These PCR kits, however, require expensive and complicated real-time PCR instruments. Serological diagnosis detecting the immunoglobulin G (IgG) and immunoglobulin M (IgM) is widely applied but is not sensitive to detect the antibodies in the early stage of infection. To date, no commercial kit that targets the CHIKV viral proteins is available. Because viral replication is accelerated at the early stage of infection, the viral proteins are abundant in the host cells. Therefore, early diagnosis can be conducted by targeting these antigens.

During the replication cycle, the envelope proteins E1 and E2 are transported and embedded into the lipid bilayer membrane of the host cell before virus assembly and budding. They are exposed on the surface of the host cells, making them the most ideal target for detection. Antibodies raised against these envelope proteins can be used to detect the presence of virus.

In this study, we developed polyclonal antibodies against the epitopes of E1 and E2 glycoproteins. The prediction of epitope peptide sequences that were exposed on the surface with antigenicity and immunogenicity was performed *in silico*. The peptides were chemically synthesized and then used for the production of the anti-peptide polyclonal antibodies. The direct enzyme-linked immunosorbent assay (ELISA) had shown that antibodies against 2 peptides were the best performing. The antibodies can potentially be

further developed into a low cost immunochromatography test strip for rapid and early detection of CHIKV.

1.2 Aims and objectives

This project was carried out to develop an antibody assay that allows early detection of CHIKV during fever onset. In order to accomplish this aim, experiments to fulfill the following objectives were carried out:

1. Cloning, expression and purification of CHIKV E2 envelope protein from bacterial system.
2. *In silico* prediction on CHIKV glycoprotein epitope and chemical synthesis of the peptides for anti-peptide polyclonal antibody production.
3. Assessment of anti-peptide polyclonal antibody and development of antibody assay for the detection of recombinant CHIKV glycoprotein.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Chikungunya outbreaks

Chikungunya was first reported in Tanzania in the year 1952-1953 (Robinson, 1955; Ross, 1956; Powers and Logue, 2007; Seneviratne *et al.*, 2007). The infection was reported as "dengue-like-illness" until the CHIKV was determined to be the true causative agent(Staples *et al.*, 2009). The neighboring country, Democratic Republic of the Congo, detected Chikungunya for the first time in 1958 and then again in 1960 (Pastorino *et al.*, 2004). Chikungunya has continued to be repeatedly detected in central Africa (Central Africa Republic), southern Africa (Zimbawe and South Africa) and western Africa (Senegal and Nigeria) in the subsequent 20 years (Schuffenecker *et al.*, 2006; Cherian *et al.*, 2009).

In 1958, it occurred in Southeast Asia for the first time in Thailand (Aikat *et al.*, 1964; Sudeep and Parashar, 2008). The first outbreak in India was reported in 1963 in Kolkata (Shah *et al.*, 1964; Arankalle *et al.*, 2007), followed by Chennai, Pondicherry, Vellore, Visakhapattanam, Rajmundry, Kakinada and Nagpur from 1964 to 1973 (Yergolkar *et al.*, 2006). The occurrence of Chikungunya in Indonesia was then reported in Kalimantan (1973), South Sumatra (1982) and Yogyakarta (1983) (Porter *et al.*, 2004; Laras *et al.*, 2005). After hundreds of thousands of cases were reported, the occurrence went down to none during the following decades. The first reported CHIKV outbreaks in global are illustrated in Figure 2.1.

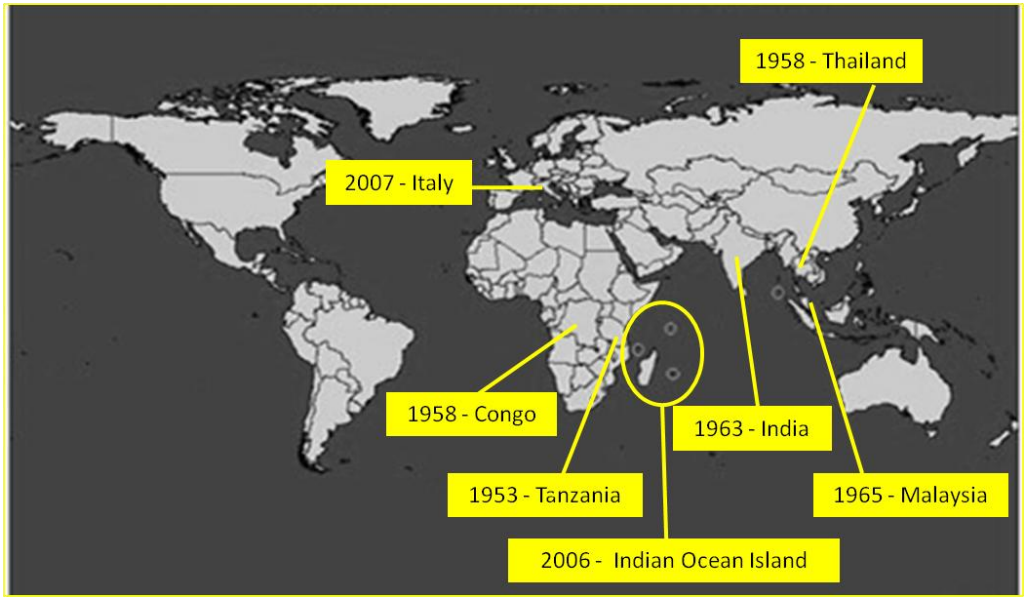

Figure 2.1 Global Chikungunya first reported outbreaks.

2.2 Reemergence of Chikungunya

After a long period of silence, Chikungunya emerged again in the Democratic Republic of Congo in early 1998-2000 and Kenya in 2004 (Pastorino *et al.*, 2004; Hapuarachchi *et al.*, 2010). During 2005–2007, enormous Chikungunya epidemics had infected almost 255,000 people out of a total population of 777,000 in the Indian Ocean islands such as Comoros islands, La Réunion, Mayotte, Mauritius, Seychelles, and Madagascar (Seneviratne *et al.*, 2007), as well as 1.3 million people in 13 states of India (Arankalle *et al.*, 2007). Death cases (237) were reported during outbreaks in La Réunion (Schwartz and Albert, 2010). Although Southeast Asian countries did not experience such destructive epidemics, remarkable reemergence occurred in Indonesia from 2001-2003 (Laras *et al.*, 2005), Malaysia in 2006 and 2007, Singapore and Thailand in 2008 (Theamboonlers *et al.*, 2009; Hapuarachchi *et al.*, 2010). Moreover, co-infection of

Chikungunya and Dengue was reported in Africa, India, Malaysia, as well as travelers who have been to India and Singapore (Nayar *et al.*, 2007; Chahar *et al.*, 2009; Schilling *et al.*, 2009; Chang *et al.*, 2010; Nkoghe *et al.*, 2012).

Chikungunya was assumed not to affect countries other than tropical areas, until hundreds of cases were reported in France (Cordel *et al.*, 2006; Seneviratne *et al.*, 2007) as well as in North Eastern Italy (Watson, 2007; Pardigon, 2009). Some Chikungunya cases were brought into France by the travelers returning from tourist destinations in Indian Ocean islands (Parola *et al.*, 2006). With the presence of compatible vectors, especially *A. albopictus*, the infected travelers spread Chikungunya fever to their neighbourhoods and slowly all over the countries.

Other factors that contribute to the reemergence of massive Chikungunya outbreaks in recent years include the changing global climate and mutation of CHIKV. Climate affects the life cycle, distribution and transmission of arbovirus vectors to vertebrate hosts as well as the dispersal pattern and evolution of arboviruses (Jansen *et al.*, 2008; Gould and Higgs, 2009). Climate plays a big role in spreading Chikungunya by favouring the breeding of mosquito vectors (*A. aegypti* and *A. albopictus*), and efficient virus reproduction in the mosquito (Mourya *et al.*, 2004; Epstein, 2007; Gould and Higgs, 2009). Indonesian Chikungunya reemergence in 2001 to 2003 happened after increased rainfall in August favouring *Aedes* vectors (Laras *et al.*, 2005).

The most significant mutation point that might contribute to the high magnitude of reemergence is the E1-A226V mutation. Any changes in the E1 gene, which is the structural gene that makes up the envelope of the virion, might influence virion assembly,

disassembly, interaction with host cell receptors and membrane fusion (Schuffenecker *et al.*, 2006; Tsetsarkin *et al.*, 2007; Kumar *et al.*, 2008). More than 90% of the isolates after September 2005 contain this particular mutation (Schuffenecker *et al.*, 2006; Jaffar-Bandjee *et al.*, 2009; Schwartz and Albert, 2010). E1-A226V was believed to contribute to cholesterol independent infection of mosquito cells leading to the increased infectivity of vector *A. albopictus* (Schuffenecker *et al.*, 2006; Tsetsarkin *et al.*, 2007; Kumar *et al.*, 2008). The mutation had changed the predominant vector from *A. aegypti* to *A. albopictus* (De Lamballerie *et al.*, 2008b). Therefore, areas that lack of *A. aegypti* but are abundant with *A. albopictus* have now become the hot spots of Chikungunya outbreaks. However, the latest study carried out by Tsetsarkin *et al.* (2011) demonstrated that cholesterol independency and increased *A. albopictus* infectivity are actually two independent phenotypes that are caused by the mutation.

2.3 Chikungunya outbreaks in Malaysia

A serological survey conducted during 1965-1969 in Peninsular Malaysia determined that Chikungunya-specific antibodies were found in citizens aged more than 20 years old staying in states bordering Thailand such as Perlis, Kedah and Kelantan. Based on the survey, Marchette *et al.* (1978) predicted the population of Selangor and Perak to be vulnerable to Chikungunya outbreaks.

However, there are only 2 documented outbreaks of Chikungunya in Malaysia until 2006. The first outbreak happened in Klang, Kuala Lumpur from 6 December 1998 to 28 February 1999 and had affected 52 citizens (Lam *et al.*, 2001; Kit, 2002). The majority of

these patients were adults living in areas with poor sanitation systems (Kit, 2002). The second outbreak reemerged in the year 2006, from 28 March to 10 April, in Bagan Panchor, Taiping and had affected more than 200 villagers (Kumarasamy *et al.*, 2006; Abubakar *et al.*, 2007; Soon *et al.*, 2007). CHIKV isolates from these 2 outbreaks are closely related, with 99% similarity (Kumarasamy *et al.*, 2006). They belonged to the Asian lineage, but are different from the Thailand, Indonesia and coinciding Indian Ocean outbreak isolates. Therefore, the reemergence of CHIKV is an endemic infection (Abubakar *et al.*, 2007).

Interestingly, infections caused by East-Central South Africa (ECSA) were also reported. An elderly woman from Batu Gajah, Perak and a middle-aged man from Kinta, Perak were infected after their discrete trips to India in August and December 2006 respectively (Noridah *et al.*, 2007; Soon *et al.*, 2007). The subsequent outbreak in Kinta affected 35 patients who never leave Malaysia before the outbreak (Noridah *et al.*, 2007).

The latest outbreak occurred during 2008-2009, started from Johor state in early February. It was not endemic and had propagated to other states, even to Sarawak in East Malaysia (Chem *et al.*, 2010; Yusof *et al.*, 2011). The outbreak is believed to be introduced from Singapore and the virus isolates are highly similar to the ECSA genotype with the A226V mutation (Sam *et al.*, 2009; Chem *et al.*, 2010). The outbreaks in Malaysia which caused by different CHIKV genotypes suggest that the spread of CHIKV was no longer limited by geographical boundaries.

2.4 Symptoms of Chikungunya

Symptoms of Chikungunya fever are very similar to Dengue fever; both of them cause fever as high as 104 °F, rash, headache, vomiting, myalgia and arthralgia (joint pain) (Ligon, 2005; Sudeep and Parashar, 2008). In the laboratory setting limited situation, Chikungunya fever might be differentiated from Dengue fever by apparent leukocytosis and arthralgia/arthritis, however, incorrect classification can lead to inappropriate case management (Lee *et al.*, 2012).

Arthritis is the most significant symptom of Chikungunya and this explains its name which means "that which bend up" in Makonde language (Seneviratne *et al.*, 2007). The viral arthritis normally recovers in a few weeks but sometimes it becomes severe and requires a long recovery period. The joint pain in CHIKV fever can last for weeks or months depending on individual physical fitness (Seneviratne *et al.*, 2007; Sourisseau *et al.*, 2007; Sudeep and Parashar, 2008). The majority of Réunion Island outbreak patients suffered at the chronic stage after the CHIKV infection. They experienced distal polyarthritis or mild monoarthritis, frequent tenosynovitides and worsening of previous injuries in joints and bones (Simon *et al.*, 2011). Although there is no specific target joint, arthritis during Chikungunya infection commonly appears at metacarpophalangeal joints, wrists, elbows, knees, ankles and metatarsal joints (Franssila and Hedman, 2006). Moreover, 12% of patients suffered the symptoms for years or experienced destructive arthritis in ankles and metatarsal heads (Brighton and Simson, 1984). Interestingly, Chikungunya is recovered in fibroblasts from muscles, skin and joints in mouse models (Couderc *et al.*, 2008a).

In 1963, a death case was reported in Calcutta because of co-infection with meningoencephalitis, haematemesis and maleana with a sudden-shock syndrome (Aikat *et al.*, 1964; Sarkar *et al.*, 1964; Sudeep and Parashar, 2008). The neurovirulence of Chikungunya became more common and severe in recent outbreaks in Réunion Island. Chikungunya might cause cell death by inducing apoptosis of immature neurons and necrotic process of mature neurons (Arpino *et al.*, 2009). The probability to suffer from encephalitis and severe conditions is age-dependant; children and the elderly are higher risk populations (Couderc *et al.*, 2008b). However, the rate of neurological manifestation of Chikungunya is about 1:200, which is lower than other arboviruses such as Eastern Equine Encephalitis (EEE) and West Nile Virus (WNV) with rates of 1:20 and 1:100, respectively (Arpino *et al.*, 2009).

2.5 Causative agent – Chikungunya virus

The causative agent of Chikungunya is known as Chikungunya virus (CHIKV) and it belongs to the family Togaviridae, genus Alphavirus. Genetic analysis identified 3 distinct clusters of CHIKV: West African (WA), East-Central South African (ECSA) and Asian (Strauss and Strauss, 1994; Seneviratne *et al.*, 2007). The naming is based on geographical distribution of its first isolation and subsequent outbreaks. On the other hand, dengue fever is caused by Dengue virus, which belongs to family Flaviviridae, genus Flavivirus, with 4 distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) has been discovered (Bulugahapitiya *et al.*, 2007).

2.5.1 Alphavirus background

Alphavirus is a small, spherical and enveloped positive single-stranded RNA virus (Strauss and Strauss, 1994). Alphavirus can be divided into "Old World Alphavirus" such as CHIKV, O'Nyong-nyong (ONN), Semliki Forest virus (SFV), Ross River virus (RRV), Sindbis virus (SIN), and Barmah Forest virus (BFV); and "New World Alphavirus" which refer to EEE and Venezuelan Equine Encephalitis (Thammapalo *et al.*) (Jaffar-Bandjee *et al.*, 2009). Most of the Old World Alphaviruses are related to symptoms of arthralgia while the New World Alphaviruses are related to encephalitis (Powers *et al.*, 2001).

The Alphavirus genus has 29 members and are grouped according to their antigenic complexes into BFV, SFV, VEE, Western equine encephalomyelitis (Sweet), Middleburg virus (MIDV), Ndumu virus (NDUV), recombinant or ungrouped (Strauss and Strauss, 1994; Powers *et al.*, 2001). Chikungunya virus and its most closely homologous virus, ONN, are under the SFV group (Powers and Logue, 2007). This group consists of members such as Getah virus (GETV), Mayoro virus (MAYV), Ross River virus (RRV), Bebaru virus (BEBV) and the SFV (Powers *et al.*, 2001). WEE, which has the most similar base ratio with CHIKV, is grouped as recombinant (Nagatomo, 1972). Newly isolated species Salmon Pancrease Disease virus (SPDV), Sleeping Disease virus (SDV) and Trocara virus (TROCV) are not yet categorized (Powers *et al.*, 2001).

Alphaviruses are widely distributed and maintained in either human or primate cycle through transmission by the arthropod vectors (Levinson *et al.*, 1990). Their distributions in different ecological environments are dependent on the availability of their vectors (Powers *et al.*, 2001). Specific host-vector interactions keep the specific virus endemic in certain geographical areas. However, movement of the host might bring the

virus to new regions. Subsequent adaptation and evolution enables the virus to survive in diverse surroundings (Levinson *et al.*, 1990).

2.5.2 Genome organization and protein functions

Although the clinical manifestation and epidemiology of CHIKV and dengue virus overlaps, the two viruses have distinct taxonomy and genome organization. About 11.8 kb of linear positive single-stranded RNA make up the genome of CHIKV (Powers and Logue, 2007). Its genome is divided into 2 open reading frames (ORFs) (Sudeep and Parashar, 2008). Whereas the approximately 11 kb single-stranded plus-sense RNA of dengue virus is non-segmented (Ligon, 2005; Bulugahapitiya *et al.*, 2007). The genome composition of CHIKV and dengue virus is illustrated in Figure 2.2.

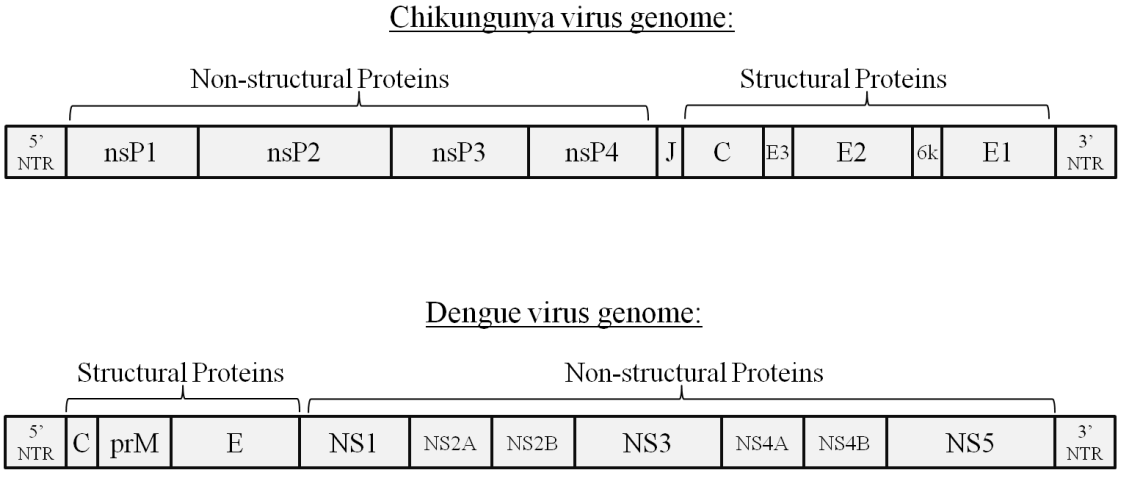

Figure 2.2 Genome composition of Chikungunya virus and Dengue virus.

The 5’ region of CHIKV genome encodes the non-structural proteins nsP1, nsP2, nsP3 and nsP4 while the 3’ region encodes the structural proteins such as capsid (C), E1, E2, 6K and E3 (Powers and Logue, 2007; Pardigon, 2009). In between the ORFs is a non-translated junction that encodes the promoter for 26S mRNA transcription (Strauss and Strauss, 1994). The conserved region of the 5’ and 3’ non-translated region is important for minus strand synthesis (Levinson *et al.*, 1990). The 3’ NTR serves as the polymerase binding site and has repeated sequences with unknown function in viral replication (Levinson *et al.*, 1990). On the other hand, the dengue virus genome encodes 3 structural proteins: capsid (C), membrane (prM) and envelope (E), and 7 non-structural proteins: NS1, NS2A NS2B, NS3, NS4A, NS4B and NS5 (Perera and Kuhn, 2008). The two viruses have no homology between their structural genes, but, the non-structural gene NS4B and nsP2 has significant similarity (Singhal and Bhandarkar, 2012).

2.5.2.1 Non-structural proteins

The translated non-structural polyprotein is cleaved into individual mature proteins with the facilitation of the C-terminal protease domain of nsP2 (Kim *et al.*, 2004). The mature proteins and the intermediate precursors take a role in synthesis of the minus strand RNA genome which is a template for subsequent 26S RNA and plus strand RNA synthesis (Schwartz and Albert, 2010).

NsP1 has 3 main known functions: initiate minus strand RNA synthesis, cap the mRNA and modulate protease activity in nsP2 (Strauss and Strauss, 1994). The study using the temperature sensitive mutant ts24 suggests that nsP1 is involved in promoter

recognition for initiation or elongation of minus strand genome template synthesis (Wang *et al.,* 1991). 5' guanosine capping of genomic and subgenomic RNA is carried out by guanine-7-methyltransferase and guanylyl transferase (Leung *et al.,* 2011). The intermediate polyprotein containing nsP1 inhibits cleavage between nsP2 and nsP3 by nsP2 protease (Strauss and Strauss, 1994).

The N-terminal domain of nsP2 consists of a nucleoside triphosphatase required in the capping of RNA and a helicase which is involved in unwinding the duplex strand in RNA replication. The C-terminus is a protease associated with transcription of 26S RNA (Strauss and Strauss, 1994; Merits *et al.*, 2001; Kim *et al.*, 2004). Two conserved residues, Cys-481 and His-558, make up the catalytic dyad of the papain-like-protease (Vasiljeva *et al.*, 2001). Deletion of 200 residues in the C-terminus can inhibit the protease activity, suggesting that this region is playing similar role like that of NS2B to the NS3 protease of Dengue virus (Pastorino *et al.*, 2008).

The crystal structure of nsP3 shows that the N-terminal has an ADP-ribose phosphatase domain (Malet *et al.,* 2009). NsP3 is highly conserved at the N-terminus but can tolerate large deletions of the C-terminus (Strauss and Strauss, 1994). This highly phosphorylated protein is speculated to actively be involved in minus strand RNA synthesis (Strauss and Strauss, 1994). Further studies have to be done to clarify the exact function of the protein.

An opal stop codon is present after nsP3 and it enables a read-through mechanism to produce polyproteins, P123 instead of P1234 (Pardigon, 2009). It is speculated that the transcription of more P123 leads to a rapid production of more protease to convert the

machinery from synthesizing the minus strand RNA to the plus strand RNA (Kim *et al.*, 2004).

NsP4 is an RNA-dependent polymerase (RdRp). It has a short half-life and is rapidly degraded because of degradation by the N-end rule pathway to regulate its amount (Strauss and Strauss, 1994). Ninety eight residues of its C-terminus forms the polymerase core catalytic domain with GDD motif, in which terminal adenylyltransferase (TATase) activity is also reported (Tomar *et al.*, 2006). This activity is facilitated by host factors for essential RNA virus polyA-tail recovery.

2.5.2.2 Structural proteins

The capsid protein can be divided into 2 domains; N-terminal 96 residues and C-terminal 97 residues. The N-terminal domain is non-conserved among alphaviruses, and is rich in proline and basic amino acids such as lysine and arginine (Strauss and Strauss, 1994; Wengler, 2009). The positive charges in this highly basic terminus neutralize the negative charges of bound RNA (Wengler, 2009). The leuzine zipper in this region assists nucleocapsid dimerization during viral particle assembly (Leung *et al.*, 2011).

In contrast, the C-terminal capsid is highly conserved, ordered in crystals and functions as a protease. The C-terminus folds into a trypsin-like protease structure in which the active site releases itself from the polyprotein precursor (Wengler, 2009). The protease is inactive as soon as the capsid protein is cleaved. Furthermore, the C-terminal hydrophobic pocket interacts with the cytoplasmic domain of the E2 glycoprotein and thus any movement of E2 might affect the arrangement of the nucleocapsid (Mukhopadhyay *et*

al., 2006). There is a short linker that connects the basic domain and protease domain (Wengler, 2009).

E3 is a short 64 amino acid protein consisting of a β-hairpin and 3 α-helices packed into a horseshoe shape (Voss *et al.*, 2010). A furin cleavage site is located at its C-terminus. Before cleavage by furin, E3 exists in the N-terminal of precursor p62 (or known as pE2) with E2 to guide translocation of p62 into the endoplasmic reticulum (ER) (Lobigs *et al.*, 1990). E3 in the precursor is also important to facilitate the proper conformation of E2 for its dimerization with E1 in the ER (Voss *et al.*, 2010). E3 is cleaved from E2 in the post Golgi compartment for protein maturation and virus assembly (Lobigs *et al.*, 1990). The uncleaved precursor can affect efficiency of virus production, however, virions with it remain capability to bind to host cells (Metz *et al.*, 2011).

E2 is 423 residues long while E1 is 439 residues. Both are the type I integral membrane proteins making up the shell enveloping the nucleocapsid (Strauss and Strauss, 1994). The E2 protein can be divided into 4 parts: N-terminal 260 residues ectodomain, 100 residues of stem region, 30 residues of transmembrane region and 33 residues of cytoplasmic domain (Mukhopadhyay *et al.*, 2006). E2 is a β protein belonging to the immunoglobulin superfamily with the ectodomain divided into A, B and C (Voss *et al.*, 2010). Domain A is folded into the center while domain B is exposed at the membrane distal end. Domain C is near the anchorage base to the viral membrane. The ectodomain and stem region are anchored to the lipid bilayer membrane by a transmembrane helix which is highly hydrophobic and palmitoylated (Strauss and Strauss, 1994; Mukhopadhyay *et al.*, 2006). The C-terminal cytoplasmic domain has Tyr-X-Leu and palmitoylated Cys in

the endodomain for interaction with core proteins in the budding process (Strauss and Strauss, 1994; Leung *et al.*, 2011).

The long thin leaf-shaped E2 with its position on the top of E1 indicates that it might be the first viral protein that makes contact with the host cell (Mukhopadhyay *et al.*, 2006). Therefore the most significant function of the E2 protein is to interact with host cell surface receptor. Receptor binding is probably in the leaf-like domain at the top of spike. The E2 protein contains the virulence determinants and is involved in the mosquito vector susceptibility range (Voss *et al.*, 2010) . Thus, E2 has most of the neutralizing epitopes on it (Powers *et al.*, 2001). Studies on E2 of various alphaviruses showed residues such as 180-220 of SIN as well as 216 and 218 of RRV are possible neutralizing epitopes (Mukhopadhyay *et al.*, 2006).

The P62-E1 heterodimer is long and twisted (150 Å long, 50 Å wide and 25 Å thick) (Voss *et al.*, 2010). After furin maturation, E1 and E2 form a stable E1-E2 heterodimer which is transported to the plasma membrane. Three heterodimers twisted counter clockwise around each other are maintained by E1-E1 interaction to form one unit of the spike (Strauss and Strauss, 1994). The trimers are positioned at quasi-3-fold axes to form the virus surface lattice, and 80 units of the spike make up the virus envelope surface (Mukhopadhyay *et al.*, 2006).

The E1 protein is more conserved than E2, therefore has cross-reactive epitopes with other alphaviruses (Powers *et al.*, 2001). It takes an active role in pH-dependent fusion with endosomal membranes (Mukhopadhyay *et al.*, 2006). The E1 ectodomain consists of three domains named domain I, II and III, which are mainly composed of β-

sheets (Voss *et al.*, 2010). The N-terminus sequence consists of domain I followed by domain II, while the C-terminus consists of domain III. The secondary structure shows that domain I is folded into the middle of domain II and III (Mukhopadhyay *et al.*, 2006). E1 forms the base of the spike with domain III adjacent to the membrane while domain I and II pose away from the membrane (Mukhopadhyay *et al.*, 2006). Domain II, the hydrophobic residues of domain III and the polar residues of domain I interact with E2 (Mukhopadhyay *et al.*, 2006). E1 interacts among itself through its domain I and its adjacent domain II (Mukhopadhyay *et al.*, 2006). It has a transmembrane helix near its C-terminus to anchor the protein (Mukhopadhyay *et al.*, 2006).

The linker between E1 and E2 is a hydrophobic peptide, named 6k, which is a multifunctional integral membrane protein that may aid in polyprotein cleavage, E1 protein translocation, membrane permeabilization and virus budding (Welch and Sefton, 1980; Loewy *et al.*, 1995; Yao *et al.*, 1996; Melton *et al.*, 2002; Sanz *et al.*, 2003). 6k is cleaved from the PE2 precursor and E1 through signalase (Strauss and Strauss, 1994). Addition of residues to the protein after residue F29 can affect the efficiency of glycoprotein precursor cleavage (Strauss and Strauss, 1994). Its C-terminus contains the translocation signal sequence for E1 (Strauss and Strauss, 1994). It comes to the plasma membrane along the spike glycoprotein complex and is speculated to be embedded into the membrane with two hydrophobic anti-parallel helices (Mukhopadhyay *et al.*, 2006). Membrane permeability is observed 4 hours post infection (hpi), in which small molecular weight ions diffuse through the host membrane. This is most probably initiated by the insertion of viroporin 6k to form pores, whereas the function is absent if the N-terminal aromatic residues are

mutated (Sanz *et al.*, 2005). The energy gradient is formed to release the mature viral particle into the medium (Sanz *et al.*, 2005).

2.5.3 Life cycle and replication in host cell

The life cycles of CHIKV consist of the stages of attachment, penetration, disassembly, replication, assembly and release(Leung *et al.*, 2011). It connects to the surface of host cells through binding with the receptor. However, the detailed identity of the host receptors are yet unknown. From the study in other alphaviruses, heparin sulfate, laminin, glycosaminoglycan, DC-SIGN, L-SIGN and integrin play important roles in this stage (Chevillon *et al.*, 2008; Schwartz and Albert, 2010).

After attachment to the host cells, the virus finds its way into the cell by endocytosis. Rearrangement of heterodimer E1-E2 occurs in the endosome, at acidic environment with pH lower than 6 (Leung *et al.*, 2011). The E1 residues initially in contact with E2 move away from the spike axes, pushing E2 out from the center, exposing itself onto the surface and form the E1 homotrimer (Mukhopadhyay *et al.*, 2006). The homotrimers then form pores in the membrane and fuse with the endosome in the presence of cholesterol to release the nucleocapsid into the cell cytoplasm (Chevillon *et al.*, 2008; Leung *et al.*, 2011). Protons flow from the pores into the cytoplasm, causing a regional low pH environment in the cytoplasm to help in the penetration of the nucleocapsid (Leung *et al.*, 2011).

Nucleocapsid uncoating occurs as soon as it is delivered into the cytoplasm within 2 minutes (Wengler, 2009). Host factors help to prime the nucleocapsid disassembly and

uncoating is ceased when the 60S subunit is saturated (Wengler, 2009; Leung *et al.*, 2011). Uncoating of the nucleocapsid releases the RNA genome for replication. The positive strand RNA resembles the mRNA and thus can be directly translated into polyprotein of replication complex by the host cell translation machinery. Minus strand RNA is synthesized as template for the 26S subgenome and 49S genome (Schwartz and Albert, 2010). The replication and translation occurs simultaneously and is regulated by the P1234 non-structural precursor.

The non-structural polyprotein P1234 and nsP4 form a RNA replication complex, to facilitate the minus strand RNA synthesis (Chevillon *et al.*, 2008; Leung *et al.*, 2011). The polyprotein is further cleaved into intermediate complex nsP1, P23 and nsP4, favouring both positive and minus strand RNA genome synthesis than subgenomic 26S mRNA synthesis (Kim *et al.*, 2004). An opal codon existing between the nsP3 and nsP4 can cause high production of P123 and the absence of nsP4 (Kim *et al.*, 2004). When the concentration of P123 is high, it is cleaved into individual mature proteins (Chevillon *et al.*, 2008). These mature proteins amplify 26S mRNA from the minus strand RNA but do not synthesize this template RNA (Kim *et al.*, 2004). Therefore, minus strand RNA synthesis stops approximately 4 hpi while positive strand RNA and 26S mRNA are continuously being produced (Tomar *et al.*, 2006).

26S mRNA, consisting of the structural polyprotein sequence, is translated into the precursor polyprotein C-pE2-6K-E1. The capsid protein releases itself from the precursor by autoproteolytic cleavage at the protease motif (Strauss and Strauss, 1994; Leung *et al.*, 2011). The remaining pE2-6K-E1 requires assistance from the host cell's post-translational modification machinery. It is processed into pE2 and E1 in the endoplasmic reticulum and

the heterodimer pE2-E1 is transported towards the plasma membrane (Chevillon *et al.*, 2008). Finally, pE2 is cleaved into E2 and E3 at the furin cleavage site, thus the heterodimer E1-E2 is presented as a spike at the cell surface (Leung *et al.*, 2011).

At the same time, capsid proteins in the cytoplasm assemble into the nucleocapsid, recognize the 5' capped complete positive strand RNA genome and pack it into the icosahedral core (Leung *et al.*, 2011). The assembled virion moves towards the plasma membrane and interacts with the cytoplasm domain of the E2 protein to bud off the host cell under the cover of the E1-E2 heterodimer glycoprotein (Wengler, 2009).

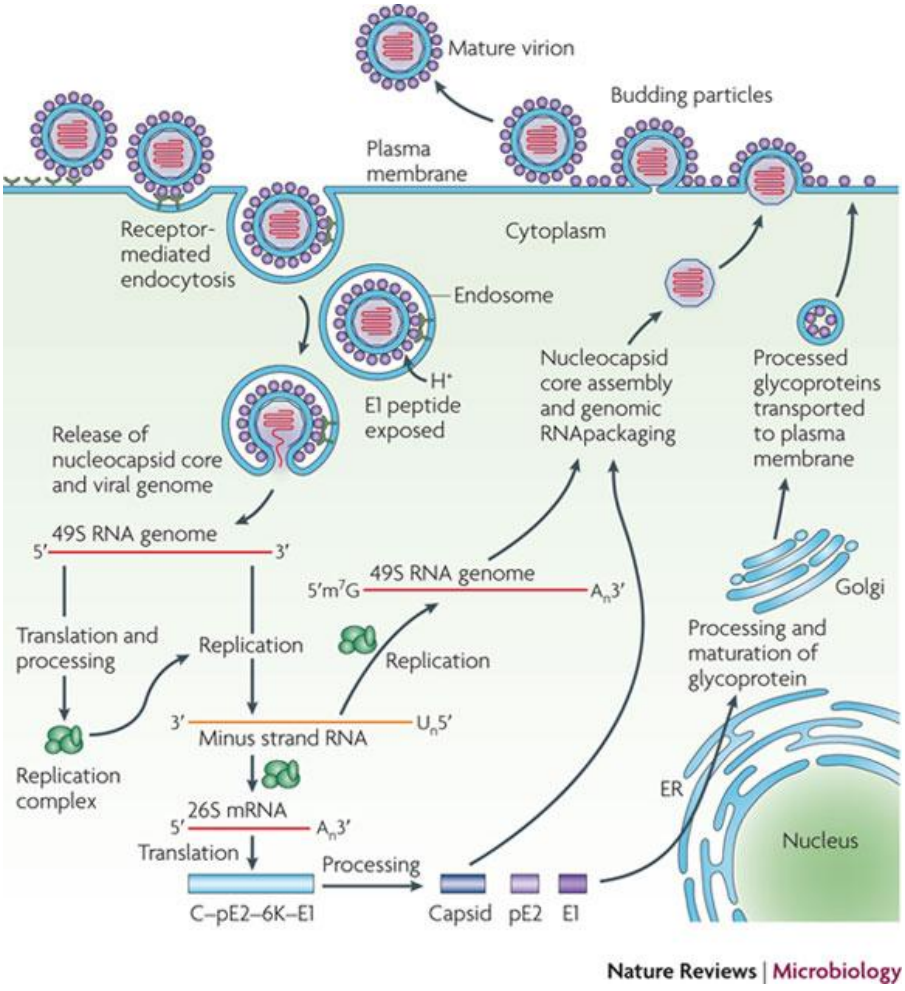

Figure 2.3 The Alphavirus life cycle. (Depicted from Biology and pathogenesis of Chikungunya virus (Schwartz and Albert, 2010)

2.6 Transmission cycle and vector

Transmission of CHIKV can be divided into sylvatic and non-sylvatic. The sylvatic cycle that involves non-humans is found only in West and Central Africa. The major species involved in sylvatic cycles are *A. furcifer*, *A. taylori*, *A. luteocephalus*, *A. africanus*, *A. dalzieli* and *A. neoafricanus* (Diallo *et al.*, 1999; Powers and Logue, 2007; Sudeep and Parashar, 2008). Continuous drought in East Africa in 2004 might had resulted in CHIKV escaping from the sylvatic cycle and reemerging in Kenya as the urban cycle. (Epstein, 2007).

In Asia, the transmission cycle is human-mosquito-human. Mosquitoes are the carrier of the virus and CHIKV can persist in the vector for a long time. Humans get infected from mosquito bites. *A. aegypti* is the main vector in Asia and outbreaks during 2004-2005 in East Africa and the Union of Comoros (Sang *et al.*, 2008). However, the priority of vector in the urban cycle had experienced some changes in recent outbreaks. *A. albopictus* (tiger mosquito), formerly known as a secondary vector, has became the major vector instead of *A. aegypti* (Vazeille *et al.*, 2007). *A. albopictus* originated from South East Asia, Western pacific and Indian Ocean Islands but its geographical distribution has extended west into India and African countries as well as east into the Oriental Indomalayan region (Knudsen, 1995; Gratz, 2004). It has also escaped from the tropical countries into temperate European countries and the United States (Gratz, 2004; Vazeille *et al.*, 2007).

Other than the normal transmission cycle, there is mother-to-child transmission reported in mothers infected near deliveries. There will be increased risk of abortion if the

mother is infected before the 22nd week of pregnancy but the infection does not increase the risk of malformation (Simon *et al.*, 2011).

2.7 Treatment and antiviral strategy

There is currently no recognized antiviral treatment available for Chikungunya. The primary symptoms are reduced by analgesics (painkiller), antipyretics, chloroquine and anti-inflammatory agents to reduce body temperature, pain and swelling of inflammation, dehydration and chronic arthritis (Brighton and Simson, 1984; De Lamballerie *et al.*, 2008b; Sudeep and Parashar, 2008). Dengue fever patient are also given analgesics and antipyretics, except aspirin, sufficient fluid replacement and bed rest, to control the febrile sickness, with no specific antiviral treatment available (Rigau-Perez *et al.*, 1998; Bulugahapitiya *et al.*, 2007).

Chloroquine, discovered 40 years ago, has been used to treat Chikungunya arthritis because of its anti-inflammatory characteristics but not antiviral feature (De Lamballerie *et al.*, 2009). Studies were unable to determine the efficacy and antiviral effect of chloroquine treatment. Treatment with chloroquine by 2400 mg in 5 days is globally tolerant with nausea and pruritis side effects (De Lamballerie *et al.*, 2008a). However, it has controversial *in cellulo* virus replication inhibition and *in vivo* virus replication enhancement (Delogu and De Lamballerie, 2011).

Other than choloquine, ribavirin has been drugs common for RNA virus. However, resistancy of alphavirus mutant strain towards ribavirin was reported (Scheidel *et al.*, 1987). In spite of that, its combination with α-inteferon can enhance the performance

(Briolant *et al.*, 2004). Azide molecule was capable of VEE inactivation and viral membrane protein inhibition with UV exposure and the 1,5 iodonapthyl azide specifically had been found to inhibit CHIKV replication (Sharma *et al.*, 2012).

Natural products are the current concern of the public, using medicinal plants for their antiviral properties. Several potential antiviral phytochemicals from plants have been studied, such as scleroglucan polysaccharides that prevent attachment of virus to glycoprotein of membrane, polyphenols that inhibit binding of virus to host cells and replication enzymes, flavonoids that inhibit polymerase, meliacine that affects virus disassembly and budding, *Aloe* polymannose that induces antibody production against non-enveloped capsid protein, sulphated polysaccharide groups that inhibit viral adsorption processes and dry Gingyo-san that significantly reduces fever and suppresses interleukin-1α (Jassim and Naji, 2003).

In silico route had been utilized to identify CHIKV inhibitors. The study based on nsP2 protein had optimized a novel compound which was very impressive in inhibiting CHIKV replication compared to chloroquine as well as simple to be synthesized (Bassetto *et al.*, 2013). Studies are ongoing to determine the accuracy of the finding on this molecule as candidate for clinical treatment.

The other strategy to overcome CHIKV is by using RNA interference (RNAi) and antisense oligonucleotides to inhibit post-transcription of mRNA (De Lamballerie *et al.*, 2008b; Lakshmi *et al.*, 2008; Sudeep and Parashar, 2008). Dash *et al.* (2008) found that 2 small interfering RNAs (siRNAs) against the conserved regions in nsP3 and E1 can inhibit