Identification of cross-neutralizing B-cell epitopes on the dengue virus envelope glycoprotein and their application in synthetic peptide based vaccine design

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by

Babu Ramanathan

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Environment and Biotechnology Centre

Swinburne University of Technology

Australia

Abstract

Dengue virus (DENV) is the causative agent for dengue fever. Classified under the flavivirus genus of the flaviviridae family and transmitted by mosquitos; DENV affects an approximately 50-100 million people per year which makes it the second most important tropical infectious disease after malaria. There is currently no vaccine or treatment for dengue fever. A key element in protection from dengue fever appears to be antibody. Development of a vaccine targeted against all 4 serotypes of dengue virus has been hampered by the potential complications following secondary infection.

Previous studies have shown that antibodies generated against the precursor-membrane protein (prM) are highly cross-reactive among the dengue virus serotypes but do not neutralize infection and potentially promote antibody dependent enhancement (ADE) of disease. This occurs as these antibodies can increase viral uptake into certain cell types, resulting in an increase in the total amount of virus produced. A means of overcoming this issue may be to design peptide vaccines that will generate a specific targeted antibody response against antigenic sequences within the Envelope (E) protein of all four dengue serotypes.

In order to discover novel neutralizing antibody epitopes, the present research involves a multi-step epitope mapping strategy using the neutralizing antibodies present in the sera of individuals who have successfully cleared a dengue fever infection. The samples of anti-dengue immunoglobulin G (IgG) purified from patient sera were used for epitope screening against an array of 70 overlapping synthetic peptides spanning the entire E protein of dengue virus serotype 2.

A combination of Enzyme-Linked Immunosorbent Assay (ELISA) and epitope extraction techniques revealed 29 epitopes recognized by anti-dengue antibodies on the E protein of DENV-2, of which nine were identified by both methods. Eight epitopes were identified in ELISA only and 12 epitopes were recognized in epitope extraction only. These epitopes span all three domains of the soluble E protein and the ectodomain of the native E protein. We have also used a multi-step computational analysis and predicted six antigenic regions on the DENV-2 E protein. These antigenic regions anchor atleast six epitopes identified by both wet-lab methods. In addition, our computational approach revealed several potential epitope candidates on the E protein of all four serotypes of DENV.

Abstract

The selected peptides were attached to a published B-cell T-helper epitope in order to serve as a vaccine candidate and evaluated in mice for their vaccine potency. Our study revealed 5 novel synthetic vaccine constructs that elicited humoral immune responses and neutralized one or more DENV serotypes *in vitro*, and are cross-reactive towards soluble recombinant E protein. The findings of this research may suggest new directions for epitope mapping and development of a much-needed anti-dengue vaccine.

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Declaration

I would like to declare that this thesis is my original work and has not previously been submitted for a degree. In addition, to the best of my knowledge, the thesis contains no material previously published or written by another person except where due reference is made in the text. Furthermore, where the work is based on joint research or publications, the thesis discloses the relative contributions of the respective workers or authors.

Signature: _____ Date: _____

(Babu Ramanathan)

Communications

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- **Ramanathan *et al.,*** Synthetic peptide-based vaccines: the next generation vaccine strategy

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List of symbols and Abbreviations

Å	Angstrom
°C	Degree Celsius
µg	Microgram
µl	Microlitre
aa	Amino acid
Ab	Antibody
Abs.	Absorbance
ADE	Antibody-dependent enhancement
BHK	Baby hamster kidney
BSA	Bovine serum albumin
C	Capsid (protein)
CDC	Centers for Disease Control and Prevention
CIGB	Centre for Genetic Engineering and Biotechnology
CMC	carboxy methyl cellulose
CNRS	Centre national de la recherche scientique
CO2	Carbon Dioxide
CPE	Cytopathic effect
CRC	Compact reaction column
DI	Domain one
DII	Domain two
DIII	Domain three
DC	Dendritic cells
DENV	Dengue virus

DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DF	Dengue fever
DHF	Dengue heamorrhagic fever
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
E	Envelope (protein)
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GSK	GlaxoSmithKline
Hg	Mercury
HI	Hemagglutination inhibition
HRP	horseradish peroxidase
IFN	Interferon
IP	Intraperitoneal
Ig	Immunoglobulin
IgG	Immunoglobulin G
IPK	Tropical Medicine Institue “Pedro Kouri”
JEV	Japanese encephalitis virus
kb	Kilobase
kDa	KiloDalton

LAV	Live attenuated vaccine
M	Molar
MAb	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MAP	Multiple antigenic peptide
ml	Millilitre
mM	Millimolar
mm	Millimeter
MVE	Murray Valley encephalitis
NGC	New Guinea-C
NIAID	National Institute of Allergy and Infectious Diseases
Nm	Nanometer
NS	Non-structural
OD	Optical density
ORF	Open reading frame
prM	Precursor membrane (protein)
PAHO	Pan American Health Organization
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.5% Tween 20
PDK	Primary dog kidney
PEG	Polyethylene glycol
PGMK	Primary green monkey kidney
Pr	Precursor
PSG	penicillin/streptomycin/glutamine solution
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid

RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase-polymerase chain reaction
sE	Soluble E protein
TBEV	Tick-borne encephalitis viruses
T_H	Helper T
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
ToF	Time of Flight
UTR	Untranslated region
v/v	Volume per volume
WHO	World Health Organisation
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
YFV	Yellow fever virus

Amino acid abbreviations and molecular mass

Amino Acid	Three letter code	Single letter code	Average mass
Alanine	Ala	A	71.08
Arginine	Arg	R	156.2
Asparagine	Asn	N	114.1
Aspartic Acid	Asp	D	115.1
Cysteine	Cys	C	103.1
Glutamine	Gln	Q	128.1
Glutamic Acid	Glu	E	129.1
Glycine	Gly	G	57.05
Histidine	His	H	137.1
Isoleucine	Ile	I	113.2
Leucine	Leu	L	113.2
Lysine	Lys	K	128.2
Methionine	Met	M	131.2
Phenylalanine	Phe	F	147.2
Proline	Pro	P	97.12
Serine	Ser	S	87.08
Threonine	Thr	T	101.1
Tryptophan	Trp	W	186.2
Tyrosine	Tyr	Y	163.2
Valine	Val	V	99.07

Chapter 1

Review of literature

Arbovirology deals with the study of arthropod-borne "Arboviruses" transmitted through arthropod vectors to vertebrate hosts. These viruses belong to viral families which possess RNA genomes comprising single-stranded positive-sense genomes (*Flaviviridae* and *Togaviridae*), negative-sense genomes (*Bunyaviridae*, *Orthomyxoviridae* and *Rhabdoviridae*) and double-stranded genomes (*Reoviridae*), with the exception of African swine fever virus (*Asfaviridae*) with a DNA genome. Yellow fever virus (YFV) and dengue virus (DENV) were the first two arboviruses to be attributed to "filterable agents" with the ability to cause human diseases via mosquito bites (Bancroft, 1906; Graham, 1903; Henchal and Putnak, 1990; Reed *et al.*, 1983). Arboviruses are largely zoonoses and before transmission to mammals these viruses infect and replicate in the invertebrate host (mosquitoes, biting flies and ticks). The common arboviruses causing human diseases belong to one of these three families: *Flaviviridae, Togaviridae* and *Bunyaviridae* (Gubler, 2002b; Lanciotti *et al.*, 1997; Whitehead *et al.*, 2007).

1.1 *Flaviviridae*

Historically, flaviviruses, also known as Group B arboviruses, were placed in the family *Togaviridae* and were separated from Group A arboviruses, the alphaviruses, based on their mode of transmission, biochemical properties and antigenic differences (Casals and Brown, 1954; Fenner *et al.*, 1974; Wang *et al.*, 2000). In 1984, the International Committee for the Nomenclature of Viruses voted to make the *Flaviviridae* a separate family (Westaway *et al.*, 1985; Goncalvez *et al.*, 2002). The family *Flaviviridae* (in Latin, *flavus* meaning yellow) comprises three genera: the pestiviruses, the hepaciviruses and flaviviruses (Ruggli and Rice, 1999; Twiddy *et al.*, 2002). The genus Pestivirus includes viruses causing livestock diseases such as border disease virus, swine fever virus and bovine viral diarrhea virus. The Hepacivirus genus mainly consists of hepatitis C virus that causes persistent hepatotrophic infections in humans. The Flavivirus genus is composed of more than 70 species including the prototype Yellow fever virus (YFV) and dengue virus

(DENV). Nucleotide sequence analysis of different flaviviruses has revealed that they have evolved from a common ancestor and are closely related (Gould *et al.*, 1985; Deubel *et al.*, 1986; Deubel *et al.*, 1990; Rice *et al.*, 1986; Lanciotti *et al.,* 1997; Wang *et al.,* 2000).

There are eight serological sub-groups within the flaviviruses recognized based on geographical distribution, the nature of host and cross neutralization tests (Calisher *et al.*, 1989; Monath and Heinz, 1996). Among all flaviviruses, the main human pathogens are the Yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and Tick-borne encephalitis virus (TBEV) (Vasilakis and Weaver, 2008; Goncalvez *et al.,* 2002; Twiddy *et al.,* 2002). Development of YFV vaccines and several vector control tactics have helped the suppression of YFV outbreaks for over a decade. However, no vaccines are currently available for dengue fever, which has emerged as the most important mosquito-borne viral disease of the 21st century (Mackenzie *et al.*, 2004). It is estimated that there are greater than 3.6 billion people at risk of dengue infection with 50-100 million cases of dengue fever, more than 2 million cases of severe dengue and approximately 21,000 deaths annually (Beatty *et al.*, 2009). A large proportion of infected population are children below 5 years of age (WHO, 2012).

1.2 Dengue virus and transmission

DENV comprises four genetically different but antigenically related serotypes (DENV-1, 2, 3, and 4) (Calisher *et al.,* 1989). Within each of the four serotypes, the viruses are further grouped into genotypes (Rico-Hesse, 1990). The genetic diversity of each genotype is described in Tables 1.1 and 1.2. All four DENV serotypes can be found in nearly all urban and peri-urban environments throughout the tropical region, which puts nearly a third of the global population at risk (Farrar *et al.*, 2007). Outbreaks are generally restricted to the tropics due to viral transmission by the *Aedes* mosquitoes that only exist in tropical climates. However, spread of this disease to non-endemic regions has occurred due to contemporary life-style trends such as rapid transportation of large numbers of people, population explosion and urbanization (Gubler, 2002a).

Table 1.1. Genetic diversity of DENV serotypes 1 and 2.

Serotype	Genotypes	Original known distribution
DENV-1	I	Japan, Hawaii (the prototype strains), China, Taiwan and Southeast Asia.
	II	Thailand.
	III	Sylvatic strains from Malaysia.
	IV	Nauru, Australia, Indonesia and the Philippines.
	V	Africa, Southeast Asia and the Americas.
DENV-2	I	Asian I strains from Thailand, Myanmar and Malaysia, and Asian II strains formerly known as subtype I and II found in China, the Philippines, Sri Lanka, Taiwan and Vietnam. Includes the New Guinea C prototype strain.
	II	Cosmopolitan strains Formerly known as genotype IV. Wide distribution including Australia, the Pacific islands, Southeast Asia, the Indian subcontinent, Indian Ocean islands, Middle East, and both East and West Africa.
	III	American strains formerly known as subtype V. Found in Latin America, old strains from India, the Caribbean, and the Pacific.
	IV	American/Asian strains formerly known as subtype III. Found in China, Vietnam, Thailand and in Latin America.
	V	Sylvatic strains isolated from non-human primates in West Africa and Malaysia

Information for this table compiled from (Lanciotti *et al.*, 1994; Lanciotti *et al.,* 1997; Wang *et al.,* 2000; Goncalvez *et al.,* 2002; Twiddy *et al.,* 2002; Wittke *et al.*, 2002).

Table 1.2. Genetic diversity of DENV serotypes 3 and 4.

Serotype	Genotypes	Original known distribution
DENV-3	I	Indonesia, Burma, the Philippines and the South Pacific islands (French Polynesia, Fiji and New Caledonia). Includes the H87 prototype strain.
	II	Thailand, Vietnam and Bangladesh
	III	Singapore, Indonesia, South Pacific islands, Sri Lanka, India, Africa and Samoa.
	IV	Puerto Rico and French Polynesia (Tahiti).
	V	Originally grouped in genotype I and isolated from China, Philippines and Malaysia
DENV-4	I	Thailand, Malaysia, the Philippines and Sri Lanka. Includes the H241 prototype strain.
	II	Indonesia, Malaysia, Tahiti, the Caribbean islands (Puerto Rico and Dominica) and the Americas.
	III	Thailand (Bangkok, specifically).
	IV	Sylvatic. Isolated from non-human primates in Malaysia.

Information for this table compiled from (Lanciotti *et al.,* 1994; Lanciotti *et al.,* 1997; Wang *et al.,* 2000; Goncalvez *et al.,* 2002; Twiddy *et al.,* 2002; Wittke *et al.,* 2002).

The principal viral transmission vector among human hosts is the peri-domestic mosquito *Aedes aegypti* of the family *Culicidae*. It prefers to breed in domestic and peri-domestic water containers. Its adaptation to human habitats and its desiccation-resistant eggs have allowed it to flourish in urban centers. The secondary vector for dengue is *Aedes albopictus* (Asian tiger mosquito) which serves as the primary vector for dengue in countries where *A. aegypti* is absent (Gratz, 2004). It is also believed that DENV follow sylvatic/enzootic transmission cycles comprising non-human primates and vector hosts living in forests. Ecological studies of dengue virus in sylvatic habitats of Asia have identified that DENV-1, -2, and -4 circulate in a sylvatic cycle between *Macaca* and *Presbytis* monkeys vectored by *A. nivues* (Peiris *et al.*, 1993). A sylvatic transmission cycle of DENV-2 in West Africa occurs between non-human *Erythrocebus patas* monkeys as reservoir hosts and arboreal, tree-hole dwelling *Aedes* species as vectors (Diallo *et al.*, 2003).

1.3 History and epidemiology

The term "dengue" is reported to have originated from the Swahili phrase "ka dinga pepo", meaning a kind of sudden cramp-like seizure from an evil spirit or plague (Christie, 1881). When the disease crossed from East Africa to the Caribbean in 1827, the phrase was popularly identified with the Spanish name "dengue" in Cuba and "dandy" in British West Indies. The name "breakbone fever", which is attributed to the severe joint pains of dengue patients, is also sometimes used in place of dengue (Rigau-Perez, 1998). Dengue fever is a very old disease with the earliest documentation of dengue-like illness found in a Chinese encyclopedia of disease symptoms and remedies first published during the Chin dynasty (265-420 AD), Tang Dynasty (610 AD) and Northern Sung Dynasty (992 AD) (Gubler, 1997; Gubler, 1999) describing a disease called "water poison" due to its association with water-associated flying insects and their clinical description included fever, rash, myalgia and hemorrhagic manifestations. A similar description of the illness did not occur until 1635 and 1699 in the French West Indies and Panama, respectively (Gubler, 1997). The first reports of possible widespread DENV outbreaks occurred an entire century later (1779-1788) during the DENV pandemics in Indonesia, Egypt, North America and Spain (Gubler, 1997). During the 19th and 20th centuries, outbreaks

have occurred in Southeast Asia, India, Philippines and in the Caribbean changing the DENV disease behavior from the sudden onset of urban epidemics to endemic disease in some areas (Smith, 1956; Brown, 1977; Gratz and Knudsen, 1996).

DENV ecology, epidemiology and distribution changed dramatically following the events of World War II. Abandonment of war materials and damage of water distribution systems created a favourable environment for the larval development of *A. aegypti*. In addition, transportation of troops and supplies to new geographical areas increased the distribution of mosquito's and their eggs resulting in greater densities of *A. aegypti* (Vasilakis and Weaver, 2008). As a result, a series of dengue epidemics were seen among military personnel during 1941-1945 in East Africa, the Caribbean, and the pacific region, from Australia to Hawaii and Guinea to Japan (Brown, 1977; Gubler, 1997; Sabin, 1952). However, the events of World War II enlightened awareness and a better understanding of dengue and increased DENV research in the scientific community. DENV-1 was first isolated from patients by Japanese scientists through a Swiss albino mice brain passage method in 1943 (Hotta, 1952), followed by isolation of both DENV-1 (Hawaii strain) and DENV-2 (NGC strain) by Albert Sabin from US soldiers in 1944 (Sabin, 1952). They were also able to identify the homotypic immunity following dengue infection, and developed a hemagglutination-inhibition (HI) test for dengue serodiagnosis (Sabin, 1952). The first hemorrhagic strains, DENV-3 (H87 strain) and DENV-4 (H241 strain), were isolated from patients presenting with hemorrhagic disease during the 1956 Philippine epidemic (Hammon *et al.*, 1960b; Hammon *et al.*, 1960a).

Though a number of epidemics occurred in Southeast Asia after World War II, no epidemics were reported in America for the next 20 years. This was mainly due to the introduction of an *A. aegypti* eradication program in 1940 by the Pan-American Health Organization (PAHO) originally aimed to control urban epidemics of yellow fever. This programme led to the eradication of the vector in 19 countries (Gubler, 1997); however, discontinuation of the program in 1970 has allowed *A. aegypti* to gradually re-establish and an increased DENV distribution has been noted. A series of DENV-1 outbreaks have been reported near the Texas-Mexico border (CDC, 1996; CDC, 2007) and in the Hawaiian Islands (Effler *et al.*, 2005).

In Asia, an increased incidence of DENV distribution was reported following outbreaks in India (Balaya *et al.*, 1969; Myers *et al.*, 1968; Myers *et al.*, 1965;

Ramakrishnan *et al.*, 1964), epidemics in Vietnam (Halstead *et al.*, 1965), Philippines (Basaca-Sevilla and Halstead, 1966), Singapore (Chan *et al.*, 1965; Lim *et al.*, 1961), Malaysia (Rudnick *et al.*, 1965) and Thailand (Halstead *et al.*, 1967). By the end of 1970, all four dengue serotypes were circulating throughout Southeast Asia and the Indian subcontinent. These observations, along with field studies in Thailand, led to the theory of antibody-dependent enhancement of dengue pathogenesis (Halstead *et al.*, 1973) which has shown the secondary antibody response patterns and the severity of dengue disease (Halstead *et al.,* 1967; Russell *et al.*, 1967).

The history of dengue in Australia extends for more than a century where records show it was a major cause of death in northern Australia before the beginning of World War I. The earliest reference described the importation of eight cases by ship from Mauritius in 1873, however the first indigenous outbreak probably occurred in Townsville, Queensland, during 1879 and Rockhampton, Queensland, in 1885 (Lumley and Taylor, 1943). Following this, a few isolated outbreaks of DENV-3 were reported during the mid-20th century in New South Wales (Russell *et al.*, 1984), Queensland (Doherty, 1957; Doherty *et al.*, 1967) and Darwin (Whelan, 1991; Mclean and Magrath, 1959). There was a decrease in DENV distribution in New South Wales (Russell *et al.,* 1984), Western Australia, Northern Territory (Whelan, 1991) and Queensland (Kay *et al.*, 1983; Sinclair, 1992) during the 1960s, which was attributed to a decline in the circulation of *A. aegypti*. Though several factors have contributed to vector reduction, the conversion of urban water supplies from household rainwater tanks to a reticulated supply has certainly been the most important single factor.

Almost more than 25 years later, dengue re-appeared in North Queensland during the 1981-82 epidemics and circulation of DENV-1 was confirmed by serological diagnosis of infected patients from Cairns, Townsville and Thursday Island (Guard *et al.*, 1984; Kay *et al.*, 1984). In 1992-93, a large outbreak of dengue was reported in Townsville and Charters Towers. A cross-sectional serological survey of 1,000 randomly selected people living in Charters Towers showed that 39.9% of the population was infected with DENV-2 and 20% of the group fulfilled the criteria for infection during 1992 outbreak (Mcbride *et al.*, 1998b). One of the largest epidemics in the last 50 years took place in 2008-2009, affecting a significant geographical area of North Queensland, with separate outbreaks in Cairns (DENV-2, -3, and -4) and Townsville (DENV-1, and -3) which confirmed 931 clinical cases, with

the majority of cases occurring in the Cairns region (CDC, 2009).

The global emergence and re-emergence of DENV may be due to a combination of several factors, including lack of effective mosquito control strategies leading to the increased distribution and density of vector, unplanned and uncontrolled rapid urbanization, inadequate wastewater management and global climate change which all favour the increased spread of the virus (Kyle and Harris, 2008; Pinheiro and Corber, 1997). In addition, phylogenetic studies of the viruses isolated in past 3 decades have shown that the reappearance of dengue infections may be due to microevolution of the virus, which leads to virulent strains replacing the less virulent/avirulent genotypes (Klungthong *et al.*, 2008; Steel *et al.*, 2010; Weaver and Vasilakis, 2009). The World Health Organization (WHO) estimates that at least 100 countries are endemic and about 40% of the world's population is at risk of infection each year as can be seen in Fig. 1.1.

1.4 Clinical manifestation

Dengue infections can vary from asymptomatic or self-limiting mild flu-like illness to classical dengue fever (DF), to the more severe disease state characterized as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Burke *et al.*, 1988). This criterion has been widely used amongst epidemiologist to classify the dengue fever. The virus is injected into the skin from the bite of an infected mosquito and the dendritic cells (DCs) are the first-cell type to encounter the infection (Wu *et al.*, 2000). In the lymph node, these infected cells present the antigen to T lymphocytes, and other cell types in the draining lymph node such as monocyte/macrophages, B-cells, and other DCs become infected. The virus enters in the bloodstream possibly through infected B-cells, which facilitates infection of secondary organs such as the liver, spleen and kidneys (Jessie *et al.*, 2004). Varying clinical features of dengue fever have been observed with increasing severity of disease in patients with increasing age and multiple infections.

1.4.1 Dengue Fever (DF)

Classical dengue fever is a self-limited febrile illness associated with fever, headache (especially in the retro-orbital area), myalgia, nausea and vomiting along

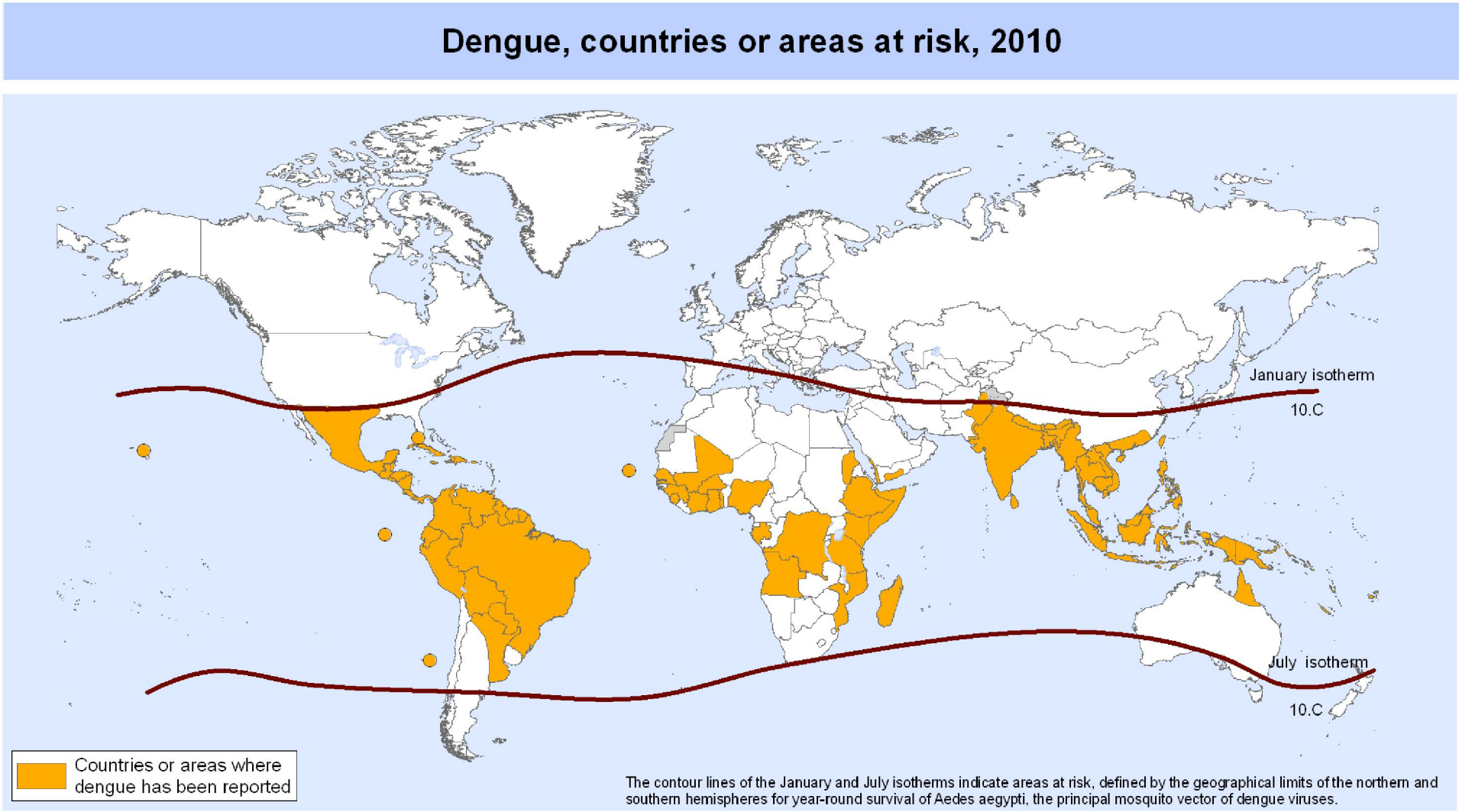


Fig. 1.1. Areas at risk of dengue infection (WHO, 2010). Approximately 3.6 billion people living in over 100 countries are at risk of dengue transmission.

with joint pains, weakness and rashes (Gubler, 1998; Whitehead *et al.,* 2007). The infection and fever generally last for 4 to 7 days (WHO, 2009), with a saddleback pattern, characterized by a drop in fever after a few days. Skin eruptions are common in children and adults following primary infection. They may also develop flushing of the face, neck and chest along with round pale areas surrounding confluent petechial rashes on the skin. Maculopapular rashes with symptoms such as pharyngeal inflammation, rhinitis and cough are indications of fever in infants and young children. However, either a mild febrile syndrome or a severe form of classical DF manifestations can be seen in old children and adults. Clearance of the virus is associated with cytotoxic T-cells (Bukowski *et al.*, 1989; Kurane *et al.*, 1989b; Yauch *et al.*, 2009) and virus neutralizing antibodies that can block virus-mediated cell membrane fusion and virus attachment by targeting domain II and domain III of the envelope (E) protein, respectively (Crill and Roehrig, 2001; Gollins and Porterfield, 1986; Kaufman *et al.*, 1987; Roehrig *et al.*, 1998; Whitehead *et al.,* 2007).

1.4.2 Dengue Hemorrhagic Fever (DHF)

DHF is defined as an acute febrile illness with high fever, hemorrhagic manifestations, thrombocytopenia ($\leq 10^5$ cells/μl) and evidence of plasma leakage due to increased vascular permeability. Most of the people infected are children under the age of ten and nearly 1 in 100 patients dies with this condition (Shekhar, 2007). DHF often follow secondary dengue infections, but may sometimes occur in primary infections, especially in infants (Dietz *et al.*, 1996; Halstead *et al.*, 2002). At this point, the patient may recover or progress to the phase of plasma leakage. DHF usually occurs around 3-7 days immediately following DF defervescence and last for 2-7 days showing a sudden rise in temperature and other symptoms resembling DF. Leakage of plasma through endothelial gaps without necrosis or inflammation of the capillary endothelium is the major indication that determines the severity of disease in DHF and differentiates it from DF which is followed by petechiae, pleural effusions, thickened gall bladder wall, bleeding from mucosa and hemorrhage in the gastrointestinal tract. A positive tourniquet test with more than 20 petechiae in a square patch of skin (2.5 x 2.5 cm) is an indication of development of DHF (Ashburn and Craig, 2004; Kalayanarooj *et al.*, 1997). In addition, a decrease in platelet count and elevation in haematocrit (erythrocyte fraction) indicates an increased probability of impending shock (Nimmannitya, 1987).

1.4.3 Dengue Shock Syndrome (DSS)

DSS is defined as DHF with signs of circulatory failure, narrow pulse pressure (≤20 mm Hg), hypotension, cold, clammy skin and frank shock. Abnormalities in liver functions are common. Sustained abdominal pain, persistent vomiting, restlessness or lethargy and a sudden change from fever to hypothermia with sweating and prostration are four warning signs of life threatening dengue shock (Rigau-Perez, 1998). Increased vascular permeability may also lead to DSS, which is associated with a very high mortality rate. Early recognition and treatment of shock is the only preventive measure and, if not managed properly, the fatality rate may be as high as 44% (Nimmannitya, 1987). Severe dengue infection may also lead to complications such as encephalitis, hepatitis, myocarditis and renal dysfunction (Pancharoen *et al.*, 2002).

Recently, a new model for dengue classification has been proposed which classifies dengue in to 3 categories, dengue without warning signs, dengue with warning signs and severe dengue (WHO, 2009). Fig. 1.2 depicts the recent criteria suggested for differentiating the dengue infections.

1.5 Antibody dependent enhancement of disease (ADE)

The antibody response to the envelope (E) glycoprotein of DENV is known to play a critical role in both protection from and enhancement of disease, especially after primary infection. Epidemiological studies have shown that infection with one serotype of DENV results in lifelong immunity to that particular DENV serotype and cross-reactive protection for a different serotype lasts for the first 6 months after primary infection. However, after this period, the patient is susceptible to the infection by the remaining three serotypes (Burke *et al.,* 1988; Guzman *et al.*, 1990; Halstead *et al.*, 1969; Kliks *et al.*, 1989; Sabin, 1952; Sangkawibha *et al.*, 1984; Thein *et al.*, 1997). Over 80% of DHF/DSS cases occur following secondary heterologous DENV infections accompanied by a high level of circulating viruses (Goncalvez *et al.*, 2007; Vaughn *et al.*, 2000; Webster *et al.*, 2009). Studies have shown that the antibodies from patients with secondary infections during large outbreaks of DHF/DSS have direct impact on dengue severity (Guzman and Kouri, 2008; San Martin *et al.*, 2010).

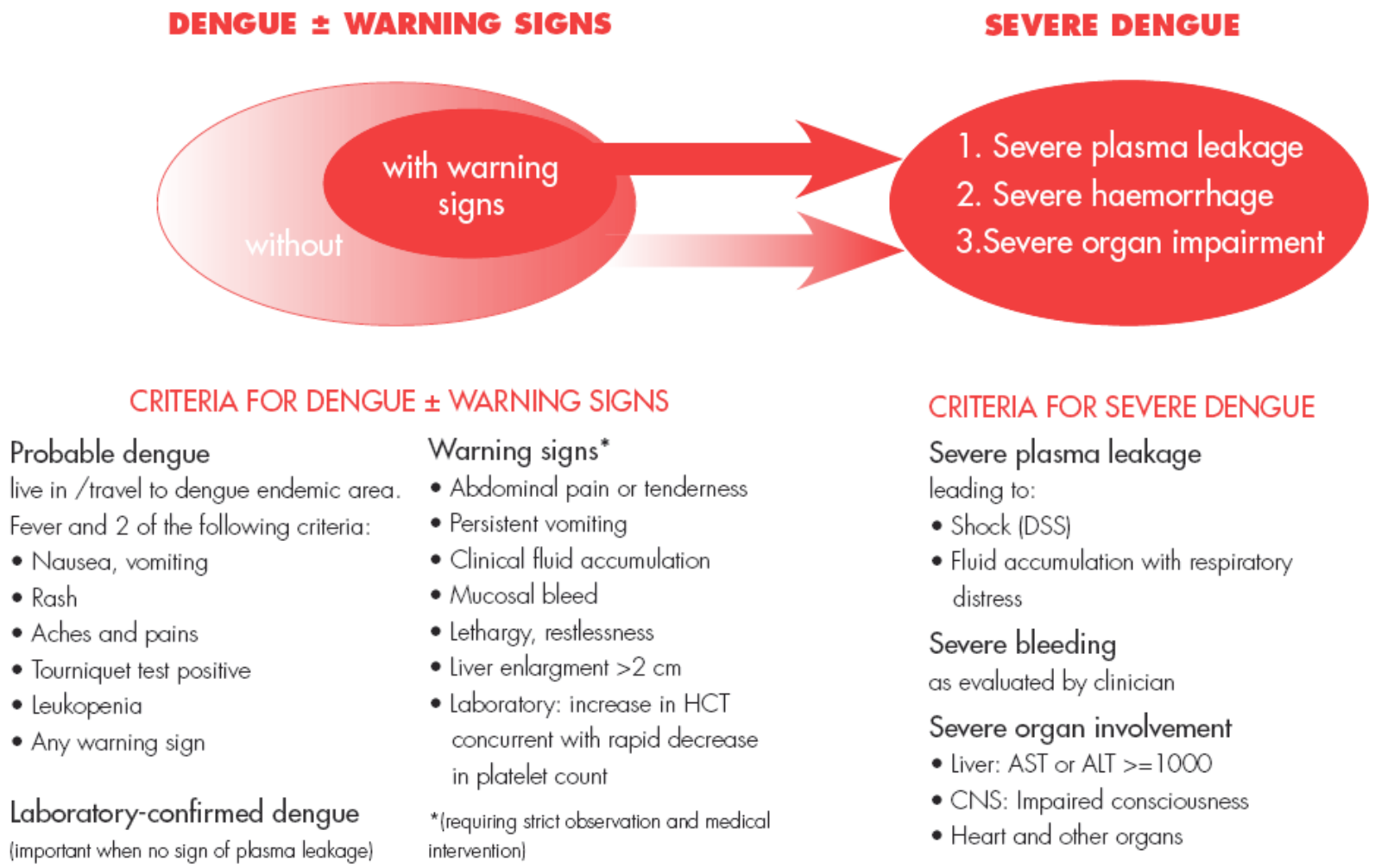


Fig. 1.2. Criteria suggested for dengue case classification. Severe dengue can be seen either with or without warning signs. (WHO, 2009)

DHF following primary infection is usually limited to infants between 4-12 months old and is reported to be caused by circulating non-neutralizing maternally-derived dengue antibodies (Chau *et al.*, 2009; Halstead, 1970; Halstead *et al.,* 2002; Kliks *et al.,* 1989).

Antibody-dependent enhancement is a phenomenon during dengue viral infection where secondary infection with a heterologous DENV serotype where pre-existing, sub-neutralizing and non-protective antibodies will bind to viruses and enhance their uptake in FcγR-bearing monocytes, resulting in enhanced infection and severity of disease. This phenomenon is depicted in Fig. 1.2. The immune system is deceived because the four subtypes are 60-80% homologous and have very similar surface antigens. Further, the immune response attracts numerous macrophages which aids infectivity of viruses which have not been inactivated (Kautner *et al.*, 1997) and promotes viral uptake into certain cell types, resulting in an increase in the total amount of virus replication. Immunoglobulin G (IgG) antibodies were found to play an important role in ADE. Mutant IgG with a 9- aa deletion at the N terminus of the CH_2 domain in the Fc region abrogated the interaction with FcγR and failed to cause enhancement (Goncalvez *et al.,* 2007).

The role of anti-prM antibodies in ADE has also been studied where the antibodies increased the infectivity of poorly infectious immature DENV to the same level as wild type virus particles in FcγR-bearing cells in a furin-dependent manner (Rodenhuis-Zybert *et al.*, 2010). Once the immature virus enters into the endosomes through FcγR mediated trafficking by prM antibodies, the acidic environment triggers conformational rearrangement of the immature virus particle. This facilitates furin cleavage by exposing the furin cleavage site of prM protein, in turn resulting in the maturation and fusion of the virus with endomembrane (Molloy *et al.*, 1999; Sariola *et al.*, 1995; Zhang *et al.*, 2003). In another study, antibodies generated against the prM protein showed highly cross-reactivity against dengue virus serotypes even at a minimal concentration and did not neutralize the virus but potentially promoted ADE (Dejnirattisai *et al.*, 2010). Research has also shown that anti-E and anti-prM monoclonal antibodies generated from DENV-2 infected mice were found to enhance DENV infection in a concentration-dependent manner mediated by the FcγRIIA pathway (Goncalvez *et al.,* 2007). In this case, increased binding of virus to the cell was mediated by the ability of the anti-prM antibodies to cross-react with host hsp60 as cells

such as BHK-21 and A-549 lack FcγR (Huang *et al.*, 2006).

In addition to an antibody response following heterologous infection, simultaneous activation of memory T-cells specific for the previous infection is proposed to delay viral clearance and increase cytokine production, which in turn affects the immune response against current infection (Mongkolsapaya *et al.*, 2003). The activated memory CD4+ T-cells may also play a role by releasing IFN-γ during infection, which helps the virus to infect the up-regulated FcγR-bearing monocytes (Pang *et al.*, 2007). Tumor necrosis factor-alpha (TNF-α) released by these infected monocytes is also strongly associated with DENV pathogenesis as they induce vascular leakage by increasing the permeability of endothelial cell monolayers (Atrasheuskaya *et al.*, 2003; Espina *et al.*, 2003; Prestwood *et al.*, 2008; Shresta *et al.*, 2006).

The genetic nature of the virus is also associated with DHF and DSS as the virus genome has direct impact on severity of the disease based on the infecting DENV serotype (Balmaseda *et al.*, 2006; Fried *et al.*, 2010) and genotype (Messer *et al.*, 2003; Rico-Hesse *et al.*, 1997; Ty Hang *et al.*, 2010). Virus genetics and ADE are major contributors towards understanding the development of DHF and DSS, and their effect should be considered in a DENV vaccine design.

1.6 Structure of dengue virus

Electron micrographs of dengue show the virion particle to be spherical, approximately 500 Å in diameter. The genome is composed of a single, positive-strand RNA genome 10.6 kb in size with a type I cape structure (m7G5'ppp5'A) that lacks a poly(A) tail at the 3' end. A single long open reading frame (ORF) is flanked by 5' (approximately 100 nucleotides) and 3' (approximately 350 nucleotides) untranslated regions (UTR's) which are important cis-acting elements for replication, transcription and translation (Rice *et al.*, 1985). The ORF is translated into a single polyprotein that is co- or post-translationally processed by host and viral proteins into distinct polypeptides; three structural proteins (at the amino terminus end) and seven non-structural proteins. The virion surface contains the envelope (E) and membrane (M) proteins, whereas the discrete nucleocapsid contains the entire viral RNA, which is packaged by the capsid protein (C) in a host-derived lipid bilayer (Kuhn *et al.*, 2002). In addition, the polyprotein contains the 7 non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. The polyprotein is depicted in Fig. 1.3 A.

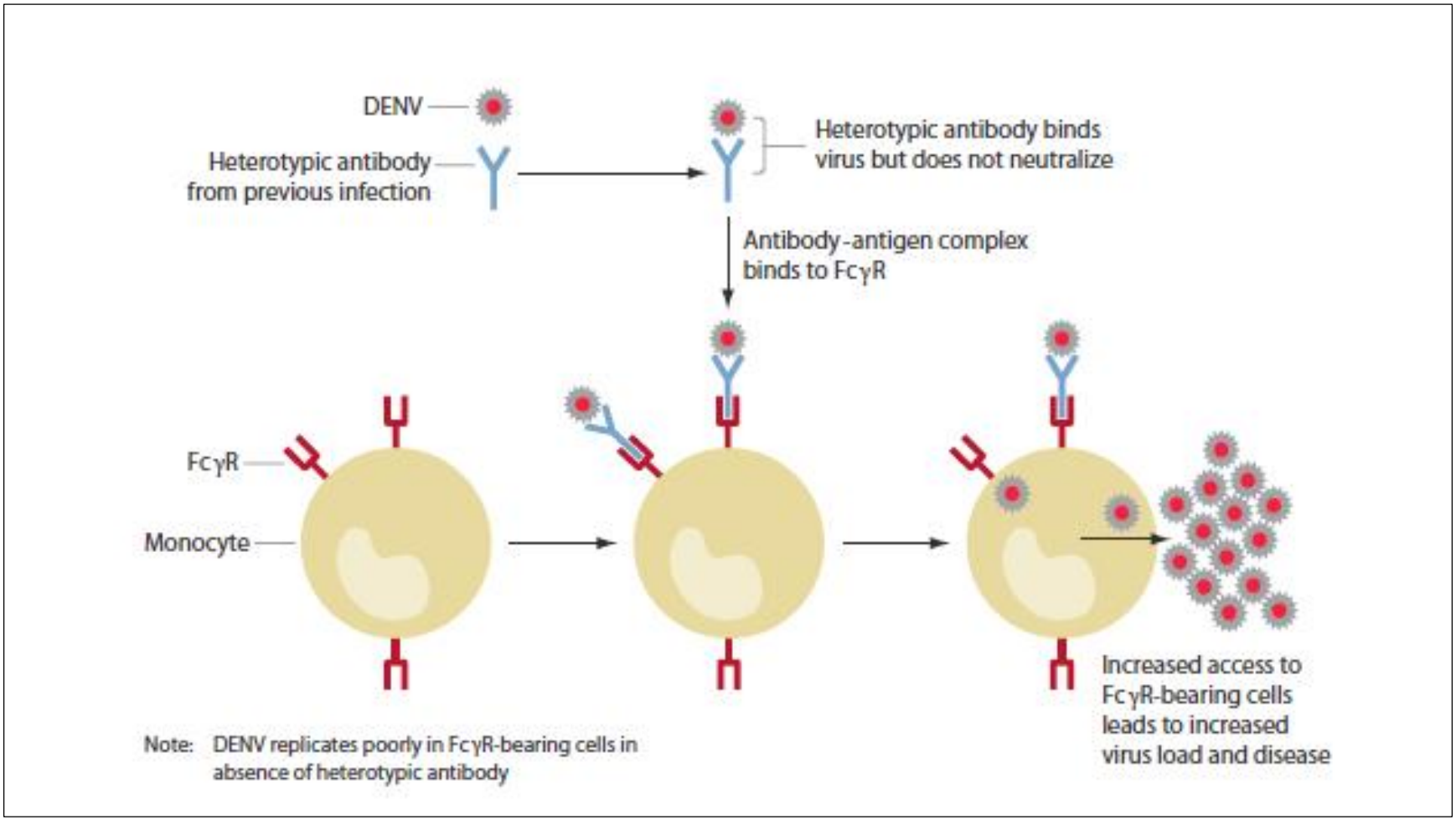


Fig. 1.3. Model for antibody-dependent enhancement (ADE) of dengue virus replication. The FcγR-bearing monocytes increase virus uptake in the presence of heterotypic antibody where as homotypic antibody do not increase the virus uptake (Murphy and Whitehead, 2011).

The non-structural proteins NS2 and NS5 induce good cytotoxic T-cell (CTL) responses and the E glycoprotein is the predominant antigenic site for antibody on the virus particles (Kurane *et al.*, 1989a). The surface of the mature virion is covered with approximately 180 copies of the E protein arranged an icosahedral scaffold of 90 herringbone orientated homodimers (Fig. 1.3 B) that lay extended and parallel to the host-cell-derived lipid bilayer (Crill and Roehrig, 2001). The E protein (495 aa) is characterized into three different domains along with a fusion peptide (Fig. 1.3. C) (Kuhn *et al.,* 2002; Lorenz *et al.*, 2002; Stadler *et al.*, 1997; Allison *et al.*, 2001). The M protein is a small proteolytic fragment of the precursor membrane protein (prM) and is produced during maturation of the viral particles in the secretory pathway. The C and prM proteins consist of 120 aa and 165 aa, respectively. Before it is cleaved during particle maturation to yield the pr peptide and the M protein (approximately 75 amino acids), the prM protein might function as a chaperone for folding and assembly of the E protein.

1.6.1 The Non-structural proteins

The major properties of the non-structural proteins (NS) are given in Table 1.4.

1.6.2 Structural proteins

1.6.2.1 Capsid protein (C)

The C protein is a small (12-14 kDa), basic, highly positively charged protein that forms the structural component of the nucleocapsid. The positive charge is due to a high proportion of lysine and arginine residues and helps to partially stabilize the negative charges of the RNA (Rice *et al.*, 1985). There is a hydrophobic stretch of uncharged amino acids in the middle of the C protein, which is conserved among all flaviviruses and functions as a signal peptide for ER translocation of prM (Rice *et al.,* 1985). This region is cleaved from the mature C protein (virion C) by the NS3/NS2B viral serine protease (Lobigs, 1993). The C protein folds into a compact dimer, with each monomer containing four alpha helices. It is not yet clear how the C protein dimers are organized within nucleocapsids, but interaction with RNA or DNA can induce isolated C protein dimers to assemble into nucleocapsid-like particles (Jones *et al.*, 2003).

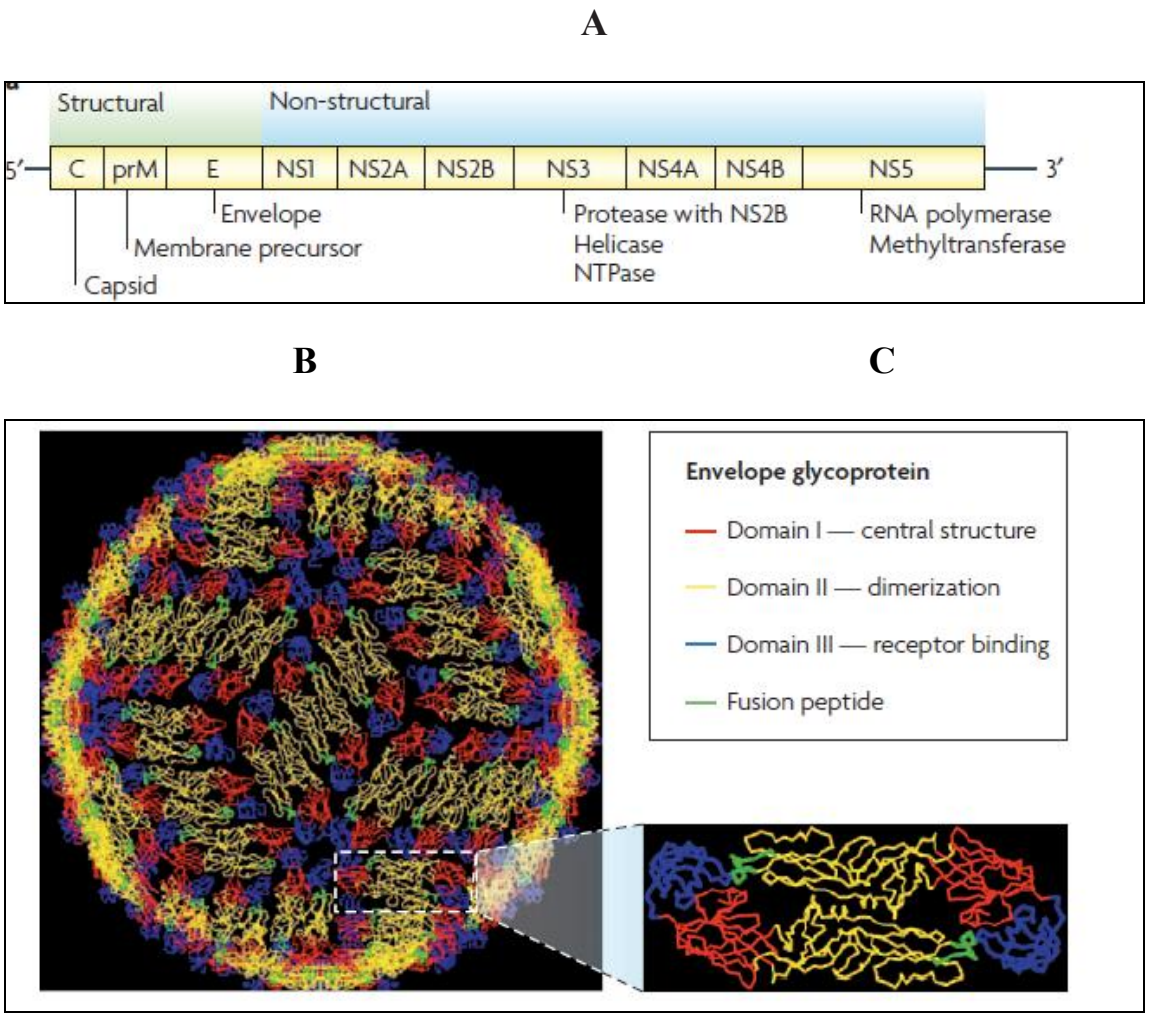


Fig 1.4. Structure and organization of the DENV genome. A) Single open reading frame is translated into a single polyprotein which is cleaved by proteases to produce the ten viral proteins: the C protein; the M protein, which is synthesized as the larger precursor protein prM; the major E glycoprotein; and seven non-structural (NS) proteins involved in viral replication. B) Arrangement of E protein on DENV surface. C) Domains I, II and III of E protein along with fusion peptide (Kuhn *et al.,* 2002; Whitehead *et al.,* 2007).

Table 1.3. Properties and major functions of the non-structural proteins

Protein	Size	Functions	References
NS1	46 kDa	• Viral RNA replication	Rice *et al.* (1985), Winkler *et al.* (1989), Lindenbach and Rice (2003)
NS2A	25 kDa	• Viral RNA replication and virus assembly • Interferon antagonist	Chambers *et al.* (1989), Mackenzie *et al.* (1998), Kummerer and Rice (2002), Leung *et al.* (2008)
NS2B	14 kDa	• Cofactor in catalytic activity of NS2B-NS3 serine protease	Clum *et al.* (1997), Falgout *et al.* (1991), Zuo *et al.* (2009)
NS3	68-70 kDa	• Viral polyprotein processing and RNA replication • Acts as a helicase for RNA unwinding • Major target of a CTL mediated immune response	Rice *et al.* (1986), Wengler and Wengler (1991), Shafee and Abubakar (2003), Yang *et al.* (2009)
NS4A/2K	150 aa	• RNA replication through a direct interaction with NS1 • Cofactor for NS3 helicase performance	Lindenbach and Rice (1999), Miller *et al.* (2007)
NS4B	248 aa	• Viral replication complex due to its co-localization along with NS3 • Enhancing helicase activity of NS3 • Interferon antagonist	Miller *et al.* (2006), Umareddy *et al.* (2006), (Munoz-Jordan *et al.*, 2003)
NS5	103-104 kDa	• 5’ capping and replication containing domains for viral RNA-dependent RNA polymerase (RdRp) • Interferon antagonist	Koonin (1993), Ackermann and Padmanabhan (2001), Best *et al.* (2005)

1.6.2.2 Membrane Protein (M)

The prM protein (18-19 kDa) is the precursor glycoprotein to the M protein (8 kDa) in all flaviviruses. This precursor undergoes a delayed cleavage to form M and the secretary N-terminal pr segment. The precursor contains one to three N-linked glycosylation sites at the N terminal region, one at Asn69 (Chambers *et al.*, 1990), and six conserved cysteine residues, all of which contribute to disulfide bridging (Nowak and Wengler, 1987). M and prM are found on extracellular and intracellular virions, respectively. Hence, the cleavage is linked to viral budding/maturation in order to prevent immature virions from fusing with host-cell membranes (Li *et al.*, 2008). The transmembrane domains of prM and E act as ER retention signals and may assist in heterodimer formation (Lin and Wu, 2005). Proteolytic cleavage of prM in prM/E heterodimers releases the pr segment and forms E homodimers (Stiasny *et al.*, 1996; Wengler and Wengler, 1989). Studies have shown the inhibitory effects of weak bases and lysosotrophic amines on prM cleavage, where prM plays an important role in maintaining the conformation of the E protein during virus passage through acidified sorting compartments (Randolph *et al.,* 1990), since flaviviruses are generally inactivated at low pH.

1.6.2.3 Envelope protein (E)

The E protein is the major structural protein (a type I membrane protein) on the surface of the virion. It is glycosylated in most flaviviruses and possesses a molecular mass of 55-60 kDa (Lindenbach and Rice, 2001). Unlike most enveloped viruses (such as influenza virus and HIV) that have protein spikes protruding from their surface, the E proteins are horizontally positioned on the flavivirus surface. The E protein is responsible for receptor-mediated attachment of virus to host cells and low pH-mediated fusion of virus to host cell membranes. It also displays the virus hemagglutination activity and is a major target for virus neutralizing antibodies (Allison *et al.*, 2001; Chambers *et al.*, 1990; Heinz and Allison, 2000). There are 12 completely conserved cysteine residues in E, and they have all been demonstrated to contribute to 6 intramolecular disulfide bridges in WNV (Nowak and Wengler, 1987; Roehrig *et al.*, 2004).

From an exterior view of the DENV virion, the E protein is packed in an icosahedral lattice covering the entire surface of the virus (Kuhn *et al.,* 2002; Zhang *et al.,* 2003). The surface is composed of three sets of nearly parallel E dimers forming a herringbone-like pattern (Kuhn *et al.*, 2002). In a dimer, the monomeric subunits are positioned anti-parallel to each other in a head-to-tail fashion (Kuhn *et al.*, 2002; Modis *et al.*, 2003; Zhang *et al.*, 2004). Studies on the atomic structure of TBEV, DENV and WNV used the membrane anchor-free ectodomain of the E protein (Modis *et al.,* 2003; Nybakken *et al.*, 2006; Rey *et al.*, 1995). These soluble E proteins (sE) were produced as recombinant proteins in *Drosophila* cells (DENV) or isolated from purified virions (TBEV) and Hi-5 cells (WNV) and were used in X-ray crystallography studies. The structural features of these proteins are identical though they shared only a 37-44% amino acid sequence homology (Modis *et al.*, 2003; Nybakken *et al.*, 2006; Rey *et al.*, 1995). It is thought that this structural homology applies to all flaviviruses E proteins (Nybakken *et al.*, 2006).

The X-ray crystallographic structure of the ectodomain (residues 1–395) of dimeric E proteins of DENV-2 and -3 has been determined and reported (Zhang *et al.*, 2004; Modis *et al.*, 2003). In a dimer of the E protein, each monomer has three β-barrel domains. The domain I (EDI) is the central structure flanked at one side by an elongated domain II (EDII: dimerization domain) that is fused with a fusion peptide at its distal end. On the other side, domain III (EDIII) is found; an immunoglobulin (Ig)-like domain that is reported to have the putative receptor binding sites (Hung, 1999; Crill and Roehrig, 2001; Kuhn *et al.*, 2002). The three DENV E protein domains are depicted in Fig. 1.4. EDI and EDII are connected by four polypeptide chains, whereas EDI and EDIII are connected by a single polypeptide. In an E dimer, the fusion peptides from each monomer are buried between EDI and EDIII of the adjacent monomer. Cryo-electron microscopy of purified DENV-2 showed a smooth outer surface of the virion particle (Kuhn *et al.*, 2002), where the E protein is arranged parallel to the lipid bilayer. Through this smooth viral surface, EDIII extends outwards which helps the virus to bind with the host cell's receptors.

EDI is a discontinuous structure located at the centre of the E protein monomer containing 120 amino acids in three distinct regions (residues 1-52, 132-192, and 280-295). EDI folds into an 8-stranded up and down β-barrel that forms two β-sheets (β sheet 1: $A_0C_0D_0E_0F_0$; β sheet 2: $B_0I_0H_0G_0$), which face each other across a tightly

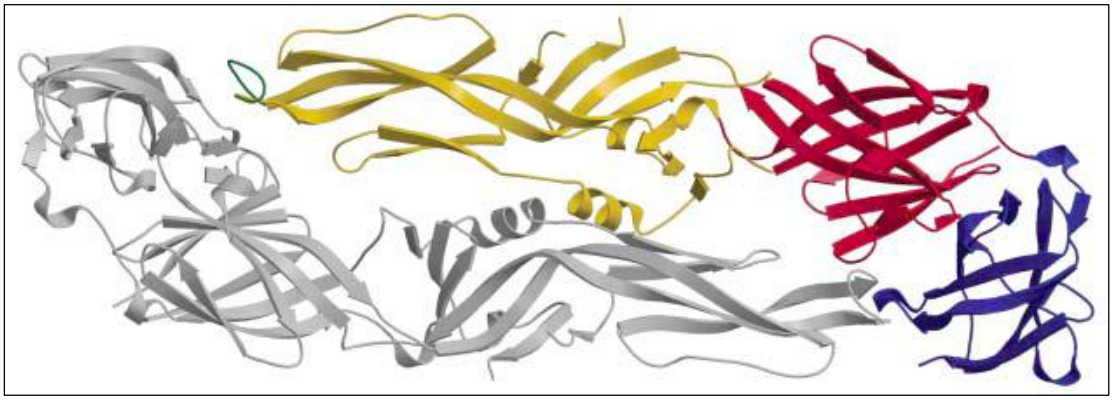


Fig. 1.5. Dimeric, pre-fusion conformation of the DENV-2 E protein. This schematic depicts the dimeric pre-fusion conformation of the DENV-2 E protein residues 1–395. The domains I, II and III are coloured red, yellow and blue, respectively, in one monomer, and the fusion peptide is shown in green. The other monomer is coloured grey (Zhang *et al.,* 2004).

packed hydrophobic interior (Rey *et al.,* 1995). EDI is connected to EDII via four peptide strands, which contain a molecular hinge region that facilitates molecular conformational changes during membrane fusion (Modis *et al.*, 2003; Rey *et al.*, 1995). It contains two disulfide bridges joining cysteine residues 3-30 and 186-290 along with a unique N-linked glycosylation site (N-X-T/S, where X = any amino acid) which carries a single carbohydrate side chain attached to the E_0F_0 loop on the external surface of the protein (Rey *et al.*, 1995). In addition, EDI acts as a flexible hinge region that is important in fusion. Monoclonal antibodies (MAbs) targeted to EDI have been reported to block the domains biological function and changes the antigenic specificity (Guirakhoo *et al.*, 1989; Roehrig *et al.*, 1998).

EDII is discontinuous, connected to EDI via the two loop hinge region and is recognized as the dimerization domain (Figure 1.6). The base of the domain contains five short strands of antiparallel β-sheet with two α-helices packed against one surface (αA and αB). It contains two segments (residues 53-131, and 193-279) along with three disulfide bridges. EDII is an elongated finger-like structure, it has a three-stranded β-sheet (Rey *et al.*, 1995) and the flavivirus conserved fusion peptide (cd-loop, residues 98-111). The three-stranded β-sheet is cross-linked by disulphide bridges, which helps with the stability of "cd-loop" at the tip of EDII (Rey *et al.,* 1995). The fusion peptide sequence DRGWGNGCGLFGGK is highly conserved amongst the flaviviruses and is necessary for membrane fusion (Rey *et al.*, 1995). In TBEV, the primary neutralization sites were reported to be exposed in the dimeric state when the cd-loop lay in a hydrophobic crevice of the E protein surrounded by hydrophilic epitopes (Rey *et al.*, 1995). This region also helps the attachment of E ectodomains to target membranes (Allison *et al.*, 2001). Studies have shown that conformational changes of E protein following low pH treatment nullified the binding ability of MAbs leading to viral mediated fusion. However, MAbs targeted to EDII at pH 6.0 can still facilitate neutralizing and anti-hemagglutination activity, thus demonstrating the flavivirus group-specific reactivity (Guirakhoo *et al.*, 1989; Roehrig *et al.*, 1998).

EDIII (residues 303-395) is located at the carboxy-terminal of the soluble E protein and has one stabilizing disulfide bond. It possesses an immunoglobulin-like (Ig) β-barrel structure connected to EDI by a single fifteen residue linker peptide (Rey *et al.,* 1995). The orientation of EDIII is different when compared to EDI and EDIl, where EDIII is perpendicular to the surface of the virus leaving its tip slightly projected away

from the other part of the E dimer. This orientation, along with the help of the linker peptide, might facilitate the movement of EDIII with respect to the rest of the molecule (Rey *et al.*, 1995). In addition, the projection of EDIll from viral surface possibly plays a role in viral attachment to the host cell receptors (Nybakken *et al.*, 2006; Rey *et al.*, 1995; Zhang *et al.*, 2004). Antibodies with high neutralizing activity have been mapped to EDIII and soluble EDIII has been used to block infection of cells with whole virus, both suggesting EDIII contains receptor-ligand epitopes and that the antibodies generated against EDIII are the most effective at preventing attachment of DENV to host cells (Abd-Jamil *et al.*, 2008; Chin *et al.*, 2007; Crill and Roehrig, 2001; Roehrig *et al.,* 1998). It has also been shown that residues E380-E389 are important in the DENV serotype-specific binding of C6/36 cells but not mammalian cells suggesting that domain III binds mainly to cell surface heparan sulfates (Hung, 2004).

1.7 Epitope mapping

The identification of epitopes involved in antibody-mediated neutralization of dengue infection has contributed greatly to our increasing understanding of disease pathogenesis and potential vaccine development. Epitopes can be classified into continuous and discontinuous epitopes (Barlow *et al.*, 1986). Continuous epitopes, also called as linear epitopes, are short peptides widely known to consist of 3-8 amino acid residues representing continuously on the primary structure of the protein sequence. Discontinuous epitopes, also known as conformational epitopes, are formed from more than 10 residues that are discrete in the primary sequence but assemble to form an antigenic determinant on the tertiary structure of the native protein (Barlow *et al.*, 1986; Laver *et al.*, 1990). Several methods for the identification of epitopes in DENV have been used, such as neutralization escape mutants, competition assays, phage display, peptide scan and computer-based epitope prediction. Antigenic epitopes of dengue virus serotypes have been reported both in structural and non-structural proteins with the majority of epitopes being found on E glycoprotein. A brief list of different methods used in epitope mapping of DENV is given in Table 1.5.

Neutralization escape mutants of viruses arise under selection pressure of neutralizing MAbs. To generate these mutants, cells are incubated with virus in the presence of neutralizing MAbs and the viral subpopulation that escaped neutralization replicate in the cells. After isolation of this subpopulation, amino acid changes can be

Table 1.4. Different techniques used for epitope mapping on DENV

Author	Virus serotype	Target protein	Mapping technique	Antibodies used	Immunogenic regions identified
Aaskov *et al.* (1989)	DENV-2	E	Pepscan, overlapping octapeptides, ELISA	Dengue immune antisera from human and rabbit MAb 1B7	Rabbit: aa 1-58, 59-297, 288-391, 392-442, 446-476, 479-495 aa 50-57, 127-134, 349-356
Innis *et al.* (1989)	DENV-2	E	Pepscan, overlapping hexapeptides, ELISA	Convalescent antisera from 7 dengue patients	22 peptides
Falconar (1999)	DENV-2	E	Pepscan, overlapping nona/decapeptides	MAbs	aa 274-283, 349-359
Henchal *et al.* (1985)	DENV-2	E	Competitive binding	MAbs	4 antigenic regions
Roehrig *et al.* (1998)	DENV-2	E	Competitive binding	MAbs	3 antigenic regions
Roehrig *et al.* (1998)	DENV-2	E	Synthetic peptides, ELISA	MAbs	aa 333-351
Falconar (2008)	DENV-2	E	Synthetic peptides, ELISA	MAbs	aa 304-313, 393-401
da Silva *et al.* (2009)	DENV-3	E	Overlapping synthetic peptides, ELISA, *in silico* epitope prediction	Immunized mice sera	aa 51-65, 131-170, 196-210, 246-260

Table 1.4. Continued.

Author	Virus serotype	Target protein	Mapping technique	Antibodies used	Immunogenic regions identified
Amexis and Young (2007)	DENV-2	E	*in silico* epitope prediction, Multiple antigenic peptides (MAPs)	Immunized mice sera	aa 80-99, 238-250, 295-307, 304-316, 333-351, 352-368, 386-397
Sanchez-Burgos *et al.* (2010)	All serotypes	E	*in silico* epitope prediction, Synthetic peptides	Immunized mice sera	aa 421-429
Li *et al.* (2011)	DENV-2	E	*in silico* epitope prediction, Synthetic peptides, ELISA	Immunized mice sera	aa 345-359, 383-397
Mason (1990)	DENV-1	E	Fusion proteins	MAbs	aa 293-403, 76-93, 298-403
Trirawatanapong *et al.* (1992)	DENV-2	E	Fusion proteins	MAbs Mouse ascites	aa 386-397 aa 386-397
Thullier *et al.* (2001)	DENV-1	E	Phage display	MAbs	aa 306-314
Beasley and Aaskov (2001)	DENV-1	E	Neutralization escape mutants	MAbs	aa 279 (Phe-Ser), 293 (Thr-Ile)
Lin *et al.* (1994)	DENV-2	E	Neutralization escape mutants	MAbs	aa 307 (Lys-Glu)

Table 1.4. Continued.

Author	Virus serotype	Target protein	Mapping technique	Antibodies used	Immunogenic regions identified
Lok *et al.* (2001)	DENV-2	E	Neutralization escape mutants	MAbs	aa 69 (Thr-Iso), 311 (Glu-Gly)
Serafin and Aaskov (2001)	DENV-2 DENV-3	E	Neutralization escape mutants	MAbs	aa 169 (Ser-Pro), 275 (Gly-Arg) aa 386 (Lys-Asn)
Lin (2012)	DENV-1	prM/E	Alanine-substitution mutants	MAbs and DENV immune polyclonal human sera	aa Q211, D215, P217
Vazquez *et al.* (2002)	DENV-2	M	*in silico* epitope prediction, Synthetic peptides	Immunized mice sera	aa 3-31, 103-124
Wu *et al.* (2001)	DENV-1	NS1	Phage display	Dengue immune antisera from human and rabbits	aa 110-117
Anandarao *et al.* (2005)	DENV-2	C, NS4A	Multi-pin peptide synthesis technology, ELISA	Dengue immune human sera	37 peptides

identified by DNA sequencing. The sequence differences are predicted to be important for the epitope of the neutralizing MAbs (Beasley and Aaskov, 2001; Serafin and Aaskov, 2001). The competition assay is used to determine whether antibodies are directed against the same or nearby epitopes. Inhibition of binding of one antibody by another may occur as a result of conformational changes of the antigen following antibody binding. Furthermore, binding of the first antibody can sterically prevent binding of the other antibody to nearby epitopes (Heinz *et al.*, 1983; Henchal *et al.*, 1985).

Phage display is a technique which involves expressing peptides as fusion proteins on the surface of bacteriophages (Thullier *et al*., 2001). Generally, this technique is used for the identification of peptides that bind to receptors for the determination of substrates or inhibitors of enzymes and for epitope mapping. The bound peptides are selected by an affinity selection technique called biopanning (Wu *et al*., 2001). The recovery of specifically bound phages is mediated by acid elution and the insert region of the phage genome can be sequenced (Abd-Jamil *et al*., 2008; Amin *et al.*, 2009). Peptide scan is a widely used method for epitope mapping. This approach involves the synthesis of multiple peptides on polystyrene pins that are attached to a plastic support. The amino acid sequence of the antigen is required for the generation of the peptide library. Generally, the peptides are overlapping and are 12 to 15 amino acids in length. The binding of the respective antibody to the synthetic peptides can be tested in enzyme linked immunosorbent assay (ELISA) (Falconar, 1999; Amexis and Young, 2007; da Silva *et al*., 2009).

Immunomics is the field of specific "omics" science for the study of epitopes for production of new vaccines. The basic tools on genomics and proteomics can provide many new data to the scientific community. The identification of linear B-cell epitopes has been based on the physiochemical properties of the amino acids such as hydrophilicity, antigenicity and flexibility (Kyte and Doolittle, 1982; Parker *et al.*, 1986). In 2006, introduction of a systematic bioinformatics approach with more appropriate algorithms such as the Hidden Markov Model (HMM) (Larsen *et al.*, 2006) and the Artificial Neural Network (ANN) models (Saha and Raghava, 2006) improved the accuracy of B-cell epitope prediction and a number of unique protein sequences required to represent complete antigenic diversity of short peptides in dengue virus have been reported (Khan *et al.*, 2006). Computational analysis of dengue E protein revealed

several epitopes suggesting that these epitope regions may be potential targets for development of dengue vaccines (Amexis and Young, 2007; Sanchez-Burgos *et al*., 2010; Li *et al.,* 2011).

Proteolytic footprinting methods of antigen-antibody complexes have been increasingly used in epitope mapping for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (Jeyarajah *et al.*, 1998; Parker and Tomer, 2002; Grollo *et al.*, 2006). Proteolytic footprinting is based on the protection of residues in the antigen that are involved in affinity binding against proteolysis or chemical modification and the high resistance of the antibody to proteolytic digestion (Davies and Cohen, 1996). The protein of interest is complexed with the antibody and then undergoes proteolytic cleavage by endopreoteinases such as carboxypeptidase Y, aminopeptidase M, Lysyl endopeptidase, or restriction factor Xa. This approach relies on the fact that antigenic regions within a protein that are bound to an antibody are more resistant to proteolytic cleavage compared to other unbound/exposed regions of the protein. Following treatment with a range of endopeptidases, mass spectrometric analysis of cleaved, acid-eluted peptides that are bound to antibodies in either liquid or solid phase will identify the corresponding epitope sequence (Parker and Tomer, 2002).

1.7.1 Immunogenicity of E protein

The E protein is found to be the major target where most of the antibody antigenic determinants have been identified so far. It elicits strong immune responses and stimulates production of neutralizing antibodies that can inhibit virus attachment to cells (Gratz, 2004). MAb mapping data identified three antigenic domains, C, A and B, that correspond to the three structural domains, EDI, EDII and EDIII, of the E protein, respectively (Guirakhoo *et al*., 1989; Roehrig *et al*., 1998; Rey *et al*., 1995). EDI comprises predominantly of serotype-specific non-neutralizing epitopes, however, neutralizing epitopes have been found to be clustered at the hinge region between EDI and EDII (fusion protein). EDIII governs structural rearrangements as immature virus is processed into mature infectious virion particle at acidic pH (Zhang *et al*., 2004). The antigenic classes of flaviviruses are mainly serotype-specific, complex cross-reactive and flavivirus group cross-reactive (Clarke, 1960). Diagnostic serology of flaviviruses has shown that the E protein plays a major role in hemagglutination inhibition (HI), complement fixation and virus neutralization (Cardiff *et al.*, 1971; Hammon and Price,

1966; Qureshi and Trent, 1973).

Murine MAbs have been widely used for dissection of the antigenic region within the E protein. MAbs can be classified into four categories based on their reactivity to DENV and other flaviviruses; (1) flavivirus group-specific antibodies which recognize multiple viruses in the genus Flavivirus, (2) dengue complex-specific antibodies which recognize all four serotypes of DENV, (3) dengue subcomplex-specific antibodies which recognize some but not all DENV serotypes, and (4) dengue type-specific antibodies which recognize only one serotype of DENV (Henchal *et al*., 1985). MAbs mapping to EDI (antigenic domain C) are more variable in their HI and neutralization activity and are mostly subtype-specific (Guirakhoo *et al*., 1989). MAbs directed against EDII (antigenic domain A) are HI active, non-neutralizing and flavivirus group cross-reactive. MAbs targeting EDIII (antigenic domain B) are HI active, neutralizing and contained complex cross-reactive as well as serotype specific epitopes (Heinz *et al*., 1983).

The antigenic domain A (EDII) displays the fusion peptide region and antibodies directed against this domain are able to block pH-dependent virus-mediated membrane fusion in endosomal compartments (Roehrig *et al*., 1998). The highly conserved nature of the fusion peptide sequences make it an important antigenic epitope determinant (Allison *et al*., 2001; Crill and Chang, 2004; Goncalvez *et al.*, 2004; Oliphant *et al.*, 2006). However, these fusion peptides were reportedly bound by the MAbs in a low pH environment. A study employing DENV-2 and anti-peptide antibodies targeted to fusion peptide region showed that the MAbs recognized the region in a structurally specific manner and bound more efficiently with low-pH treated virus than the neutral pH treated virus. Studies using DENV-infected human polyclonal sera have shown that antibodies targeting the fusion peptide are cross-reactive and non-neutralizing towards the heterologous serotypes with a highly variable proportion of the antibody response (Crill *et al.*, 2009; Lai *et al.*, 2008; Throsby *et al.*, 2006).

The antigenic domain B (EDIII) is responsible for host cell attachment and contains complex cross-reactive, receptor-ligand epitopes as well as serotype-specific protective epitopes. It has been extensively reported that murine MAbs targeting EDIII identified several neutralizing epitopes, both serotype-specific and serocomplex-specific. Potential neutralizing epitopes have been identified in a truncated DENV-2 EDIII (aa 386-397) by employing serotype-specific MAbs (Trirawatanapong *et al*.,

1992). DENV-2 serotype-specific neutralizing MAbs have identified the conserved epitopes on residues K305, P384 (Gromowski, 2007), E383 and P384 of EDIII (Sukupolvi-Petty *et al.*, 2007). In addition, serotype-specific, conserved DENV-1 epitopes on EDIII have also been reported to reside in the residues 307-312, 387, 389 and 391 (Lisova *et al.*, 2007). However, studies on EDIII antibody depleted human sera from DENV infected patients suggest that antibodies against EDIII may play a smaller role in total DENV neutralization as the EDIII depleted sera retained a relatively high neutralization titer (Wahala *et al.*, 2009). EDIII also elicits serocomplex cross-reactive neutralizing antibodies of DENV (Crill *et al*., 2009; Gromowski *et al.*, 2008; Matsui *et al.*, 2009; Rajamanonmani *et al.*, 2009; Sukupolvi-Petty *et al*., 2007).

The antigenic domain C helps in viral fusion to the host cell but a detailed antigenic mapping study of EDI is not yet completed. The first ever study on EDI epitopes involved in neutralization was reported in DENV-1 (Beasley and Aaskov, 2001). There were 3 MAbs identified in this study by using DENV-1 neutralization-resistant mutants, D1-M10 and D1-M17, which had single amino acid substitutions at E279 (Phe-Ser) and E293 (Thr-Ile), respectively. All three neutralizing MAbs reacted with spatially related epitopes on the E protein of dengue 1, which were also recognized by antibodies in sera from dengue patients. A similar study with DENV-4 neutralization escape mutants identified residues 174 and 176 in EDI as important serotype-specific neutralizing epitopes (Lai *et al.*, 2007).

Alternatively, DNA shuffling and screening technology has been used to develop a single recombinant dengue E antigen capable of inducing neutralizing antibodies against all four antigenically distinct dengue serotypes. The chimeric antigens protected mice against a lethal DENV-2 virus challenge suggesting that DNA shuffling and associated screening can lead to the selection of multi-epitope antigens against closely related dengue virus serotypes (Apt *et al.*, 2006). Recombinant flavivirus E proteins produced using different expression systems such as *E. coli*, vaccinia and baculoviruses (Delenda *et al.*, 1994; Deubel *et al*., 1990; Mason, 1990) have elicited variable degrees of protective immunity in animal models suggesting that specific vaccine targets may be uncovered within the E protein.

1.7.2 Epitopes on other structural and non-structural proteins

The NS1 protein is an important target of antibodies against DENV. NS1 is expressed on the surface of infected cells and is also secreted into the circulation as a soluble multimer (Rothman, 2004). B-cell epitopes of NS1 glycoprotein and anti-NS1 antibody responses following DENV-2 infection were identified using a series of 15-mer synthetic peptides from the predicted B-cell linear epitopes of DENV-2 NS1. Testing these peptides against from sera of dengue patients using ELISA showed one positive peptide from DENV-2 NS1 (amino acids 1–15) as the immunodominant epitope (Huang *et al.*, 1999). Simultaneously, identification of a DENV-1 serotype-specific B-cell epitope of NS1 using a random peptide library showed that the epitopes reacted with a high degree of specificity with serum samples obtained from both DENV-1-infected rabbits and patients. The study suggested that the DENV-1 epitope-based serologic tests could be useful in laboratory diagnosis and in understanding the pathogenesis of DENV-1 (Wu *et al*., 2001). However, the presence of high levels of secreted NS1 in the sera of patients experiencing secondary DENV infections suggests that NS1 may contribute significantly to the formation of the circulating immune complexes that are suspected to play an important role in the pathogenesis of severe dengue disease (Young *et al.*, 2000).

B-cell epitopes on two small DENV proteins, C and NS4a, were identified using a multi-pin peptide synthesis strategy. Several linear, immunodominant epitopes on both these proteins have been identified and almost all these epitopes mapped to regions predicted to be hydrophilic in nature. This study suggested that the immunodominant epitopes of these two dengue proteins might have the potential to be used as a part of a recombinant multi-epitope protein containing carefully chosen E and NS1 epitopes for the detection of dengue infections with a high degree of sensitivity and specificity (Anandarao *et al*., 2005). In addition, the antibody response of five synthetic peptides from the prM protein of DENV-2 was evaluated and two of them elicited neutralizing antibodies against all four DENV serotypes suggesting the role of synthetic peptides from pr and M antigens in the development of anti-flaviviral vaccines (Vazquez *et al*., 2002).

1.8 Dengue vaccine development

In recent decades, the incidence of dengue has been reported widely, making it a global health concern. The WHO reports that two-fifths of the world's population is at risk of dengue infection, with an increase in the annual number of cases, however, no licensed vaccine is currently available (WHO, 2012). Development of a vaccine targeted against all four serotypes of dengue virus has been hampered by the potential complications following secondary infection (Murrell *et al.*, 2011). A key element in protection from dengue fever appears to be the antibody-mediated immune responses. However, many of the antibodies generated are cross-reactive but cannot neutralise the virus and, therefore, may lead to ADE. Thus, the immunogenicity induced by the vaccine should be such that the level of neutralizing antibodies produced is high enough to provide complete protection against all four serotypes. Development of a safe and effective vaccine against a disease with such strong immunological complications poses considerable challenges. In addition, a greater understanding of dengue pathogenesis is crucial in order to develop a successful dengue vaccine (Murrell *et al*., 2011).

The most effective way to test the basic immunology of dengue infections is to use animal models. Mice are most commonly used as an animal model before testing in non-human primates. However, this has proven to be an obstacle as wild-type mice are resistant to dengue-induced diseases. The difficulty seems to lie in developing a mouse model in which human viral isolates of DENV strains are able to replicate well and in which the model mice can develop signs of human DENV-infection. This has led to the development of a variety of different mouse models, including intercerebral infection, chimeric mice transplanted with human cells, immunocompromised mice and immunocompetent mice (Shresta *et al*., 2006; Yauch and Shresta, 2008). Additionally, non-human primate models have been shown to be the most appropriate for human vaccine development (Onlamoon *et al.*, 2010). Mouse models as well as non-human primates are essential to test the efficacy and safety of potential vaccine candidates before them using in human clinical trials.

Conventional vaccines have played a major role in combating flavivirus diseases, such as yellow fever, Japanese encephalitis and tick-borne encephalitis. These have provided hope that a safe and effective dengue vaccine could be developed (Stephenson, 2005). So far, strategies to develop a DENV vaccine have focused mainly on live attenuated virus vaccines, chimeric vaccines, inactivated virus vaccines, DNA

vaccines and recombinant subunit protein vaccines (Durbin, 2011). A comprehensive list of these vaccine constructs can be seen in Table 1.6.

1.8.1 Live attenuated vaccines (LAV)

Live attenuated vaccines are among the most rigorously followed methods for DENV vaccination. LAV comprise of an avirulent form of a live virus that elicits antibodies to both the structural and non-structural proteins of the dengue virus. In addition, LAV also induces cellular immunity. LAV tend to mimic the natural infection and induce long lasting humoral and cellular responses, often from a single dose of vaccine. Attenuation of dengue virus was first achieved by Sabin in 1945 by intracerebral passaging of DENV-1 or DENV-2 in mouse brain (Sabin, 1952). The degree of attenuation varied between strains leading to development of rashes in humans and an alternative method was proposed to propagate and attenuate the dengue virus by serial dilutions in primary dog kidney (PDK) cells (Halstead and Marchette, 2003). This study demonstrated a moderate reactogenicity and high seroconversion rates (89%). However, reactogenicity was highest after the first dose but not after subsequent doses. Results from a small study alleviated the fear that pre-existing dengue antibodies induced by live-attenuated dengue vaccine could result in more severe disease after natural exposure.

The potential for developing vaccines using live attenuated strains of all four serotypes has been widely accepted, considering this method was successful in YFV (Xie *et al.*, 1998). However, the main problems related to live vaccines are; (i) reversion to wild-type strains, (ii) mutations which lead to other virulent forms of the virus, (iii) spread of vaccine strains to non-vaccinated people; and, (iv) development of disease in immunocompromised individuals (Seligman and Gould, 2004). Managing viral interference and balancing attenuation, in order to produce acceptable tetravalent immunogenicity with minimal reactogenicity, is another challenge for live vaccines (Kitchener *et al.*, 2006).

During the 1980s, the Center for Vaccine Development at Mahidol University in Bangkok, Thailand, and the Walter Reed Army Institute of Research (WRAIR) in Washington, DC, started developing live attenuated DENV vaccines via tissue culture derived methods (Bhamarapravati and Sutee, 2000). The original viruses were isolated from DENV-infected patients and serially passaged in PDK cells (DENV-1, DENV-2

Table 1.5. Candidate dengue vaccines in development (Adopted from Webster *et al.* 2009; Durbin and Whitehead, 2011)

Vaccines	Details	Phase of clinical trial	Comment
Chimeric			
ChimeriVax (Acambis/Sanofi Pasteur)	Recombinant infectious cDNA clone of yellow fever 17D vaccine strain as a backbone, substituting membrane precursor protein and envelope protein genes with those of dengue viruses	3	Leading candidate; safe and immunogenic in human trials
Live attenuated			
Mahidol University (Sanofi Pasteur)	Passage in primary cell culture	2*	Monovalent vaccines show good immune responses; difficulties with tetravalent formulations
WRAIR (GSK)	Passage in primary cell culture	2	Monovalent vaccines show good immune responses; difficulties with tetravalent formulations
Infectious clone			
rDEN4Δ30 (NIAID)	30 nucleotide deletion from DENV4 3' untranslated region as genetic backbone for vaccines with structural genes from other serotypes	1	Monovalent vaccines show promise; tetravalent formulations to be evaluated
rDEN4Δ30-200,201 (NIAID)	Further mutation in rDEN4Δ30 construct	1	Retained immunogenicity of rDEN4Δ30, but with improved safety profile

- Development suspended

Table 1.5. Continued.

Vaccines	Details	Phase of clinical trial	Comment
Inactivated			
WRAIR	Whole purified inactivated virus	Preclinical	Safe and immunogenic in rhesus macaques with evidence of efficacy
Replication-incompetent			
RepliVax (Novartis)	Capsid gene-deleted WNV with membrane precursor and envelope protein genes substituted for dengue genes	Preclinical	Immunogenicity and efficacy shown in mice
Protein			
r80E (Hawaii Biotechnology)	Amino-terminal 80% of the DENV-2 envelope with adjuvants	Phase 1 completed	Monovalent vaccines show promise; tetravalent vaccines to be evaluated
cEDIII (IPK/CIGB)	Consensus dengue virus envelope protein domain III of all four serotypes	Preclinical	Immunogenic in mice
DNA			
US Navy	Several encoding membrane precursor protein and envelope protein genes	Preclinical	Immunogenic with very short lived protection
Virus vector			
Adenovirus (GenPhar Inc)	Tetravalent formulation combining two bivalent adenovirus constructs	Preclinical	Neutralising antibody, short and long-term protection against challenge from each serotype in rhesus macaques
Measles virus (CNRS)	Expression of a DENV1 antigen by a vector derived from live attenuated Schwarz measles vaccine	Preclinical	Long-term production of dengue neutralising antibody in mice

and DENV-4) or primary green monkey kidney (PGMK) cells (DENV-3). These LAV candidates were tested as monovalent, bivalent, trivalent and tetravalent formulations in adult flavivirus-naive Thai and American volunteers. Monovalent, bivalent and trivalent formulations using DENV-1, -2 and -4 vaccine candidates were found to be generally safe, with fever, rash and elevated liver enzymes being the most common side effects. The vaccines elicited seroconversion rates between 90 and 100%. However, the combined tetravalent vaccine resulted in a predominant response to the DENV-3 serotype (Kanesa-Thasan *et al.*, 2001). Reverse transcriptase-polymerase chain reaction (RT-PCR) assays performed with sera from volunteers challenged with tetravalent vaccines indicated extensive viremia with DENV-3. It was postulated that the preferential replication of DENV-3 observed in tetravalent vaccines might have been due to competitive interference between the four attenuated DENV serotypes (Kanesa-Thasan *et al*., 2001). The vaccine was reformulated using lower doses of DENV-3 and, though the immunogenicity of the vaccine appeared promising, further clinical development of this vaccine was put on hold because of unacceptable reactogenicity caused by the DENV-3 component (Webster *et al*., 2009).

WRAIR has developed several live attenuated DENV vaccine candidates through serial PDK passage. However, several of these candidates were found to be unacceptably reactogenic in human trials, or over attenuated and non-immunogenic, hence they were no longer continued (Bancroft *et al.*, 1984; Eckels *et al.*, 1984; Innis *et al.*, 1988). Increasing PDK cell passage of monovalent DENV vaccine candidates resulted in increased attenuation for volunteers, but decreased immunogenicity, leading to the difficulty of selecting appropriate attenuated serotype viruses for tetravalent formulations (Kanesa-Thasan *et al.*, 2003). These studies indicated that each of the four components of the vaccine must induce a balanced neutralizing antibody response and coadministration of four live DENV serotypes is associated with competition among serotypes, with respect to replication and the ability to stimulate neutralizing antibodies. An increase in seroconversion and neutralization titers with appropriate reactogenicity was observed by adjusting the PDK passage for individual serotypes (Sun *et al.*, 2003). Several tetravalent formulations of the DENV vaccine candidates that elicited an acceptable balance of reactogenicity and immunogenicity have been identified for expanded Phase 2 human clinical trials in collaboration with GlaxoSmithKline.

1.8.2 Chimeric vaccines

The strategy to build a chimeric DENV vaccine utilizes a molecular genetics approach. The objective is to retain the attenuation properties from the "backbone" viral vaccine and incorporate dengue antigenicity. A chimeric DENV vaccine was designed using the YFV 17D as the genetic backbone and replacing the E and prM protein genes of YFV with DENV1-4 (Guirakhoo, 2002; Guirakhoo *et al.*, 2004). The resulting four DENV chimeric vaccines (ChimeriVax-DENV1-4) were produced in Vero cells. Preclinical trials in non-human primates demonstrated that ChimeriVax induced low level, detectable viremia in YFV immune and non-immune monkeys when compared to monkeys given wild-type DENV. The vaccine was shown to be attenuated, efficacious, safe and highly unlikely to be transmitted by arthropod vectors. Sero-conversion to 3 out of 4 serotypes was observed after the initial inoculation; however, ChimeriVax-DENV-2 appeared to be immunodominant (Guirakhoo *et al*., 2004). Lowering the dose of ChimeriVax DENV-2 in the tetravalent formulation resulted in a more balanced neutralizing antibody response to DENV-1, -2, and -3 with modestly higher titers to DENV-4.

Challenging non-human primates with the four-dose formulation (ChimeriVax DENV1-4) showed nearly complete protection and reduced neurovirulence when compared to YF-Vax (Guirakhoo *et al*., 2004). Phase I clinical trials of ChimeriVax DENV-2 in flavivirus-naïve and YFV immune volunteers resulted in mild side effects, similar to those of YF-Vax (Guirakhoo, 2006). All volunteers vaccinated with high dose ChimeriVax DENV-2 seroconverted to DENV-2 and no volunteers seroconverted to DENV-1, -3, -4, or YFV. In addition, YFV immunity did not interfere with ChimeriVax DENV-2 immunization and increased heterologous DENV seroconversion. In an analysis of potential replicative or immune interference between the four serotypes, it was demonstrated DENV-1 and DENV-4 were dominant in neutralizing antibody titers regardless of dose or route of administration (Guy, 2009). When tetravalent ChimeriVax was given to non-human primates or adult volunteers, a longer time span between primary immunization and boost increased the seroconversion rates was observed, suggesting interference could be prevented with a longer dose schedule (Morrison, 2010). Phase 2 clinical studies of ChimeriVax by Sanofi Pasteur in DENV endemic regions showed that a three-dose vaccination regimen induced an acceptable immune response against all four serotypes in the majority of vaccines. Preexisting flavivirus

immunity favors quicker and higher immune responses without adversely effecting clinical safety or increasing vaccine viremia. The vaccine is currently in Phase III clinical trial (Guy, 2011).

1.8.3 Whole virus inactivated vaccines

Whole virus inactivated vaccines are advantageous over LAV as the viruses cannot revert to the virulent phenotype, they do not interfere with other serotypes in a tetravalent format (since they cannot replicate) and immune compromised individuals can receive these vaccines. However, these vaccines show lower immunogenicity than LAV, hence require additional doses and boosters to reach protective levels as neutralizing antibody titers may decrease over time. WRAIR developed a DENV-2 vaccine by sucrose gradient purification followed by formalin inactivation (Putnak *et al.*, 1996a; Putnak *et al.*, 1996b). High levels of neutralizing antibody titres were observed in vaccinated mice and macaques and the vaccine provided partial protection against viral challenge. However, the neutralizing antibody response was not stable over a prolonged period in macaques suggesting that a higher dose of vaccine or additional boosting may be required to maintain protective antibody levels (Putnak *et al.*, 2005).

1.8.4 DNA vaccines

DNA vaccines are stable, resistant to temperature extremes and induce intracellular antigen processing for adaptive immunity. Unlike LAV, they do not have the complications of immunodominance when combining monovalent vaccines to form di, tri and tetravalent vaccine formulations. DNA vaccines typically consist of a bacterial plasmid with a strong promoter, the gene of interest and polyadenylation/termination sequence. The plasmid DNA is engineered synthetically or by PCR for optimal expression in eukaryotic cells (Danko, 2011). Vaccines based on recombinant DNA technologies have undergone animal trials. A DNA vaccine with the plasmid encoding prM and E genes of DENV-2 elicited high neutralizing antibodies in mice, and further viral challenge showed an anamnestic antibody response with higher neutralizing antibody titres (Konishi *et al.*, 2000).

Viral vector based DENV vaccines have also been studied. Two bivalent dengue virus vaccines were formulated by incorporating the genes expressing prM and E

proteins of DENV-1 and -2 (cAdVax-D1-2) or DENV-3 and -4 (cAdVax-D3-4) in a complex adenovirus vector. Rhesus monkeys were vaccinated by intramuscular inoculation of a tetravalent dengue vaccine formulated by combining these two bivalent vaccine constructs. Vaccinated animals produced high-titer antibodies that neutralized all four serotypes of dengue viruses *in vitro*. Two separate live-virus challenges administered at 4 and 24 weeks after the final vaccination shown the ability of the vaccine to induce rapid protective immune responses (Raviprakash *et al.*, 2008). However, the main disadvantage of DNA vaccines includes low immunogenicity elicited in immunized human hosts. In addition, DNA stays at the site of inoculation and is broken down before it can be used effectively (Danko, 2011). Concerns arise in regards to the ability of a DNA vaccine to induce immunity to the inactive viral proteins that would be used to make the vaccine.

1.8.5 Recombinant subunit protein vaccines

Recombinant protein vaccine development mainly focuses on the expression of E protein of DENV as the major antigen. Similar to whole virus inactivated vaccines, subunit protein vaccines are also hindered by lower immunogenicity and require administration with an adjuvant. Recombinant E proteins have been produced using different expression systems such as *E. coli*, vaccinia virus and baculoviruses (Deubel *et al.*, 1991; Delenda *et al*., 1994). The purified subunit vaccine candidates elicited variable degrees of protective immunity in animal models. The expression of 80% of recombinant E protein (r80E) can assist the E protein secretion in cultured cells. The r80E protein of DENV-2 has been successfully expressed in fruit fly cells and the vaccine construct was tested using four different adjuvants in macaques (Putnak *et al*., 2005). Regardless of adjuvant used, these vaccines elicited high levels of DENV neutralizing antibodies. Expression of both prM and E genes of DENV-2 in a single vaccine construct has also been reported and this system requires prM protein as a molecular chaperone to help the correct folding of the E protein. The vaccine elicited serotype-specific neutralizing antibodies against DENV-2 as well as antigen-specific cytotoxic T lymphocyte responses in mice. Two-dose immunization in rhesus macaques induced comparable levels of neutralizing antibodies and the animals showed protection against live DENV-2 challenge (Putnak *et al.*, 2003). Studies have also focused on recombinant EDIII expressed in *E. coli* and yeast that elicited neutralizing antibodies

against DENV and protected mice against lethal DENV challenge (Srivastava *et al.*, 1995; Simmons *et al.*, 2001; Hermida *et al.*, 2006; Jaiswal *et al.*, 2004; Etemad *et al.*, 2008; Leng *et al.*, 2009).

1.9 Synthetic peptides as potential B-cell epitope vaccine candidates

Antibodies recognize relatively short epitopes (containing <15 residues of a polypeptide) within larger macromolecular structures and play an important role in immune activation (Black, 2010). The peptide must be immunogenic to be considered as a vaccine candidate. Synthetic peptides have a number of potential advantages over conventional vaccines such as (i) the absence of infectious viral material in vaccine formulations, (ii) the ability to exclude deleterious protein sequences, (iii) easy introduction of lipid, carbohydrate and phosphate groups increasing stability and immunogenicity of vaccine constructs, (iv) easy characterization and purification using well-established laboratory techniques, (v) economical large-scale production, (vi) ability to store in lyophilized form, eliminating the need for cold-chain, (vii) no risk of reversion or recombination, (viii) no risk of genetic material integration, (ix) safety and ease of production, and (x) the ability to include multiple vaccine antigenic peptides (epitopes) from several different or the same pathogen (Purcell *et al.*, 2007). Peptide epitopes can induce antibody responses and can be rapidly adapted to genetic changes where the conventional whole virus vaccine formulations may face genetic restrictions due to mutations leading to complex immune responses.

An effective antibody response is mainly dependent on the activation and proliferation of two different cell populations. B-cells are activated via the direct interaction of cell surface anchored antibody molecules with exogenously presented antigen. Helper T-cells (TH-cells) are activated through recognition of defined antigenic peptide epitopes in the context of MHC molecules and these activated helper T-cells are necessary for providing appropriate cytokine help in the activation and proliferation of B-cells into antibody secreting plasma cells. The provision of T-cell help is usually in the form of various carrier proteins and adjuvants (Grollo *et al*., 2006; Li *et al*., 2011).

In 1992, Roehrig and his co-workers used MEV E protein based synthetic peptide approach to investigate the TH-cell response to the DENV-2 E protein. An increase in antibody response was observed when the B-cell epitopes were linked to TH-cell epitopes (Roehrig *et al.*, 1992). In addition, better responses were seen when the B-

and TH-cell epitopes were synthesized co-linearly (Roehrig *et al*., 1992). This was confirmed in another study using DENV-2 E synthetic peptides as TH–cell epitopes (Roehrig *et al.*, 1994). Furthermore, these epitopes were consistent in location and activity between two distantly related flaviviruses, DENV-2 and MEV, suggesting that these TH-cell epitopes may be conserved among all flaviviruses (Roehrig *et al*., 1994).

In the last two decades a number of synthetic approaches have been investigated to address problems associated with peptide vaccine design in DENV (Aaskov *et al*., 1989; Innis *et al*., 1989; Roehrig *et al*., 1998; Falconar, 1999; Vazquez *et al*., 2002; Amexis and Young, 2007; Falconar, 2008; da Silva *et al*., 2009; Sanchez-Burgos *et al*., 2010; Li *et al*., 2011). Overlapping synthetic octapeptides of the DENV-2 E protein revealed 6 potential binding sites (aa 1-58, 59-297, 288-391, 392-442, 446-476, 479-495) when tested in ELISA with dengue immune antisera from rabbits (Aaskov *et al*., 1989). Innis *et al.* (1989) reported 22 overlapping hexapeptides recognized in ELISA using convalescent antisera from 7 dengue patients. MAbs targeted to overlapping synthetic peptides of DENV-2 E protein revealed aa 274-283, 304-313, 349-359, and 393-401 as potential immunogenic regions (Falconar, 1999; Falconar, 2008).

Synthetic peptides of the prM protein have been investigated as potential B-cell epitopes, and two peptides (aa 3-31, 103-124) elicited a strong antibody response protecting mice against a lethal DENV-2 challenge (Vazquez *et al*., 2002). Screening the peptide library of DENV-3 E protein against serum from infected patients revealed several immunodominant IgG-specific epitopes (da Silva *et al*., 2009). Multiple antigenic peptides (MAPs) derived from DENV-2 E protein using a computer-based *in silico* epitope prediction software (MacVector™) have shown 7 neutralizing DENV-2 epitopes suggesting that the MAP platform can be used as an antigen-presenting platform for dengue vaccine development. Recently, a multi-epitope based strategy combining both B- and T-cell epitopes of DENV-2 EDIII showed multiple neutralizing epitopes and induced cell-mediated immune response in the mouse (Li *et al*., 2011). It appears that the use of synthetic peptide-based vaccine constructs eliminates some of the complexities involved in vaccine design by delivering a precise, chemically-defined epitope and eliciting better neutralizing antibody response, an option to be considered in DENV vaccine design.

1.10 Aim of the thesis

Dengue infection has emerged as a leading public health concern in the world with more than 3.6 billion people at risk globally. Developing a vaccine against DENV has been in progress for several decades, however no vaccine is currently licensed for human use. Developing a vaccine against dengue fever has been a challenging task due to the complex pathology of the illness and the need to control four serotypes simultaneously in a single vaccine formulation. A key factor in protection appears to be neutralizing antibody-mediated protection. However, dengue infections can lead to DHF/DSS in individuals who have dengue antibodies acquired either passively through maternal transmission or actively from a prior DENV exposure with a heterologous serotype. Hence protection against a single serotype of DENV may increase the risk of ADE.

An effective DENV vaccine must be tetravalent and provide simultaneous protection against all four DENV serotypes. Clinical trials of live attenuated tetravalent dengue vaccine formulations have shown unbalanced immune response due to immunodominance. Studies have also shown that antibodies targeted against the prM protein are cross-reactive among all DENV serotypes but do not neutralize the virus, leading to ADE. While efforts are underway to optimize the tetravalent formulations, the risk of viral interference and inadequate immunity levels suggest alternative strategies are required. One such strategy is investigating the possibility of using synthetic peptides as potential vaccine candidates. Therefore, the objectives of this study are to identify the neutralizing antibody epitopes recognized by sera of previously unstudied cohort of dengue-infected individuals from Australia through multiple epitope mapping techniques.

Sera from volunteers who were infected with a single DENV strain or multiple strains will be used in this study to evaluate the antibody neutralization potential against four DENV prototype strains. The sera neutralizing one or more DENV serotypes will be further exploited to identify potential antibody epitopes in the DENV E protein. Three distinctively different techniques will be used for epitope mapping:

1) A panel of overlapping synthetic peptide library representing entire E protein of DENV-2 will be used to screen anti-dengue human antibodies by common solid phase binding ELISA technique.

2) The binding profile of each peptide against the antibody will be tested in epitope extraction, although probing of the peptides will be carried out in solution as opposed to the solid-phase ELISA. Identification of the antibody bound peptides will be performed through mass spectrometry, making this technique more sensitive.
3) A multi-step computational approach will be used to identify the potential B-cell epitopes for all four DENV serotypes.

It is anticipated that a combination of these three strategies will reveal several novel linear epitopes. Selected epitope candidates will thus be co-synthesized with a previously published T_H–cell epitope and the resulting synthetic peptide based vaccine constructs will be tested for their ability to elicit a humoral immune response in mice. Potential DENV binding and neutralizing activity will be determined by ELISA and *in vitro* virus neutralization assays. Overall, the present study will assess whether the use of a multiple epitope mapping technique combined with the synthetic peptide-based vaccine strategy is a promising platform for future DENV vaccine development.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Equipment

The equipment used in this study is listed in Table 2.1

Equipment Type	Equipment Name	Brand/Manufacturer
Mass spectrometer (Ion-trap)	LC/MSD Trap XCTplus 3D iontrap	Agilent Technologies, Palo Alto, CA
Mass spectrometer (MALDI-ToF)	Bruker microFLEX MALDI-ToF	Bruker Daltonics, Germany
Mass spectrometer (Q-ToF)	QSTAR Elite hybrid quadrupole time-of-flight (QqTOF)	Applied Biosystems/MDS Sciex, Foster City, CA
Liquid chromatography	Agilent 1100 series nanoLC	Agilent Technologies, Palo Alto, CA
Liquid chromatography	Shimadzu Prominence nano LC system	Shimadzu Corporation, Kyoto, Japan
Spectrophotometer	Ultrospec 1100 Pro UV/ visible spectrophotometer	Amersham Pharmacia, Uppsala, Sweden
Spectrophotometer	Cary 100 Bio UV-visible spectrophotometer	Varian, Palo Alto, CA
Plate Reader	iMark Microplate Reader	BioRad Laboratories, Hercules, CA
Inverted microscope	Olympus inverted microscope	Olympus Imaging Corp, China

Table 2.1 Equipment used in this study

2.1.2 Culture media

Cell growth medium: RPMI-1640 medium (Invitrogen, U.S.A) supplemented with 10% v/v heat inactivated (56^{o} C for 30 min) fetal calf serum (FCS; Invitrogen, U.S.A) and 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml L-glutamine solution (PSG) (Invitrogen, U.S.A). Media was sterilized by membrane filtration (pore size 0.45 μm, Millipore, U.S.A) and stored at 4°C.

Cell freezing medium: RPMI-1640 medium (Invitrogen, U.S.A) supplemented with 30% v/v heat inactivated fetal calf serum (FCS; Invitrogen, U.S.A) and 10% v/v dimethyl sulphoxide (DMSO; Sigma, U.S.A).

Virus growth medium: Serum-free RPMI-1640 medium (Invitrogen, U.S.A) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml L-glutamine solution (PSG) (Invitrogen, U.S.A). Media was sterilized by membrane filtration (pore size 0.45 μm, Millipore, U.S.A) and stored at 4°C.

CMC-overlay medium: RPMI-1640 medium (Invitrogen, U.S.A) supplemented with 2% v/v heat inactivated fetal calf serum (FCS; Invitrogen, U.S.A), 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml L-glutamine solution (PSG) (Invitrogen, U.S.A), and 1.5% w/v carboxy-methyl cellulose (CMC; Sigma-Aldrich, U.S.A).

2.1.3 Buffers

PBS (1X): 5g sodium chloride (NaCl) (Merck, Kilsyth, Australia), 0.2g potassium chloride (KCl) (Univar, U.S.A), 1.44g di-sodium hydrogen phosphate (Na_2HPO_4) (AnalaR, UK), and 0.24g potassium di-hydrogen phosphate (KH_2PO_4) (AnalaR, UK) were dissolved in 800 ml distilled water. The pH was adjusted to 7.3 using sodium hydroxide (NaOH) (Merck, Kilsyth, Australia) and the volume of the solution was made up to 1 litre.

Protein A loading buffer (1M potassium phosphate, pH 9.0): 17.42g di-potassium hydrogen orthophosphate (K_2HPO4) (Merck, Kilsyth, Australia) dissolved in

distilled water and the volume was made up to 100 ml. The solution was sterilized by membrane filtration (pore size 0.45 μm, Millipore, U.S.A) and stored at 4°C.

Elution buffer (0.1M citric acid): 2.11g citric acid (Sigma-Aldrich, U.S.A) was dissolved in distilled water and the volume was made up to 100 ml.

1.5M Tris-HCl (pH 7.0): 18.17g Tris base (Trizma®) (Sigma-Aldrich, U.S.A) was dissolved in 75 ml distilled water. The pH was adjusted to 7.0 with conc. hydrochloric acid (HCl) (Univar, U.S.A) and the final volume of the solution was made up to 100 ml.

3.7% formaldehyde/PBS: 100 ml v/v of 37% formaldehyde (Sigma-Aldrich, U.S.A) was mixed with 900 ml of 1x PBS pH 7.3.

0.1% Nonidet P-40/PBS: 1 ml v/v Nonidet P-40 (Sigma-Aldrich, U.S.A) was mixed with 999 ml of 1x PBS pH 7.3.

2% skimmed milk/PBS: 20g of skimmed milk powder (Nestle, Australia) was mixed with 900 ml of 1x PBS pH 7.3 and the volume was made up to 1 litre.

TMB substrate solution: Equal volumes of solution A (hydrogen peroxide) and solution B, 3,3′,5,5′-tetramethylbenzidine (TMB) (BD Biosciences, Australia) were mixed 10 minutes prior to addition in assays.

Sodium carbonate buffer (50mM): 2.93g sodium bi-carbonate ($NaHCO_3$) (AnalaR, UK), and 1.59g sodium carbonate (Na_2CO_3) (AnalaR, UK) were dissolved in 900 ml of distilled water. The pH was adjusted to 9.6 using HCl and the volume of the solution was made up to 1 litre.

PBST: 0.5 ml v/v Tween 20 (Merck, Kilsyth, Australia) was added to PBS (pH 7.3) and the solution was made up to 1 litre.

BSA_{10}PBS: 10 mg/ml of bovine serum albumin (BSA) (Sigma-Aldrich, U.S.A) in PBS (pH 7.3).

BSA_5PBST: 5 mg/ml of BSA in PBST (pH 7.3).

1M Sulphuric acid: 5.5 ml of conc. sulphuric acid (H_2SO_4) (Sigma-Aldrich, U.S.A) was added to 94.5 ml of distilled water.

50 mM Tris-HCl (pH 7.4): 3.028g Tris base was dissolved in 475 ml distilled water. The pH was adjusted to 7.4 with conc. HCl and the final volume of the solution was made up to 500 ml.

Washing buffer for epitope extraction: 0.605g of Tris base (Trizma®) (Sigma-Aldrich, U.S.A) was dissolved in 75 ml of distilled water and the pH was adjusted to 7.4 with conc. HCl. To this, 2.92g of NaCl and 500 µl of N-octyl-D-glucoside (Calbiochem, San Diego, U.S.A) were added and the final volume of the solution was made up to 100 ml.

Elution buffer for Epitope extraction: 500 µl of formic acid (Fluka, Buchs, Switzerland) was mixed with 99.5 ml of distilled water.

Crystal violet-formalin staining solution: 1.3 g of crystal violet (Sigma-Aldrich, U.S.A) in 50 ml of 95% isopropyl alcohol (Sigma-Aldrich, U.S.A), 300 ml of 37% formaldehyde (Merck, Kilsyth, Australia) and the stock solution was made up to 1 litre with distilled water. Final solution is prepared with a 1:1 volume of PBS at pH 7.3.

2.1.4 Cells

Baby hamster kidney cells (BHK-21 clone 15) (Morens *et al.*, 1985) and *Aedes albopictus* mosquito cells (C6/36) (Igarashi, 1978) were kindly provided by Prof. John Aaskov, WHO collaborating Centre for Arbovirus Reference and Research, Queensland University of Technology, Australia.

2.1.5 Viruses

Dengue virus prototypes DENV-1 (Hawaii), DENV-2 New Guinea C (NGC), DENV-3 (H87) and DENV-4 (H241) were kindly provided by Prof. John Aaskov, WHO collaborating Centre for Arbovirus Reference and Research, Queensland University of Technology, Australia.

2.1.6 Monoclonal antibodies

Anti-dengue virus complex specific monoclonal antibody 2H2 was kindly provided by Prof. John Aaskov, WHO collaborating Centre for Arbovirus Reference and Research, Queensland University of Technology, Australia. The anti-flavivirus E protein specific monoclonal antibody 4G2 (clone D1-4G2-4-15) was purchased from Chemicon, Millipore (Merck Millipore, U.S.A).

2.1.7 Human sera

Convalescent DENV immune sera were obtained from volunteers who had experienced natural DENV infections. All volunteers were enrolled following an informed consent process. A total of 40 serum samples were collected. Four uninfected human sera were also included in the study as negative control. Approximately 20 ml of blood was obtained from each volunteer via peripheral venipuncture and the tubes were incubated at 4°C overnight. The following day, the blood clots were removed using a toothpick and the sera were centrifuged at 8000 rpm for 5 min. The clarified supernatant was transferred to fresh vials, heat-inactivated at 56°C for 1 hour and stored at -20°C. For long-term storage, duplicate human sera vials were stored untreated at -80°C. The protocol for blood collection and use was approved by the Cairns and Hinterland Health Service District Human Research Ethics Committee and the Human Research Ethics Committee of Swinburne University of Technology (Protocol No. HREC/10/QCH/17-646; SUHREC- 2010/158). The sera samples were tested for the presence of dengue IgG and IgM antibodies using PanBio dengue indirect IgG and capture IgM tests following manufacturer's instruction by Prof. John McBride, Cairns Base Hospital, James Cook University, Queensland.

2.1.8 Peptide library

The peptide library consisted of 70 synthetic peptides (PEPscreen, Sigma-Aldrich, U.S.A) spanning the entire E protein (495 aa) of dengue virus serotype 2 (GenBank Accession No: ABW06583.1). Peptide synthesis by the manufacturer was performed by Fmoc chemistry under continuous flow conditions using polyethylene glycol (PEG)-Polystyrene resins. Upon synthesis, the peptides were cleaved from the resin, precipitated purified by High pressure liquid chromatography (HPLC) and

subjected to lyophilization. The length of the each peptide was 18 amino acids and they overlapped 11 amino acids with the subsequent peptide (Table 2.1). The peptides were initially dissolved in 10-20 µl of DMSO (Sigma, U.S.A) and further diluted in sterile MilliQ water. The dissolved peptide stock solutions were aliquoted in small volumes and stored at -20°C for medium-term storage and at -80°C for long-term storage.

2.1.9 Vaccine constructs

The peptides identified as potential dengue B-cell epitopes were synthesized in line with an 18 amino acid T helper epitope (LITVNPIVTEKDSPVNIE) (Roehrig et al., 1992) from dengue serotype 2 envelope glycoprotein. The vaccine constructs were synthesized commercially (GL Biochem, Shanghai, China). Seven vaccine constructs were used in this study (Table 2.2).

2.1.10 Recombinant proteins

The recombinant proteins representing the envelope protein of dengue serotypes 1, 2 and 3 were purchased commercially from Hawaii Biotech, USA. The proteins were dissolved in PBS buffer at the concentration of 1 mg/ml and stored at 4°C as suggested by the manufacturer. DENV-4 recombinant protein was not included in the study as the majority of sera samples collected from volunteers were limited to DENV-1, -2 and -3 infection following recent outbreaks in Australia.

2.1.11 Mice

Male 6-8 week old BALB/c mice (5 animals per group) were obtained from the Animal Facility, Department of Microbiology and Immunology, The University of Melbourne. The protocol used in this study was reviewed and approved by the Animal Ethics Committee (SAEC) of Swinburne University of Technology (Protocol No. SAEC Project 2011/02).

Peptide number	Amino acid sequence	Amino acid position	Molecular weight (Daltons)
1	MRCIGISNRDFVEGVSGG	1-18	1897.2
2	NRDFVEGVSGGSWVDIVL	8-25	1949.2
3	VSGGSWVDIVLEHGSCVT	15-32	1845.1
4	DIVLEHGSCVTTMAKNKP	22-39	1943.3
5	SCVTTMAKNKPTLDFELI	29-46	2011.4
6	KNKPTLDFELIKTEAKQP	36-53	2100.4
7	FELIKTEAKQPATLRKYC	43-60	2139.5
8	AKQPATLRKYCIEAKLTN	50-67	2048.4
9	RKYCIEAKLTNTTTESRC	57-74	2117.4
10	KLTNTTTESRCPTQGEPS	64-81	1950.1
11	ESRCPTQGEPSLNEEQDK	71-88	2047.1
12	GEPSLNEEQDKRFICKHS	78-95	2117.3
13	EQDKRFICKHSMVDRGWG	85-102	2192.5
14	CKHSMVDRGWGNGCGLFG	92-109	1924.2
15	RGWGNGCGLFGKGGIVTC	99-116	1782.1
16	GLFGKGGIVTCAMFTCKK	106-123	1861.3
17	IVTCAMFTCKKNMEGKVV	113-130	2002.5
18	TCKKNMEGKVVLPENLEY	120-137	2095.5
19	GKVVLPENLEYTIVITPH	127-144	2022.4
20	NLEYTIVITPHSGEEHAV	134-151	2009.2
21	ITPHSGEEHAVGNDTGKH	141-158	1886.0
22	EHAVGNDTGKHGKEIKIT	148-165	1934.1
23	TGKHGKEIKITPQSSITE	155-172	1954.2
24	IKITPQSSITEAELTGYG	162-179	1908.1
25	SITEAELTGYGTVTMECS	169-186	1892.1
26	TGYGTVTMECSPRTGLDF	176-193	1935.2
27	MECSPRTGLDFNEMVLLQ	183-200	2083.4
28	GLDFNEMVLLQMEEKAWL	190-207	2166.5
29	VLLQMEEKAWLVHRQWFL	197-214	2326.8
30	KAWLVHRQWFLDLPLPWL	204-221	2318.8
31	QWFLDLPLPWLPGADTQG	211-228	2054.3
32	LPWLPGADTQGSNWIQKE	218-335	2097.3
33	DTQGSNWIQKETLVNFKN	225-242	2122.3
34	IQKETLVNFKNPHAKKQD	232-249	2138.5
35	NFKNPHAKKQDVVVLGSQ	239-256	2009.3

Table 2.2. List of synthetic peptides used in this study

Table 2.2 cont.

Peptide number	Amino acid sequence	Amino acid position	Molecular weight (Daltons)
36	KKQDVVVLGSQEGAMHTA	246-263	1898.2
37	LGSQEGAMHTALTGATEI	253-270	1787.0
38	MHTALTGATEIQMSSGNL	260-277	1862.1
39	ATEIQMSSGNLLFTGHLK	267-284	1947.2
40	SGNLLFTGHLKCRLRMDK	274-291	2089.5
41	GHLKCRLRMDKLQLKGMS	281-298	2114.6
42	RMDKLQLKGMSYSMCTGK	288-305	2077.5
43	KGMSYSMCTGKFKIVKEI	295-312	2050.2
44	CTGKFKIVKEIAETQHGT	302-319	1990.3
45	VKEIAETQHGTIVIRVQY	309-326	2084.4
46	QHGTIVIRVQYEGDGSPC	316-333	1959.2
47	RVQYEGDGSPCKIPFEIM	323-340	2069.4
48	GSPCKIPFEIMDLEKRHV	330-347	2099.5
49	FEIMDLEKRHVLGRLITV	337-354	2169.6
50	KRHVLGRLITVNPIVTEK	344-361	2073.5
51	LITVNPIVTEKDSPVNIE	351-368	1981.3
52	VTEKDSPVNIEAEPPFGD	358-375	1944.1
53	VNIEAEPPFGDSYIIIGV	365-382	1933.2
54	PFGDSYIIIGVEPGQLKL	372-389	1946.2
55	IIGVEPGQLKLNWFKKGS	379-396	2014.4
56	QLKLNWFKKGSSIGQMFE	386-403	2141.5
57	KKGSSIGQMFETTMRGAK	393-410	1957.0
58	QMFETTMRGAKRMAILGD	400-417	2056.5
59	RGAKRMAILGDTAWDFGS	407-424	1952.2
60	ILGDTAWDFGSLGGVFTS	414-431	1843.0
61	DFGSLGGVFTSIGKALHQ	421-438	1834.1
62	VFTSIGKALHQVFGAIYG	428-445	1908.2
63	ALHQVFGAIYGAAFSGVS	435-452	1795.0
64	AIYGAAFSGVSWTMKILI	442-459	1928.3
65	SGVSWTMKILIGVIITWI	449-466	2017.5
66	KILIGVIITWIGMNSRST	456-473	2002.5
67	ITWIGMNSRSTSLSVSLV	463-480	1951.3
68	SRSTSLSVSLVLVGVVTL	470-487	1817.2
69	VSLVLVGVVTLYLGAMVQ	477-494	1861.3
70	SLVLVGVVTLYLGAMVQA	478-495	1833.3

Vaccine	Amino acid sequence
B2	LITVNPIVTEKDSPVNIENRDFVEGVSGGSWVDIVL
B16	LITVNPIVTEKDSPVNIEGLFGKGGIVTCAMFTCKK
B29	LITVNPIVTEKDSPVNIEVLLQMEEKAWLVHRQWFL
B38	LITVNPIVTEKDSPVNIEMHTALTGATEIQMSSGNL
B45	LITVNPIVTEKDSPVNIEVKEIAETQHGTIVIRVQY
B64	LITVNPIVTEKDSPVNIEAIYGAAFSGVSWTMKILI
B19	LITVNPIVTEKDSPVNIEGKVVLPENLEYTIVITPH

Table 2.3. List of vaccine constructs used in this study

2.2 Methods

2.2.1 Cell propagation

Cells were grown in RPMI-1640. For cell passaging, the cell monolayer was washed twice with PBS (pH 7.3) and 3 ml of 0.25% trypsin-EDTA (Invitrogen, U.S.A) solution was added. The dislodged cells were centrifuged at 400g for 5 minutes and the cell pellet was resuspended in fresh growth medium. BHK21 cells were incubated at 37°C with 5% v/v CO_2/air for 2-3 days and C6/36 cells were propagated at 30°C for 3-4 days.

2.2.2 Freezing cells

For cell freezing, the confluent cell monolayer was trypsinized and centrifuged at 400g for 5 minutes. The cell pellet was then resuspened in 5 ml of chilled RPMI supplemented with 30% heat inactivated FCS and 10% v/v DMSO. Cell counts were performed using a haemocytometer and the volume of media was adjusted to 1.5 X 10^6 cells/ml. The cells were aliquoted into 1 ml cryotubes (Nunc, Denmark) and stored at -80°C. The next day, the frozen cells were moved to liquid nitrogen.

2.2.3 Preparation of virus stock

Stocks of DENV prototype viral strains were diluted 10 fold in serum-free RPMI-1640. 10 ml of the diluted virus inoculum was used to infect a confluent monolayer of C6/36 cells in a 75cm^2 tissue culture flask (Becton Dickinson, BD, North Ryde, NSW, Australia). The flasks were incubated at 30°C for 2-3 hours to facilitate virus attachment. Following this, the virus inoculum was discarded and 10 ml of serum-free RPMI-1640 containing PSG was added to the cells. The cultures were incubated at 30°C for 5-7 days and examined daily for the presence of cytopathic effect (CPE). Cell debris was pelleted by centrifuging at 400g for 5 minutes at 4°C and the virus supernatant was collected. Heat-inactivated FCS was added to the clarified supernatant to bring the final concentration of FCS to 30 % v/v and stored in 1 ml cryotubes at -80°C. The virus titre was measured by plaque assay.

2.2.4 Titration of virus

Plaque titration was performed on BHK-21 cells as previously described by Morens *et al.* 1985. Briefly, cell monolayers were prepared by seeding 24 well tissue culture plates (Becton Dickinson, BD, North Ryde, NSW, Australia) with 1 ml of cells at a concentration of 2 x 10^5 cells/ml in RPMI-1640 containing 2% heat-inactivated FCS with PSG and grown at 37°C with 5% v/v CO_2/air. The following day, serial ten-fold dilutions (10^{-1} to 10^{-6}) of virus were prepared in RPMI-1640 containing 2% heat-inactivated FCS with PSG. The media in the wells was discarded and 200 µl of virus dilutions were added in duplicate to the BHK-21 cell monolayers. Plates were incubated at 37°C with 5% v/v CO_2/air to allow the virus to absorp to cells. After 2 hours, the wells were overlaid with 1 ml of 1.5% w/v CMC and RPMI-1640 containing 2% heat-inactivated FCS with PSG. The plates were incubated at 37°C with 5% v/v CO_2/air for 7-9 days for DENV-1 and DENV-3, 5 days for DENV-2 and 6 days for DENV-4.

After incubation, the wells were fixed and stained for 10 to 15 minutes with 1 ml of crystal violet (Sigma-Aldrich, USA) -formaldehyde (Merck, Kilsyth, Australia) staining solution (Thomas *et al.*, 2009). The plates were then gently rinsed with water and air-dried. The viral plaques were counted and the titre was calculated as plaque forming units per ml (PFU/ml).

2.2.5 Immunoglobulin G purification

The immunoglobulin G (IgG) fractions were purified from human sera using Protein A-Sepharose Fast Flow affinity chromatography (Sigma-Aldrich, U.S.A) following manufacturer's instructions. Briefly, 250 mg of Protein A-Sepharose beads were mixed with 1 ml of Protein A loading buffer (1M potassium phosphate, pH 9.0) and the slurry was transferred in to a 10 x 85 mm column. The sera were diluted 10-fold in Protein A loading buffer and filtered through 0.45 µm pore size membrane filters (Millipore, U.S.A). The filtered sera were applied onto the Protein A-Sepharose column and the unbound fractions were slowly allowed to pass out of the column. The unbound residual proteins from the column were washed 3 times each with 10 ml of PBS (pH 7.3). The bound IgG fractions were eluted with 2 ml of elution buffer (0.1M citric acid) and the pH of the eluted fractions was neutralized with equal volume of 1.5M Tris-HCl (pH 7.0). The absorbance of the IgG fractions was measured in a UV spectrophotometer

(Amersham Pharmacia, Uppsala, Sweden) at 280 nm and the antibody concentrations were calculated using the following formula:

Antibody concentration = $A_{280}/1.36$

where, 1.36 is the constant factor as 1 mg/ml of IgG was expected to give an absorbance of approximately 1.36 in a 1 cm light path cuvette.

2.2.6 Virus neutralization assay

2.2.6.1 Log Neutralization Index (LNI)

The neutralizing ability of purified IgG from human sera was measured by a constant antibody-varying virus plaque-reduction neutralization test (Morens *et al.,* 1985). Briefly, BHK-21 cell monolayers were prepared in 24 well tissue culture plates (Becton Dickinson, BD, North Ryde, NSW, Australia) with 1 ml of cells at a concentration of 2 x 10^5 cells/ml in RPMI-1640 containing 2% heat-inactivated FCS with PSG and grown at 37°C with 5% v/v CO_2/air. DENV serotypes 1-4 with a working stock of approximately 10^6 PFU/ml were diluted ten-fold (10^{-1} to 10^{-6}) in RPMI-1640 containing 2% heat-inactivated FCS with PSG. 10 µg/ml of purified human IgG was diluted in RPMI-1640 containing 2% heat-inactivated FCS with PSG, 100 µl of this was mixed with an equal volume of serially diluted viral suspensions in a 96 well round bottom plate (Becton Dickinson, BD, North Ryde, NSW, Australia). For a negative control, 100 µl of RPMI 1640 was mixed with equal volume of viral suspension.

Following incubation at 37°C for 2 hours, the virus-antibody mixture was added in duplicate to the monolayers of confluent BHK-21 cells and incubated at 37°C with 5% v/v CO_2/air for 1 hour to allow the virus to absorb to the cells. The wells were then overlaid with 1 ml of 1.5% w/v CMC and RPMI-1640 containing 2% heat-inactivated FCS with PSG. The plates were incubated at 37°C with 5% v/v CO_2/air for 7-9 days for DENV-1 and DENV-3, 5 days for DENV-2 and 6 days for DENV-4. After incubation, the overlay medium was removed from all wells, and the cells were fixed and stained for 10 to 15 minutes with 1 ml of crystal violet-formaldehyde staining solution (Thomas et al., 2009). The plates were then gently rinsed with water and air-dried. The viral plaques were counted and the virus titre was recorded as PFU/ml. Virus neutralization was indicated by the reduction in virus titre following the addition of purified IgG to virus culture. Neutralization was expressed as a Log Neutralization Index (LNI).

$$\text{Neutralization Index: } \log_{10} \frac{\text{titre of virus + RPMI}}{\text{titre of virus + IgG}}$$

2.2.6.2 Focus reduction neutralization test (FRNT)

The neutralizing antibody titres of immune mice sera were determined by FRNT as previously described (Leng *et al.,* 2009). Briefly, BHK-21 cell monolayers were prepared in 24 well tissue culture plates with 1 ml of cells at a concentration of 2 x 10^5 cells/ml in RPMI-1640 containing 2% heat-inactivated FCS with PSG and grown at 37°C with 5% v/v CO_2/air. The medium in the wells was discarded immediately prior to performing the FRNT. Pooled pre-immunization or post-immunization mice sera were heat-inactivated at 56°C for 30 min and diluted 1:10 with RPMI-1640 containing 2% heat-inactivated FCS and PSG. Serial two-fold dilutions of this inactivated mice sera were prepared in RPMI-1640 containing 2% heat-inactivated FCS and PSG, and 100 µl of the dilutions were mixed with an equal volume of virus suspension in a 96 well round bottom plate. The virus titer prior to mixing was about 50 FFU (focus forming units) per well.

Following incubation at 37°C for 2 hours, the virus-antibody mixture was added in duplicate to the monolayers of confluent BHK-21 cells and incubated at 37°C with 5% v/v CO_2/air for 2 hour to allow the virus to absorb to the cells. The wells were then overlaid with 1 ml of 1.5% w/v CMC in RPMI-1640 containing 2% heat-inactivated FCS with PSG. The plates were incubated at 37°C with 5% v/v CO_2/air for 72 hours (for DENV-2, -3, and -4) or 96 hours (for DENV-1). After incubation, the overlay medium was removed from all wells, and the cells were washed with cold PBS (pH 7.3). The cells were fixed for 15 min with 1 ml of 3.7% formaldehyde (Sigma-Aldrich, U.S.A) in PBS. After washing with PBS, the cells were permeabilized with 1 ml of 0.1% Nonidet P40 in PBS for 15 min and blocked with 1 ml of 2% w/v skimmed milk (Nestle, Australia) in PBS for 1 hour. The contents of all wells were discarded and washed with PBS.

Infected cells were labeled with 200 µl of an anti-flavivirus E protein specific monoclonal antibody (4G2) (Chemicon, Merck Millipore, U.S.A) at the dilution of 1:1000 in PBS containing 0.05% Tween-20 and 0.5% BSA. Following incubation at

37°C for 1 hour, unreacted 4G2 was removed by washing 2 times with PBS and the antibody-labeled cells were detected using 200 µl of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich, Castle Hill, Australia) at the dilution of 1:1,000 in PBS containing 0.05% Tween-20 and 0.5% BSA at 37°C for 1 hour. The contents of all wells were discarded and washed three times in PBS to eliminate unbound conjugated antibody. 200 µl of TMB substrate solution was added in all wells and incubated at room temperature for 15-30 min. After foci of infected cells were visible, the cell monolayers were rinsed once with distilled water and foci were counted. The neutralizing antibody titer was calculated as the reciprocal of the highest serum dilution that produced a 50% reduction of 50 focus forming units compared to control samples containing the virus and pre-immunization sera.

2.2.7 Enzyme Linked Immunosorbent Assay (ELISA)

Antibody titres were determined by ELISA as described previously (Zeng *et al.*, 2005). Briefly, Immulon 4 HB (High Binding) 96 well ELISA plates (In Vitro Technologies, Australia) were coated overnight at room temperature with 10 µg/ml of peptides or 5 µg/ml of DENV recombinant proteins in 50 µl of 50 mM sodium carbonate buffer pH 9.6. Wells were then blocked with 1% BSA in PBS pH 7.3 (BSA_{10}PBS) for 1 hour. The wells were washed once with PBST and rinsed with PBS. Purified IgG was diluted to 20 µg/ml in BSA_5PBST and 50 µl added per well. For individual or pooled mice sera, serial half-log dilutions were prepared in BSA_5PBST and 50 µl added per well. The plates were incubated for 1 hour and the sera or purified IgG dilutions were removed and the wells were washed twice. 50 µl (1:1000 dilution) of HRP-conjugated goat anti-human IgG (Sigma-Aldrich, USA) or HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich, Castle Hill, Australia) diluted in BSA_5PBST was then added to each well and plates were incubated for 1 hour. Unbound antibody was removed, wells washed twice with PBST and rinsed with PBS. The bound antibody was detected by the addition of 100 µl of TMB substrate solution. The reaction was stopped after 15 mins by addition of 1M sulphuric acid. The optical density (OD) of individual wells was detected by an iMark Microplate Reader (BioRad Laboratories, Hercules, CA) at a wavelength of 450nm. Antibody titres were expressed as the reciprocal of the

logarithm of that dilution of serum that gave an OD four times above that obtained in wells with preimmune control sera.

2.2.8 Epitope extraction

The extraction of bound peptides to purified human IgG was done as previously described by Grollo *et al.* 2006. 70 peptides spanning the sequence of the E protein were arranged in to 14 groups containing 5 peptides each at a concentration of 0.2 mg/ml in PBS. None of the 5 peptides in a group had the same molecular mass and care was taken to make sure there was minimal or no peptide sequence overlaps in each group. To 50 µl of each peptide pool, 50 µl of purified IgG (50 µg) from either dengue patients or non-infected controls was added and allowed to react for 30 min at room temperature. The peptide-antibody mixture was then added to compact reaction columns (CRC's) (USB, Cleveland, OH) containing 50 µg of Protein A-sepharose beads in 50 µl of 50mM Tris-HCl (pH 7.4) and incubated for 30 min at room temperature. The unbound peptides were washed once with washing buffer containing 50mM Tris-HCl (pH7.4) in 0.5M NaCl and 0.5% N-octyl-D-glucoside (Calbiochem, San Diego, USA) followed by two washes each with 0.5M NaCl and H_2O. The peptides bound to the antibody were eluted from Protein-A sepharose beads by addition of 50 µl of elution buffer (0.5% formic acid, Fluka, Buchs, Switzerland).

2.2.9 Mass spectrometry

2.2.9.1 Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

The eluted peptides were analysed in a Bruker microFLEX (Bruker Daltonics, Germany) matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF). Briefly, 1 µl of eluted peptide was dried on a MALDI sample stage (Bruker Daltonics, Germany) with 1 µl of MALDI matrix containing α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, U.S.A) in 50% acetonitrile (Sigma-Aldrich, U.S.A) and 0.1% Tri-fluro acetic acid (Sigma-Aldrich, U.S.A). Detection was performed in positive reflector mode with m/z range of 1500-3500 using flexControl software (version 3.0, Bruker Daltonics. Germany). Peak lists were generated in

flexAnalysis software (version 3.0, Bruker Daltonics, Germany) using the Snap peak detection algorithm with default settings.

2.2.9.2 Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Peptides were identified using LC-ESI-MS/MS. 10 µl of eluted peptides were loaded in glass vials (Waters, U.S.A) and separated by Agilent 1100 series nanoLC (Agilent Technologies, Palo Alto, CA) on a 5 µm (150 mm x 75 µm) Zorbax 300SB-C18 (Agilent Technologies, Palo Alto, CA) chip column using ChipCube interfaced at the front end of a LC/MSD Trap XCTplus 3D iontrap mass spectrometer (Agilent Technologies, Palo Alto, CA). Solvent in mobile phase A consisted of 0.1% formic acid (Fluka, Buchs, Switzerland) in water and mobile phase B consisted of 95% acetonitrile (Sigma-Aldrich, U.S.A) with 0.1% formic acid (Fluka, Buchs, Switzerland). The elution gradient for the chip column was from 15% to 50% buffer B over 19 minutes. The mass spectrometer was operated with electrospray ionisation in the positive ion mode with m/z range of 1500-3500. Data was acquired using the 6300 Series ion Trap LC/MS Software 6.1 (Agilent Technologies, Palo Alto, CA). Selected ions were subject to further MS-MS analysis to confirm peptide identity.

Alternatively, 15 µl of the eluted peptides were loaded onto a 300 µm x 5 mm Zorbax 300SB-C18 (Agilent Technologies, Palo Alto, CA) reversed-phase precolumn attached to a Shimadzu Prominence nano LC system (Shimadzu Corporation, Kyoto, Japan). The precolumn was washed with 0.1% formic acid in 5% acetonitrile for 15 min before placing in-line with a 150 mm x 75 µm Zorbax 300SB-C18 (Agilent Technologies, Palo Alto, CA) reversed-phase column. Peptides were eluted using a gradient of 5–65% (v/v) acetonitrile in 0.1% formic acid over 60 min, at a flow rate of 0.25 µl/min. Peptides were analyzed via electrospray ionization on a QSTAR Elite hybrid quadrupole time-of-flight (QqTOF) mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The mass spectrometer was operated in the positive ion mode with ion source voltage of 2,200 V. Analyst QS 2.0 software (Applied Biosystems/MDS Sciex, Foster City, CA) was used to collect data in a data-dependent acquisition mode for the three most intense ions fulfilling the following criteria: m/z between 450 and 2,000; ion intensity 40 counts; and charge state between +2 and +3. After MS/MS analysis, these ions were dynamically excluded for 18 s, using

a mass tolerance of 50 mDa. MS scans were accumulated for 0.5 s, and MS/MS scans were collected in automatic accumulation mode for a maximum of 2 s.

2.2.10 Database search

The peptide mass fingerprints (PMFs) from MALDI-ToF were searched against a local copy of the non-redundant database SWISS-PROT (http://www.expasy.ch/sprot) using the MASCOT search program (Perkins *et al.*, 1999). The MS/MS peak lists (.mgf) from the ion trap were generated using default parameters in DataAnalysis version 3.4 (Agilent Technologies, Palo Alto, CA). Peak lists for QSTAR Elite data were made using ProteinPilot™ software version 3.0 (Applied Biosystems/MDS Sciex) and searched against the dengue virus database (Swissprot/Uniprot) using the Paragon algorithm (Shilov *et al.*, 2007). The Paragon algorithm search parameters were: Sample type: identification; Cys alkylation: none; Digestion: none; Instrument: LTQ; Search effort: Thorough ID; Detected protein thresh hold: >0.05 (10%). The false positive rate determined was 0.2%.

2.2.11 *in silico* epitope prediction

2.2.11.1 Bioinformatics sequence approach

The dengue virus envelope (E) protein regions were computationally analyzed for hydrophobicity, solvent accessibility, surface accessibility of residues, polarity and spatial distance orientation relationships (Hopp and Woods, 1981; Kyte and Doolittle, 1982; Emini *et al.*, 1985; Parker *et al.,* 1986). The sequences were obtained via NCBI Genbank and scored for the aforementioned key antigenic attributes against the BLASTP (Altschul *et al.*, 1997) query algorithm. Alignment of protein regions were compiled against multiple publicly available database sets and sorted via the ClustalW alignment program (Chenna, *et al.,* 2003). Conserved sequences demonstrating homology within the protein data bank listings PDB ID: 1K4R Dengue virus; PDB ID: lOAN Dengue 2 virus envelope protein; and PDB ID: 1UZG Dengue 3 virus envelope protein were used to construct and verify the model. The predicted epitopes were displayed on a three dimensional structure of DENV-2 E protein (PDB ID-1OAN) using PDB viewer software. The epitopes sequences identified from 3 different epitope mapping strategies were coloured in green.

2.2.11.2 Bioinformatics structure approach

Sequence alignment models demonstrating more than 40% structural conservation with the PDB were used to generate a three-dimensional structural model for the DENV E protein assemblies, using the Chimera (Pettersen *et al.*, 2004) interface to modeller (Eswar *et al.*, 2007). The crystallographic atomic coordinates were reconstructed and uploaded to the epitopia server to estimate the rate of amino acid substitutions at each position in the alignment of homologous proteins.

2.2.12 Mice immunization

Male 6-8 week old BALB/c mice (5 animals per group) were obtained from the Animal Facility, Department of Microbiology and Immunology, The University of Melbourne. 50μg of peptide immunogen was suspended in PBS and emulsified in a 1:1 ratio of complete Freund's adjuvant (Sigma-Aldrich, USA) for the first dose or incomplete Freund's adjuvant (Sigma-Aldrich, USA) for the boost. The animals were injected subcutaneously at the base of the tail with the vaccines in 100uL of solution. There were 28 days between the prime and boost of the vaccine and the animals were bled on day 0, 10 and 38. Sera was separated and stored at -20°C until further use.

2.2.13 Statistical analysis

Statistical analyses of all data were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Data derived from at least two independent assays are presented as mean ± standard error of the mean (SEM). A P value of <0.05 was considered statistically significant for all parameters and the confidence interval (CI) was 95%. Mean LNI's were compared to detect significant differences between antibody titres to viruses using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. The statistical analyses of the mice antibody titres were carried out using two-tailed Student's t –test and each of the resultant P values for a particular comparison is shown in the appropriate text or in the figure legend. Mean $FRNT_{50}$ values were compared by one-way ANOVA followed by Tukey's honestly significant difference (HSD) multiple comparison test with significance level alpha (P) set at 0.05.

Chapter 3

Serological surveillance and neutralization pattern of convalescent sera from primary and secondary dengue patients

3.1 Introduction

Dengue virus (DENV) is a medically important arthropod-borne virus, causing the most prevalent re-emerging infectious disease of 21st century, dengue fever. Approximately 100 countries are endemic and one third of world's population is at risk of becoming infected by DENV due to the distribution of their principal vector, *A. aegypti* (Gubler, 1998). Vector control measures, better understanding of epidemiological surveillance and advances in post exposure treatment helped to greatly reduce the dengue disease burden following World War II. However, the last three decades have seen a dramatic increase in disease severity due to endemic DENV outbreaks along with other pathogenic flaviviruses (Mackenzie *et al.,* 2004; Solomon and Mallewa, 2001). Over 3.5 billion people, 40% of the world's population, living in DENV-endemic areas are at risk of infection, resulting in an estimated 50-100 million infections annually (WHO, 2012).

DENV's global emergence and re-emergence may be a result of multiple factors including increased distribution and density of the vector, global climate change, increased movement of people to endemic areas as tourists and microevolution of the more virulent viral strains replacing the less virulent genotypes. All of these contributing factors have led to overlapping co-circulation of the four DENV serotypes, resulting in a greater number of re-infections. Dengue vaccine development has been in progress over the last 30 years, however, the pathological complexity of disease and the need to control four virus serotypes concurrently have hindered the progress. Though promising vaccine candidates are in development and a few of them in early phase clinical trials, a licensed vaccine for human use appears to be some time away (Whitehead *et al.,* 2007; Murrell *et al.,* 2011).

Consisting of four related but antigenically distinct serotypes (DENV-1, -2, -3, and -4), infection with any single serotype appears to provide life-long immunity, however cross-protection of other DENV serotypes is limited and temporary (Calisher

et al., 1989; Sabin, 1952; Tesh *et al.*, 2002). Disease severity associated with DENV secondary infection makes dengue vaccine development challenging, as such a vaccine should induce a robust immune response against the four serotypes in naive as well as individuals with previous infections. DENV infections in humans range from asymptomatic to acute self-limiting febrile illness known as dengue fever (DF) or a more severe life-threatening dengue hemorrhagic fever (DHF) and an acute vascular permeability syndrome, leading to dengue shock syndrome (DSS) (Gubler, 1997). The severity of DSS is age-dependent with vascular leakage being most severe in young children. Primary infections in adults with each of the four DENV serotypes often result in DF. However, dengue infections are frequently observed with bleeding that can lead to severe haemorrhage (Guzman *et al.*, 2010).

The DENV genome is a single-stranded positive-sense RNA molecule of approximately 11 kb in size. It is translated into a single polyprotein, which is cleaved by proteases into three structural proteins: capsid (C), pre-membrane/membrane (prM/M) and envelope (E) proteins, and seven non-structural proteins (Lindenbach and Rice, 2003; Kuhn *et al.,* 2002). Mature virions contain an ER-derived lipid bilayer covered with a dense lattice of membrane-bound prM/M and E proteins, organized into dimers on its surface (Kuhn *et al.,* 2002). DENV and all flavivirus E proteins contain three structural and functional domains, EDI, EDII and EDIII (Modis *et al.,* 2003; Rey *et al.,* 1995). The E protein is the major antigenic structural protein, which elicits a humoral immune response and therefore plays a major role in antibody-mediated neutralization of the virus. In addition, the E protein contains a highly conserved internal fusion peptide and the cellular receptor-binding motifs; both are essential for viral infectivity via receptor-mediated endocytosis (Allison *et al.,* 2001; Kuhn *et al.,* 2002; Lindenbach and Rice, 2003; Chin *et al.,* 2007).

Infection with any DENV serotype leads to the production of a wide spectrum of immunoglobulins that can be, 1) cross-reactive to all flaviviruses, 2) recognize serocomplexes of different subsets of flaviviruses, 3) DENV-group specific, 4) DENV serotype-specific. The broadly cross-reactive antibodies do not demonstrate cross-protection between the flaviviruses and this is classified as non-neutralizing (Chambers *et al.,* 1989; Oliphant *et al.*, 2007; Sabin, 1952; Apt *et al.,* 2006; Stiasny *et al.*, 2006; Tesh *et al.,* 2002). DENV complex and sub-complex cross-reactive antibodies vary in their neutralizing capabilities (Gromowski *et al.,* 2008; Roehrig *et al.,* 1998; Sukupolvi-

Petty *et al.,* 2007) and these antibodies can provide cross-protection between DENV serotypes (Sabin, 1952). However, secondary infection with a heterologous DENV serotype stimulates a broadly cross-reactive anamnestic immune response, which is not efficiently cross protective and sometimes can lead to DHF/DSS via a phenomenon called antibody-dependent enhancement of disease (ADE) (Thein *et al.,* 1997; Fried *et al.,* 2010; Takada and Kawaoka, 2003).

Though the first appearance of dengue in Australia is reported to have occurred in 1873 in eight infected travellers from Mauritius, epidemics were reported for the first time in 1905 in the state of New South Wales (Lumley and Taylor, 1943). For the next 70 years, a few isolated outbreaks have been reported in New South Wales, the North Coast, Western Australia and the Northern Territory (Russell *et al.,* 1984; Doherty, 1957; Doherty *et al.,* 1967; Whelan, 1991; Mclean and Magrath, 1959). In 1981-82, a DENV-3 outbreak in North Queensland was reported with several hundred serologically confirmed cases from Cairns, Townsville and Thursday Island (Guard *et al.,* 1984; Kay *et al.,* 1984). The occurrence of DENV-1 was reported during an outbreak in 1990-91, which was followed by a large outbreak of DENV-2 in Townsville and Charters Tower during 1992-93 with more than 900 cases of dengue fever reported (Streatfield *et al.*, 1993; Mcbride *et al.*, 1998a). Several isolated outbreaks of all four serotypes of DENV occurred over the following years, however, large outbreaks of DENV-2 have been reported in Queensland in1996-97 with 208 confirmed cases and 2003-04 with more than 800 confirmed cases (Hanna *et al.*, 1998; Hanna *et al.*, 2003; Hanna *et al.*, 2006; Mcbride, 2005). The most recent and one of the largest epidemics in the last 50 years occurred in Cairns, Townsville and surrounding suburbs during 2009 with more than 950 cases reported with DENV-1, -2 and -3 (CDC, 2009).

The above epidemiologic data along with the fact that candidate DENV vaccines must eventually be evaluated in adult populations in DENV-endemic regions highlight the need to generate more information on DENV virus infections in adults. Despite the fact that DENV vaccines are entering large scale clinical testing, little is known about the relationship between the binding properties of DENV antibodies in human immune sera and the functional outcome of these interactions.

The presence of cross-reactive immunoglobulins in patient sera can create difficulty for serodiagnosis and serosurvey, especially in disease endemic regions where multiple flaviviruses are co-circulating and demonstrate a cross-reactive

immunoglobulin G (IgG) response accumulated over a lifetime. Antibody cross-reactivity is most problematic following secondary infections, as it is difficult to differentiate between antibodies elicited by primary and secondary infections (Kuno, 2003; Roberson *et al.*, 2007). In addition, ADE is of greatest *in vivo* concern with secondary DENV infections, where sub-neutralizing levels of heterologous antibodies lead to severe pathogenic manifestations (DHF/DSS) (Gubler, 2002a; Guzman and Kouri, 2008; Onlamoon *et al.,* 2010). An important research agenda necessary to successfully address the DENV global public health challenge is to improve our understanding of humoral immune responses to DENV infection. The neutralizing antibody response and the relative quantities of such antibody populations after viral exposure and their role in protection needs to be addressed (Farrar *et al.,* 2007; Goncalvez *et al.,* 2007; Halstead, 2007; Sukupolvi-Petty *et al.,* 2007).

Our current understanding of the interactions between DENV and neutralizing antibody level is mostly based on studies with mouse monoclonal antibodies (MAbs). DENV-neutralizing mice MAbs have been used to map all three domains of the E protein (Crill and Roehrig, 2001; Gromowski, 2007; Lai *et al.,* 2008; Roehrig *et al.,* 1998; Sukupolvi-Petty *et al.,* 2007; Gromowski *et al.,* 2008). The response of human antibody repertoire to DENV has been well documented in literatures (Beltramello *et al.,* 2010; Dejnirattisai *et al.,* 2010; de Alwis *et al.,* 2012). In addition, the epidemiology of dengue fever and its relationship with neutralizing antibody levels in sera from DENV-infected local populations following outbreaks must be studied to have a better understanding of the humoral immune responses with respect to dengue infections. As an initial step towards this goal, a retrospective study was performed to measure the level and specificity of DENV neutralizing antibodies from adults living in Cairns and surrounding areas who had recovered from either a primary and/or secondary DENV infection(s).

3.2 Results

3.2.1 Study population

People identified as having previous infections based on their infection history or during past outbreaks at Cairns, Queensland, Australia and/or during foreign travel were asked to participate in the study. The study was approved by the Cairns and Hinterland Health Service District Human Research Ethics Committee, and the Human Research Ethics Committee of Swinburne University of Technology (Protocol No. HREC/10/QCH/17-646; SUHREC- 2010/158). Where possible, the infecting serotype of the virus was determined during previous outbreaks by the presence of NS1 antigen in acute blood samples and RT-PCR, and the patient history was recorded in the Cairns Base Hospital.

Volunteers identified as having previous infections based on hospital records and previous history were included in the study following written consent, and the sera were collected and tested for the presence of dengue IgG and IgM antibodies using PanBio dengue indirect IgG and capture IgM tests following manufacturer's instruction by Prof. John McBride, Cairns Base Hospital, James Cook University, Queensland. A total of 40 sera from DENV infected humans were used to examine the neutralization pattern of pre-existing DENV antibodies against all 4 prototype strains of DENV. Negative control sera were included in all our studies. The age of the study subjects ranged from 35 - 86 with a mean age of 60.8. The age distribution of different dengue cases included in this study is shown in Table 3.1. The majority of the study subjects were more than 50 years old (80% of total study population). The interval between time of infection and sample collection varied from 1 to 68 years.

3.2.2 Clinical features

Clinical manifestations were mainly DF except two subjects who had been diagnosed with DHF. WHO set criteria has been followed for the classification of disease (WHO, 2009). The DENV serotypes identified were DENV-2 (4), DENV-3 (10) and DENV-4 (1); DENV-3 was found to be the major serotype identified with 66.6% of the test subjects followed by DENV-2 with 26.6 % (Table 3.2). A secondary DENV infection was seen in 30 % (12 cases) of study subjects. Among 28 patients (70%)

diagnosed to have primary infections, 8 (28.5%) were identified to have been infected with DENV-3 and 3 (7%) had a DENV-2 infection.

Table 3.1. Age distribution of dengue cases

Category	Age, years			
	35-45	46-55	56-65	>66
Dengue cases	4	12	10	14
Percentage (%)	10	30	25	35

Table 3.2. Dengue fever pattern by type of infecting dengue serotype

Virus type	Primary	Secondary	Total
DENV-2	3	1	4
DENV-3	8	2	10
DENV-4	1	0	1
Unknown	16	9	25
Total	28	12	40

3.2.3 Antibody response pattern of cases with DENV-2 infection

The IgG from all sera were purified to study the cross neutralization ability in different DENV serotypes. Neutralizing antibodies to DENV were measured by a constant antibody-varying virus plaque-reduction neutralization test. The prototype DENV-1 to -4 working stock was determined based on viral plaque assay. Working dilutions of the purified antibody were experimentally standardized and a concentration of 10 μg/ml was seen to produce clear and countable plaques. The same IgG concentration was used in all experiments. Seroconversion was defined as Log_{10} Neutralization Index (LNI) and mean LNI of ≥ 1.000 from two independent assays was considered to indicate positive neutralization.

All 4 volunteers representing the DENV-2 assay panel had classical dengue fever. The age of the volunteers ranged from 35-57 years with a mean of 48.7, and the maximum interval between year of infection and sample collection was 17 years (Table 3.3). All but one had a primary dengue infection. The mean LNI against DENV-2 (log_{10} 2.340) was significantly different ($p < 0.05$) when compared to DENV-1, -3, and -4. All samples were able to show neutralizing activity against DENV-2 with a maximum neutralization index of log_{10} 3.107 (sample no. 3), followed by log_{10} 3.069 (sample no. 15). This was equivalent to a 1000-fold reduction in the virus titre. In addition, sample no. 3 also neutralized DENV-3 by 2.171 log_{10}; this result was expected as the volunteer was diagnosed with a secondary DENV infection, therefore the primary infection may have been with DENV-3. The interval between time of DENV-2 infection and sample collection did not have any influence on antibody neutralization, however this factor correlated to the year of previous DENV-2 outbreaks. This was supported by the neutralizing ability of sample no. 19 to neutralize DENV-2 by log_{10} 2.013 and cross neutralize DENV-3 by log_{10} 1.016, even though the infection occurred 17 years earlier.

Table 3.3. Neutralizing antibody response of DENV-2 infected patient's IgG against 4 DENV prototype strains

Sera sample Id	Age (years)	Infection/ sample collection Interval (years)	Place of infection	Infected DENV serotype	Nature of disease DF/DHF*	Log_{10} neutralization index** against virus serotype				Primary or Secondary Infection
						DENV-1	DENV-2	DENV-3	DENV-4	
3	57	5	Cairns	2	DF	0.968	3.107	2.171	0.269	Secondary
15	51	1	Borneo	2	DF	0.124	3.069	0.928	0.894	Primary
19	52	17	Townsville	2	DF	0.360	2.013	1.016	0.128	Primary
33	35	1	Mooroobool	2	DF	0.919	1.939	0.928	0.384	Primary

* DF- Dengue fever, DHF- Dengue hemorrhagic fever

** A log neutralization index of 1.000 was considered as a cut-off value for positive neutralization

3.2.4 Antibody response pattern of cases with DENV-3 infection

There were 10 volunteers with DENV-3 infection and 8 of them had a primary infection. The age of the volunteers varied from 35-85 years with an average of 56.5. The neutralization index against DENV-3 ranged from $\log_{10}$ 1.257 to 2.970 (Table 3.4). The interval between infection and sample collection for all volunteers was within 1-2 years. None of the volunteers with primary infection showed cross neutralization against DENV-1 and DENV-4; however sample no. 12 showed neutralization against DENV-2 by $\log_{10}$ 1.083. Among the primary infections, the mean LNI against DENV-3 ($\log_{10}$ 1.978) was significantly different ($p < 0.05$) than that of DENV-2 ($\log_{10}$ 0.934), DENV-1 ($\log_{10}$ 0.3158) and DENV-4 ($\log_{10}$ 0.547) (Fig.3.1). Though the number of samples was inadequate to draw a clear pattern of neutralization levels corresponding to age of the volunteers, the observation indicated that patients with age 50±10 showed considerable seroconversion when compared to other age groups. Within volunteers who had a heterotypic infection, sample no. 16 had neutralizing antibodies against all four serotypes. Interestingly, seroconversion against DENV-3 ($\log_{10}$ 1.818) was slightly lower than DENV-2 ($\log_{10}$ 1.859) and significantly different against DENV-1 and -4.

Table 3.4. Neutralizing antibody response of DENV-3 infected patient's IgG against 4 DENV prototype strains

Sera sample Id	Age (years)	Infection/ sample collection Interval (years)	Place of infection	Infected DENV serotype	Nature of disease DF/DHF*	Log_{10} neutralization index** against virus serotype				Primary or Secondary Infection
						DENV-1	DENV-2	DENV-3	DENV-4	
1	50	2	Cairns	3	DF	0.158	0.928	2.970	0.943	Primary
4	84	1	Cairns	3	DF	0.230	0.874	1.691	0.096	Primary
8	45	2	Cairns	3	DF	0.770	0.972	1.990	0.435	Primary
9	57	1	Cairns	3	DF	0.322	0.988	2.045	0.928	Primary
12	42	1	Cairns	3	DF	0.481	1.083	2.951	0.451	Primary
13	85	1	Cairns	3	DF	0.212	0.888	1.344	0.491	Primary
16	46	1	Cairns	3	DF	1.100	1.859	1.818	1.161	Secondary
20	64	1	Cairns	3	DF	0.750	1.262	1.496	1.111	Secondary
36	57	2	Whitfield	3	DF	0.272	0.920	1.573	0.176	Primary
40	35	2	Cairns North	3	DF	0.081	0.818	1.257	0.859	Primary

* DF- Dengue fever, DHF- Dengue hemorrhagic fever ** A log neutralization index of 1 was considered as a cut-off value for positive neutralization

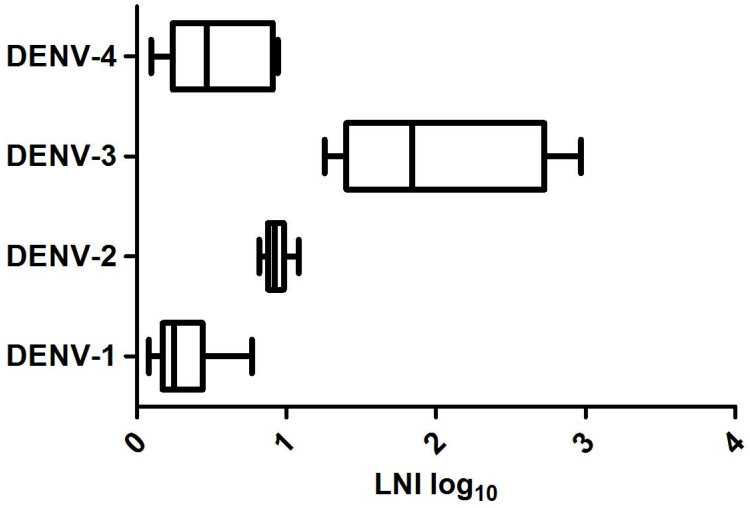


Fig. 3.1. Seroconversion pattern of subjects with primary DENV-3 infection against 4 DENV prototypes. Purified IgG (2 μg) from sera of 8 volunteers with primary DENV-3 infection was used to test the virus neutralizing ability against 4 DENV prototype viral strains, DENV-1 (Hawaii), DENV-2 New Guinea C (NGC), DENV-3 (H87) and DENV-4 (H241) in an *in vitro* constant antibody-varying virus neutralization test employing BHK-21 cells. The neutralization was expressed as the log neutralization index (LNI) with mean LNI value against each serotype represented as a vertical line. One-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test has shown that the mean LNI against DENV-3 was greater than other serotypes ($p<0.5$).

3.2.5 Seroconversion of volunteers having primary and secondary infection with unknown serotype of infection

Twenty-five volunteers were included in this study where the infecting DENV serotype was not known. Of all 25 cases, 2 were diagnosed with DHF. The age of the volunteers varied from 47-86 years with an average of 64.8. Apart from cases infected in Australia, we had subjects with possible infection sites from Singapore, Thailand and Papua New Guinea. The interval between year of infection and sample collection varied from 1 year to 68 years. There were 16 volunteers classified to have had a primary infection and 9 had secondary infections. Among the volunteers with primary infection, the number shown to have seroconversion against DENV-1, -2 and -3 were 1, 7 and 6, respectively (Table 3.5). None of the samples were found to be neutralizing against DENV-4. The volunteers diagnosed with DHF exhibited neutralizing antibodies ($\log_{10}$ 1.161) against DENV-3. This may be associated with the 1973 DENV outbreak in Singapore as the prevalent serotype circulating during this particular outbreak was DENV-3. Seroconversion levels of volunteers from Papua New Guinea were limited to DENV-2, which was related to the major DENV-2 outbreaks during 1976 and 1983. Those volunteers did not show cross-neutralizing activity against any other DENV serotypes. Two volunteers (samples no. 10 and 11) who had the greatest interval between year of infection and sample collection (65 and 68 years) did not show a positive LNI for any DENV serotypes tested suggesting that the antibody level to the infecting serotype had diminished. However, when compared to all other serotypes, the LNI against DENV-1 was higher in both samples ($\log_{10}$ 0.849 for sample no. 10 and $\log_{10}$ 0.911 for sample no. 11).

Table 3.5. Antibody neutralization profile of primary infection volunteers with unknown serotype of infection

Sera sample Id	Age (years)	Infection/sample collection Interval (years)	Place of infection	Infected DENV serotype	Nature of disease DF/DHF*	Log_{10} neutralization index** against virus serotype				Primary or Secondary Infection
						DENV-1	DENV-2	DENV-3	DENV-4	
2	53	9	Cairns	Unknown	DF	0.125	2.300	0.902	0.204	Primary
5	62	37	Singapore	Unknown	DHF	0.420	0.818	1.161	0.161	Primary
7	64	1	Cairns	Unknown	DF	0.230	0.952	1.868	0.169	Primary
10	86	65	Brisbane	Unknown	DF	0.849	0.161	0.232	0.420	Primary
11	83	68	Cairns	Unknown	DF	0.911	0.190	0.377	0.168	Primary
18	55	7	Cairns	Unknown	DF	0.146	1.941	0.844	0.191	Primary
21	51	26	Freshwater	Unknown	DF	0.448	0.267	0.924	0.613	Primary
22	68	2	Port Douglas	Unknown	DF	0.190	0.411	1.118	0.587	Primary

* DF- Dengue fever, DHF- Dengue hemorrhagic fever

** A log neutralization index of 1 was considered as a cut-off value for positive neutralization

Table 3.5. Cont.

Sera sample Id	Age (years)	Infection/ sample collection Interval (years)	Place of infection	Infected DENV serotype	Nature of disease DF/DHF*	Log10 Neutralization index** against virus serotype				Primary or Secondary Infection
						DENV-1	DENV-2	DENV-3	DENV-4	
23	66	2	Port Douglas	Unknown	DF	0.176	0.522	1.134	0.363	Primary
24	67	6	Portsmith	Unknown	DF	0.204	3.358	0.892	0.463	Primary
27	71	33	Papua New Guinea	Unknown	DF	0.096	1.795	0.493	0.192	Primary
28	69	1	Edgehill	Unknown	DF	0.130	0.839	3.390	0.389	Primary
29	47	17	Bungalow	Unknown	DF	0.449	2.129	0.412	0.190	Primary
31	62	25	Papua New Guinea	Unknown	DF	0.283	2.356	0.397	0.096	Primary
35	52	3	Stratford	Unknown	DF	0.146	0.543	3.601	0.602	Primary
37	56	24	Tahiti	Unknown	DF	2.000	0.348	0.285	0.558	Primary

* DF- Dengue fever, DHF- Dengue hemorrhagic fever

** A log neutralization index of 1 was considered as a cut-off value for positive neutralization

Among the volunteers with secondary infection, the mean LNI was higher against DENV-3 (log_{10} 1.341) and DENV-2 (log_{10} 1.203) when compared to DENV-1 (log_{10} 0.323) and DENV-4 (log_{10} 0.641) (Fig 3.2). Though the variance was not statistically significant, it was observed that the predominant neutralizing antibodies were targeted to DENV-2 and DENV-3 (Table 3.6). When excluding the volunteers infected in Thailand, and Papua New Guinea, the mean LNI against DENV-3 slightly increased by log_{10} 1.492. Considering the recent DENV-3 outbreaks in Queensland, this could be associated to DENV-3 infections; however the sero-prevalence did not appear to have switched to DENV-3.

The neutralizing activity of IgG from a volunteer infected with DENV-4 during 2004 was also examined, and the primary infection sera showed a neutralizing antibody index of log_{10} 2.573 against DENV-4, the highest level against DENV-4 of all the volunteers. Overall, among all the volunteers infected in Queensland irrespective of infected DENV serotype, the mean neutralizing antibody index after secondary infection was log_{10} 0.477 (DENV-1), log_{10} 1.422 (DENV-2), log_{10} 1.463 (DENV-3), and log_{10} 0.692 (DENV-4). Although the number of volunteers tested was limited, the results indicated that the seroconversion was predominantly against DENV-2 and -3 as seen in Figure 3.2.

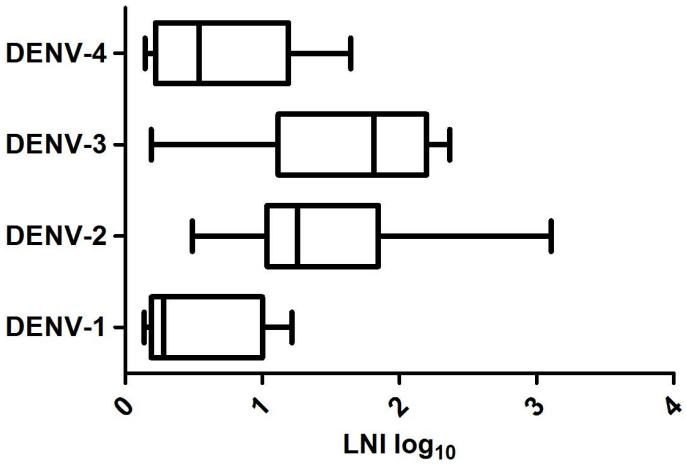


Fig. 3.2. Neutralizing antibody levels of secondary infection volunteers with unknown serotype of infection. Purified IgG (2 μg) from sera of 9 volunteers with secondary dengue infection was used to test the virus neutralizing ability against 4 DENV prototype viral strains, DENV-1 (Hawaii), DENV-2 New Guinea C (NGC), DENV-3 (H87) and DENV-4 (H241) in an *in vitro* constant antibody-varying virus neutralization assay employing BHK-21 cells. The neutralization was expressed as the log neutralization index (LNI) with mean LNI value against each serotype represented as a vertical line.

Table 3.6. Antibody neutralization profile of secondary infection volunteers with unknown serotype of infection

Sera sample Id	Age (years)	Infection/ sample collection Interval (years)	Place of infection	Infected DENV serotype	Nature of disease DF/DHF*	Log_{10} Neutralization index** against virus serotype				Primary or Secondary Infection
						DENV-1	DENV-2	DENV-3	DENV-4	
6	79	2	Cairns	Unknown	DF	0.272	1.176	1.272	0.176	Secondary
14	71	6	Cairns	Unknown	DF	0.139	1.257	1.859	1.285	Secondary
17	84	7	Cairns	Unknown	DF	0.286	1.110	0.190	0.235	Secondary
25	50	10	Thailand	Unknown	DF	0.146	1.242	0.543	1.146	Secondary
26	66	40	Papua New Guinea	Unknown	DF	0.282	1.457	1.082	0.060	Secondary
30	78	1	Earlville	Unknown	DF	0.190	0.491	2.286	0.587	Secondary
32	65	1	North Cairns	Unknown	DF	1.216	1.844	2.367	0.146	Secondary
34	51	1	Kewarra Beach	Unknown	DHF	0.190	0.805	1.817	0.493	Secondary
39	65	5	Machans Beach	Unknown	DF	0.190	1.449	0.655	1.645	Secondary

* DF- Dengue fever, DHF- Dengue hemorrhagic fever

** A log neutralization index of 1 was considered as a cut-off value for positive neutralization

3.3 Discussion

There has been increased attention recently to examine the complex polyclonal human immune responses to flavivirus infection (de Alwis *et al.*, 2012). It is important to understand the epidemiology of DENV infections in an adult population where the majority of individuals have been exposed to one or more DENV serotypes. The results presented in this study add to this nascent body of work with DENV-specific antibody responses among people infected with different DENV serotypes. Earlier studies measuring DENV-2-specific IgG titres from six DENV-2 infected patients revealed antibody levels ranging from 10^4 to 10^6 (Stiasny *et al.,* 2006). These sera had low to no IgM, consistent with theirs being convalescent phase sera (Stiasny *et al.,* 2006). Another study involving 2 different groups of convalescent sera collected after 4-8 and 22 years of infection had a significant increase in homologous neutralizing antibody levels (Guzman *et al.*, 2007). Similar magnitudes and variation were seen in this study.

The predominant neutralizing antibodies identified in the current study were against DENV-2 and DENV-3 suggesting that these were the prevalent serotypes circulating in the region where our samples were collected (Hanna *et al.,* 2006). In addition, the most recent outbreak in Cairns during 2009 was due to DENV-3 (CDC, 2009). It is also interesting that the primary infection with DENV-3 led to one DHF case, which is not a common phenomenon in dengue infection Vaughn *et al.,* 2000). This may be associated with the fact that specific virus serotypes and genotypes may replicate more readily in specific population groups to cause DHF even in primary infections (rare) or enhance more readily in the presence of pre-existing antibody (Vaughn *et al.,* 2000).

Sera from volunteers who had recovered from both primary and secondary DENV infections were investigated and IgG titres against primary infection were found to be greater than secondary infections. Similar results were reported in a study with late acute-early convalescent phase sera from DENV-2-infected patients from Taiwan although total immunoglobulin was assayed and the authors did not distinguish between IgM and IgG in their assays (Lai *et al.,* 2008). The improvement in homotypic neutralizing antibody titre and decrease in heterotypic neutralizing antibody titre in the

current results may be due to affinity maturation (Halstead and Marchette, 2003; Guzman *et al.,* 2007).

The neutralizing antibody levels of sera from secondary infections (Sera no. 3 and 20) were higher against secondary infecting virus. However, it has been shown earlier that the neutralizing antibody dominance to the primary infecting virus occurs within a week to multiple weeks after secondary infection with a heterologous DENV serotype (Kuno, 2003). Although the numbers are too small to make any significant conclusions, this can be attributed to the argument that the patients might still be in the early convalescent phase and have not yet switched to antibody dominance (Kuno, 2003). Interestingly, the two volunteers with a history of unknown primary DENV infection 65-68 years earlier did not show positive neutralization against any DENV serotypes tested but the LNI against DENV-1 was higher when compared to other serotypes. These observations may be attributed to the fact that both the volunteers might have been infected during 1942-1945 outbreak reported common among U.S. army soldiers stationed in Queensland and Northern Territory (Lumley and Taylor, 1943) and their movement through steam trains. In addition, it may also be correlated to DENV-1 serotype isolated from U.S. soldiers after World War II (Sabin, 1952).

An earlier study of serum samples from US military personnel with Japanese encephalitis virus (JEV) infection shown that the LNI increased from a mean of 1.7 to 3.5 (Halstead and Russ, 1962). They measured the antibody levels of convalescent sera 1-5 years after infection. This earlier study and the present study are unique in that they measured qualitative attributes of human antibodies for long intervals after infection with wild-type flavivirus. The preliminary data presented here suggest a continuous process of selection of populations of dengue virus antibodies with increasing homologous reactivity and a concurrent decrease in heterotypic cross-reactions. Clearly, more analyses will be needed to determine if this is a general phenomenon.

The present study has some limitations. First, relatively small numbers of DF and DHF cases did not allow a more detailed survey of the pattern of disease occurrence between different age groups, though majority of volunteers 75 years of age and above showed low levels of neutralizing antibodies. Second, because convalescent sera from several different previous outbreaks dated back as far as 1942-43 were used, the infecting DENV serotype was not known in several volunteers, which did not allow a detailed serotype-wide study between primary and secondary infections. The

conclusions and questions stemming from the results presented in this thesis begin to unravel the complex polyclonal humoral immune responses to primary and secondary DENV infections and provide a direction for future studies in this field that will be essential both for improving our understanding of DENV pathogenesis and for the development and testing of candidate DENV vaccines.

Chapter 4

Epitope mapping of DENV-E protein

4.1 Introduction

The envelope protein (E) of DENV elicits the majority of the protective immune response and is the site where the majority of antigenic determinants reported so far have been located (Kuhn *et al.,* 2002; Lindenbach and Rice, 2003; Oliphant *et al.,* 2006). The epitopes harboured within distinct regions of the E protein stimulate production of neutralizing antibodies that can inhibit virus attachment to cells (Gratz, 2004). The E protein contains cellular receptor-binding motifs and a highly conserved internal fusion peptide; both are essential for viral infectivity via receptor-mediated endocytosis (Allison *et al.,* 2001; Kuhn *et al.,* 2002; Lindenbach and Rice, 2003). Murine monoclonal antibodies (MAbs) have been widely used for dissection of epitope specificity and identifying biological characteristics of antibody responses to the E protein (Crill and Roehrig, 2001; Stiasny *et al.,* 2006; Gromowski, 2007; Sukupolvi-Petty *et al.,* 2007; Gromowski *et al.,* 2008).

DENV and all flavivirus E proteins contain three structural and functional domains (Rey *et al.,* 1995; Modis *et al.,* 2003). E protein domain I (EDI) is the central domain containing virus-specific cross-reactive epitopes and the neutralizing antibody epitopes of DENV-1 and -4 have been reported on EDI (Beasley and Aaskov, 2001; Lai *et al.,* 2007). EDII is the dimerization domain, which contains the internal fusion peptide. The highly conserved fusion peptide forms the epicentre of a series of overlapping immunodominant cross-reactive epitopes eliciting predominantly non- or weakly neutralizing antibodies (Allison *et al.,* 2001; Crill and Chang, 2004; Oliphant *et al.,* 2006; Lai *et al.,* 2008; Crill *et al.,* 2009). EDIII is an immunoglobulin-like structure responsible for virus-host cell attachment and contains serotype-specific, highly protective neutralizing epitopes and DENV complex cross-reactive epitopes (Crill and Roehrig, 2001; Crill and Chang, 2004; Stiasny *et al.,* 2006; Gromowski, 2007; Sukupolvi-Petty *et al.,* 2007; Gromowski *et al.,* 2008).

Serological studies of dengue patients have shown that binding of most primary polyclonal anti-E antibodies were cross-reactive and specific, whereas secondary infections leads to broad spectrum of anti-E antibody binding (Lai *et al.,* 2008). Efforts

to isolate large panels of DENV-reactive antibodies from human donors indicated that the majority of EDI and EDII-reactive antibodies isolated from primary infections were serotype-specific, whereas those isolated from secondary infections were all cross-reactive (Beltramello *et al.*, 2010). Studies of DENV-infected immune sera have also shown a prevalence of EDI and EDII-specific neutralizing antibody epitopes and a much lower abundance of EDIII-specific epitopes (Oliphant *et al.,* 2007; Lai *et al.,* 2008; Crill *et al.,* 2009; Wahala *et al.,* 2009). In addition, depletion of EDIII-binding antibodies from DENV and WNV immune human sera made only a minor impact to the total neutralizing antibody titre (Oliphant *et al.,* 2007; Wahala *et al.,* 2009; de Alwis *et al.,* 2012).

Human antibody responses to prM in both primary and secondary infection patients were highly cross-reactive and non-neutralizing even at high concentrations, but potently enhanced the infectivity of non-infectious immature DENV over a broad range of antibody concentrations (Huang *et al.,* 2006; Beltramello *et al.,* 2010; Dejnirattisai *et al.,* 2010). These findings may be attributed to the fact that EDI/EDII-specific antibodies mainly contribute to the neutralization activity of human immune sera and EDIII/prM antibodies may not play a large role in DENV neutralization. Although invaluable insights were gained through these studies, the human antibody responses elicited by DENV infections and the target epitopes involved are not completely understood.

Epitope identification through short synthetic peptides has drawn much attention and a number of synthetic peptide-based approaches have been investigated to identify the antigenic determinants in DENV and other related flaviviruses (Leclerc *et al.*, 1987; Aaskov *et al.,* 1989; Roehrig *et al.,* 1994; Vazquez *et al.,* 2002; Amexis and Young, 2007; da Silva *et al.,* 2009; Li *et al.,* 2011). Synthetic peptides have been used to map the prM protein of DENV-2, with at least three peptides shown to be potential B-cell epitopes with a strong antibody response (Vazquez *et al.,* 2002). Screening the peptide library of DENV-3 E protein against serum from infected patients revealed five immunodominant immunoglobulin G (IgG)-specific epitopes at amino acids positions 51–65, 71–90, 131–170, 196–210 and 246–260 (da Silva *et al.,* 2009). Multiple antigenic peptides (MAPs) derived from DENV-2 E protein using a computer based *in silico* epitope prediction (MacVector^{TM}) method have shown seven neutralizing DENV-2 epitopes suggesting that the MAP platform can be used as a potential technique for

epitope identification (Amexis and Young, 2007). Recently, a multi-epitope-based strategy combining both B and T-cell epitopes of DENV-2 EDIII showed multiple neutralizing epitopes and induced cell-mediated immune response in mice (Li *et al.,* 2011).

With the advent of computational biology, some new research studies have been carried out on predictive pathobiology of dengue. Indeed, there are many bioinformatics tools that can be applied to dengue research. The identification of linear B-cell epitopes has been based on the physiochemical properties of the amino acids such as hydrophilicity, antigenicity and flexibility (Kyte and Doolittle, 1982; Parker *et al.,* 1986). Attempts were made to improve the accuracy of B-cell epitope prediction by designing more appropriate algorithms such as the Hidden Markov Model (HMM) (Larsen *et al.,* 2006) and the Artificial Neural Network (ANN) models (Saha and Raghava, 2006). Based on the aforementioned algorithms, a number of attempts to use bioinformatics tools for prediction of B-cell epitopes revealed potential antigenic epitopes on the E protein of DENV-2 and -3 at regions 80-99, 238-250, 295-307, 304-316, 333-351 and 352-368 (Amexis and Young, 2007; Mazumder *et al.*, 2007; Tambunan *et al.*, 2009; Sanchez-Burgos *et al.,* 2010; Li *et al.,* 2011). However, B-cell epitope prediction using a single predictive method is usually not sufficient to identify epitopes at a scale greater than random and a multiple step epitope prediction scheme can help increase the probabilistic odds of binding interactions in predicting candidate epitopes.

Recently, proteolytic footprinting methods such as epitope excision and epitope extraction techniques have been increasingly used in epitope mapping of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (Jeyarajah *et al.,* 1998; Parker and Tomer, 2002; Grollo *et al.,* 2006). In epitope excision methods, the protein of interest is complexed with the immobilized antibody and then subjected to enzymatic digestion. Because the antigen is bound to the antibody in its native conformation under physiological conditions, this approach allows the identification and characterization of linear and discontinuous epitopes (Parker and Tomer, 2002). In epitope extraction, a protein is first subjected to enzymatic digestion and then the peptide fragments are presented to either an immobilized antibody or an antibody in solution (Jeyarajah *et al.,* 1998; Parker and Tomer, 2002). Alternatively, a peptide library representing the entire protein of interest can be made synthetically and be probed by the antibody (Aaskov *et*

al., 1989; Roehrig *et al.,* 1994; Vazquez *et al.,* 2002; Grollo *et al.,* 2006; da Silva *et al.,* 2009; Li *et al.,* 2011). These epitope excision/extraction techniques have been used in combination with matrix-assisted laser desorption (MALDI)-time of flight (TOF) mass spectrometry for the characterization of linear epitopes. A similar epitope extraction approach to identify the epitopes recognized by antibodies from immune sera has not yet been performed for DENV.

In the present study, a panel of 34 polyclonal human sera from DENV-infected volunteers was used to study the peptide binding profile. The majority of the volunteers had been infected during recent outbreaks at North Queensland, Australia, and an epitope mapping study using these sera samples has not yet been performed. This sera panel was found to be neutralizing either a single DENV serotype or all four DENV serotypes as determined by earlier neutralization studies. The purified IgG from these sera was used to screen an overlapping peptide library of 70 synthetic peptides representing the entire E protein of DENV-2, the predominant infecting serotype in Queensland at the commencement of this study. The binding profile of each peptide against the antibody was tested in a combination of ELISA and epitope extraction. Concurrently, a multi-step computational approach was used to identify the potential B-cell epitopes for all four DENV serotypes. Most *in silico* programs available today rely on a single predictive algorithm to identify epitopes either in a single protein sequence or in a single structural protein model. Here, a sequence-based approach was used in conjunction with a structural-based approach to predict the potential epitope candidates. A combination of ELISA, epitope extraction and improved computational strategies revealed several novel linear epitopes, which provide new insights for future epitope-based dengue vaccines.

4.2 Results

4.2.1 Synthetic peptides and sera panel

An overlapping peptide library of 70 synthetic peptides (each 18 amino acids long, overlapping by 11 amino acids) representing the entire 495 amino acids of E protein of DENV serotype 2 (GenBank Accession No: ABW06583.1) was used in this study. The polyclonal human sera collected from convalescent DENV patients were used to purify the IgG and tested for their ability to cross neutralize different DENV serotypes. Those IgG samples exhibiting neutralizing ability (Log Neutralization Index ≥ 1) to either one or all four DENV serotypes (Chapter 3.2) were screened against the synthetic peptide library by ELISA and epitope extraction. The particulars of the 34 different sera samples used in this study are shown in Table 4.1.

Table 4.1. Summary of DENV immune patient sera used in this study

Infecting DENV serotype	Nature of infection	Sample ID's
DENV-2	Primary	15,19,33
DENV-3	Primary	1,4,8,9,12,13,36,40
DENV-4	Primary	38
Unknown	Primary	2,7,18,21,22,24,27,28,29, 31,35,37
DENV-2	Secondary	3
DENV-3	Secondary	16
Unknown	Secondary	6,14,25,26,30,32,34,39

4.2.2 Identification of peptide-antibody binding profile through ELISA

The survey of antibody response to DENV-2 E synthetic peptide library through direct binding ELISA showed a total of 17 of the 70 peptides (peptide 2, 16, 19, 29, 33, 38, 40, 43, 45, 47, 48, 53, 54, 64, 68, 69 and 70) with high cross reactivity against the DENV immune human polyclonal IgG. The IgG from four DENV negative individuals were used as a control and the mean absorbance of negative control plus 3 times the standard deviation (OD-0.128) was used as the cut-off line to identify the positive peptides. The reaction pattern of 34 DENV immune IgG samples against peptide 16 is shown in Fig 4.1 and the list of peptides that reacted against IgG from volunteers with DENV-2 infection are shown in Table 4.2. Among the peptides reacted, peptides 2, 16, 19, 40, 43, 45, 48, 54, 64 and 69 reacted against IgG from all DENV-2 volunteers. The IgG from a volunteer with a secondary DENV-2 infection reacted against all 17 peptides.

Among the peptides that cross-reacted against IgG from DENV-3 volunteers, peptides 2, 16, 19, 28, 40, 45, 53, 54, 64, 69 and 70 reacted with most of the samples (Table 4.3). An interesting observation was that the peptide 28 reacted against IgG from DENV-3 volunteers only. IgG from a volunteer with secondary DENV-3 infection cross-reacted with more peptides than IgG from a primary DENV-3 infection. On the other hand, the IgG from a volunteer with primary DENV-4 infection cross-reacted with only five peptides (Table 4.4). The reaction patterns of IgG from volunteers with unknown serotype of infection were also tested. The majority of the volunteers with primary DENV infection reacted against peptides 2, 16, 19, 28, 40, 45, 53, 54 and 64 (Table 4.5). IgG from volunteers with secondary infection shown a broader cross-reactivity when compared to the primary infection (Table 4.6).

Overall, 17 peptides were identified which reacted against most of the IgG samples analysed through ELISA. The relative position of these peptides on the DENV-2 E protein is shown in Fig. 4.2. These peptides were distributed in ten continuous regions along the entire E protein of DENV-2 at amino acids 8-25, 106-123, 127-144, 190-207, 225-242, 260-291, 295-347, 365-389, 442-459 and 470-495. The first 8 antigenic regions were located within the soluble E (sE) protein and antigenic regions 9 and 10 are located at the “stem” region outside sE. Peptides 2 and 16 were found within the EDI and EDII of sE, respectively. Peptide 19, corresponding to aa 127-144, is

located within the “hinge” region between EDI and EDII, and earlier studies have shown that the partial sequence (aa 127-134) of peptide 19 as an immunodominant epitope (Aaskov et al., 1989). P40 (aa 274-291) was located within the “hinge” region between EDI and EDIII, and earlier studies showed a strong reaction of MAb 4G2 against the amino acid sequences found within “hinge” region (Innis et al., 1989). Among all, the peptides 29, 33 and 38 reacted to only a few IgG samples tested and all three peptides are located within EDII.

The antigenic region corresponding to amino acids 295-347 (peptide 43, 45, 47 and 48) appeared to be the longest region identified in this study. This is adjacent to the region representing aa 365-389 (peptide 53 and 54). Both these regions are located within the EDIII domain of the sE protein. Apart from epitopes harbored on soluble E protein, four peptides were identified in two different antigenic regions representing the membrane proximal “stem” region of the E ectodomain corresponding to aa 442-459 (peptide 64) and aa 470-495 (peptide 68, 69 and 70). Interestingly, the antibody-binding pattern of the three different overlapping peptides (peptide 68, 69 and 70) was consistent against most of the IgG samples tested. The ELISA data (OD values) of each DENV immune IgG against all positive peptides identified in this study are shown in Appendix II.

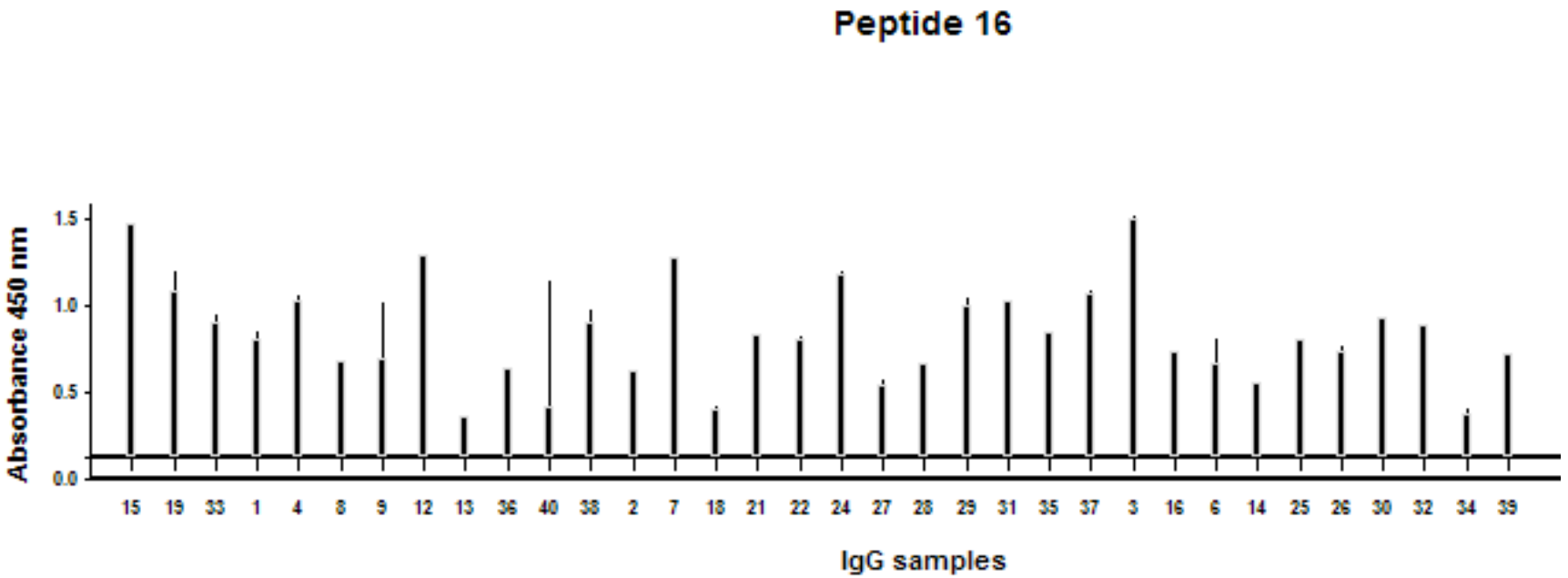


Fig. 4.1. Antibody binding profile of peptide 16 against different DENV immune patient IgG. Samples of IgG at a concentration of 20 µg/ml were used in direct binding ELISA to test the cross reactivity against peptides and the results are presented as the mean optical density of triplicates. IgG from 4 non-infected individuals were used as a negative control and the horizontal line in each figure shows the cut-off value of negative control (OD-0.128). The positive peptides were identified based on the antibody reaction above the cut-off value.

Table 4.2. Peptides reactive against DENV-2 immune human IgG

Sample Id	Nature of infection	Peptides reacted
15	Primary	2, 16, 19, 29, 38, 40, 43, 45, 47, 48, 54, 64, 68, 69, 70
19	Primary	2, 16, 19, 29, 33, 38, 40, 43, 45, 53, 54, 64, 68, 69, 70
33	Primary	2, 16, 19, 40, 43, 45, 48, 54, 64, 69
3	Secondary	2, 16, 19, 29, 33, 38, 40, 43, 45, 47, 48, 53, 54, 64, 68, 69, 70

Table 4.3. Peptides cross-reactive against DENV-3 immune human IgG

Sample Id	Nature of infection	Peptides reacted
1	Primary	2, 16, 19, 28, 40, 45, 46, 53, 54, 64, 69, 70
4	Primary	2, 16, 19, 28, 40, 45, 46, 47, 48, 54, 64, 68, 69, 70
8	Primary	2, 16, 19, 28, 40, 45, 47, 53, 54, 64, 68, 69, 70
9	Primary	2, 16, 19, 28, 40, 45, 54, 64, 69, 70
12	Primary	2, 16, 19, 28, 40, 43, 45, 46, 53, 54, 64, 69, 70
13	Primary	16, 28, 45
36	Primary	2, 16, 19, 28, 32, 38, 40, 43, 45, 53, 64, 69, 70
40	Primary	2, 16, 19, 28, 32, 33, 40, 43, 45, 48, 64
16	Secondary	2, 16, 19, 28, 29, 38, 40, 45, 46, 47, 48, 53, 54, 64, 68, 69, 70

Table 4.4. Peptides cross-reactive against DENV-4 immune human IgG

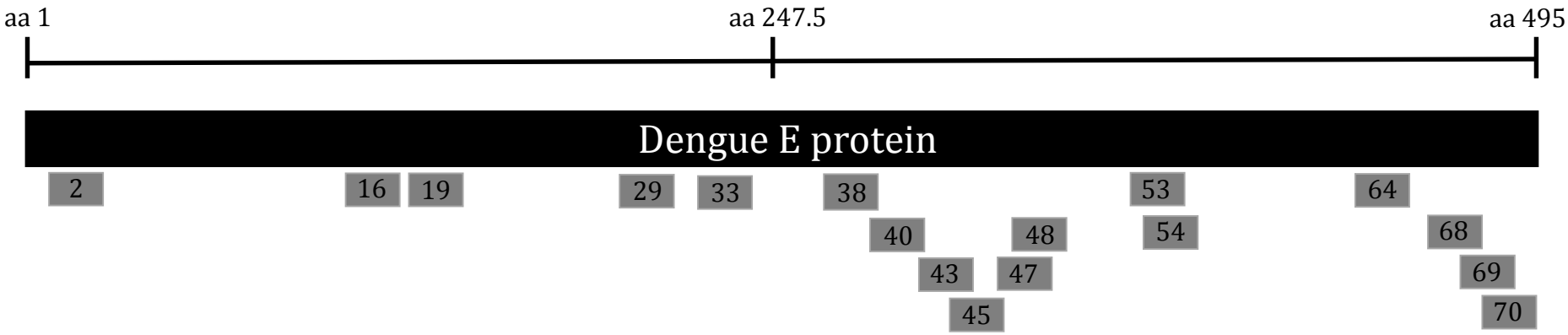
Sample Id	Nature of infection	Peptides reacted
38	Primary	2, 16, 40, 45, 64

Table 4.5. Peptides reactive against immune IgG from primary infection volunteers with unknown serotype of infection

Sample Id	Nature of infection	Peptides reacted
2	Primary	2, 16, 19, 28, 38, 40, 45, 46, 47, 48, 53, 54, 64, 68, 69, 70
7	Primary	2, 16, 19, 28, 40, 41, 45, 46, 47, 48, 53, 54, 64, 68, 69, 70
18	Primary	16, 19, 40, 45, 47, 48, 53, 54, 64, 68, 69, 70
21	Primary	2, 16, 19, 40, 45, 47, 54, 64
22	Primary	2, 16, 19, 40, 47, 54, 64
24	Primary	2, 16, 19, 28, 32, 33, 38, 40, 53, 54, 64, 68, 69, 70
27	Primary	16, 19, 45, 54, 64
28	Primary	2, 16, 19, 28, 40, 45, 53, 54, 64
29	Primary	2, 16, 19, 40, 45, 53, 54, 64
31	Primary	2, 16, 19, 28, 40, 45, 54, 64
35	Primary	2, 16, 19, 28, 40, 45, 53, 54, 64
37	Primary	2, 16, 19, 28, 40, 45, 53, 54, 64, 68, 69, 70

Table 4.6. Peptides reactive against immune IgG from secondary infection volunteers with unknown serotype of infection

Sample Id	Nature of infection	Peptides reacted
6	Secondary	16, 19, 28, 29, 40, 43, 45, 46, 47, 48, 53, 54, 64, 68, 69, 70
14	Secondary	2, 16, 19, 28, 33, 40, 45, 53, 54, 64, 69, 70
25	Secondary	2, 16, 19, 40, 43, 45, 46, 47, 53, 54, 64, 68, 69, 70
26	Secondary	2, 16, 19, 33, 40, 43, 45, 53, 54, 64, 69, 70
30	Secondary	2, 16, 19, 40, 43, 45, 46, 47, 53, 54, 64, 69, 70
32	Secondary	2, 16, 19, 28, 40, 43, 45, 46, 47, 48, 53, 54, 64, 68, 69, 70
34	Secondary	2, 16, 19, 33, 40, 43, 45, 46, 64, 69, 70
39	Secondary	2, 16, 19, 28, 40, 43, 45, 46, 47, 64



PEPTIDE NUMBER	PEPTIDE SEQUENCE
2	NRDFVEGVSGGSWVDIVL
16	GLFGKGGIVTCAMFTCKK
19	GKVVLPENLEYTIVITPH
29	VLLQMEEKAWLVHRQWFL
33	DTQGSNWIQKETLVNFKN
38	MHTALTGATEIQMSSGNL
40	SGNLLFTGHLKCRLRMDK
43	KGMSYSMCTGKFKIVKEI
45	VKEIAETQHGTIVIRVQY

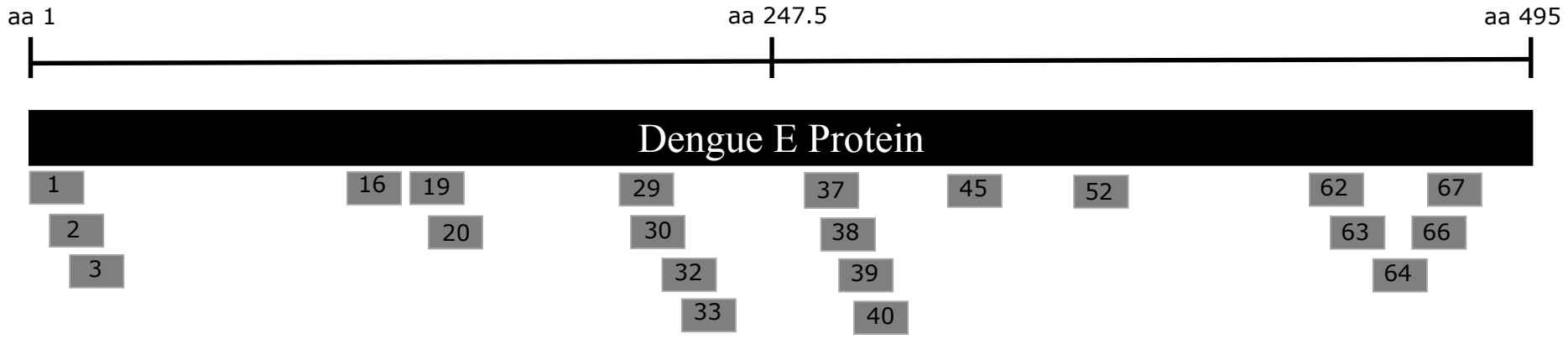
PEPTIDE NUMBER	PEPTIDE SEQUENCE
47	RVQYEGDGSPCKIPFEIM
48	GSPCKIPFEIMDLEKRHV
53	VNIEAEPPFGDSYIIIGV
54	PFGDSYIIIGVEPGQLKL
64	AIYGAAFSGVSWTMKILI
68	SRSTSLSVSLVLVGVVTL
69	VSLVLVGVVTLYLGAMVQ
70	SLVLVGVVTLYLGAMVQA

Fig. 4.2. Linear epitope map of peptides identified through ELISA. The sequence of E protein is indicated with the relative positions of 17 epitopes that were recognized by immune sera in ELISA shown as short grey bars. The peptide sequences for each of the epitopes are shown in the corresponding table.

4.2.3 Epitope extraction

The synthetic peptides were grouped into 14 pools with each pool comprising 5 peptides. In contrast to ELISA, these peptides were allowed to react with purified IgG in solution at neutral pH. The subsequent mass spectrometry showed that at least 21 peptides were able to bind to most of the IgG samples used in this study, whereas IgG from DENV negative human sera did not show reactivity against the peptides. The position of the 21 peptides on the E protein of DENV-2 is shown in Fig. 4.3. A complete list of peptides that reacted against various DENV immune IgG samples is shown in Appendix III. Based on the pattern of antibody reaction among the overlapping peptide sequences, 7 peptides were selected as immunoreactive epitopes to display the corresponding mass spectrum. For example, if there were antibody reactions against 3 consecutive overlapping peptides, the peptide in middle was selected as a representative of that particular epitope since the other two peptides would share an 11 amino acid sequence overlapping with the selected epitope. In addition, the antibody response against the selected peptides in ELISA was also considered as a criterion to select the positively reactive peptides.

Peptide 2 (NRDFVEGVSGGSWVDIVL) corresponding to aa 8-25 of E protein (EDI) reacted against all immune IgG's tested. Mass spectrometry showed the corresponding m/z value of 1949 with possible b and y ion coverage both as single and/or doubly charged ion (Fig. 4.4). A similar pattern of antibody reaction against all IgG's was seen with peptide 16 (106-GLFGKGGIVTCAMFTCKK-123) with an m/z value of 1861 (Fig. 4.5) and peptide 45 (309-VKEIAETQHGTIVIRVQY-326) with an m/z value of 2084 (Fig. 4.6). The immunodominant peptide 19 located in the "hinge" region between EDI and EDII (127-GKVVLPENLEYTIVITPH-144) and peptide 64 representing the membrane proximal "stem" region (442-AIYGAAFSGVSWTMKILI-459) of E protein also showed a positive reaction against antibodies in solution with an m/z value of 2022 (Fig. 4.7) and 1928 (Fig. 4.8) respectively. In contrast to ELISA, peptides 29 (197-VLLQMEEKAWLVHRQWFL-214) and 38 (260-MHTALTGATEIQMSSGNL-277) showed an ability to bind to several immune IgG samples in epitope extraction (Figs. 4.9 and 4.10).



PEPTIDE NUMBER	PEPTIDE SEQUENCE	PEPTIDE NUMBER	PEPTIDE SEQUENCE
1	MRCIGISNRDFVEGVSGG	38	MHTALTGATEIQMSSGNL
2	NRDFVEGVSGGSWVDIVL	39	ATEIQMSSGNLLFTGHLK
3	VSGGSWVDIVLEHGSCVT	40	SGNLLFTGHLKCRLRMDK
16	GLFGKGGIVTCAMFTCKK	45	VKEIAETQHGTIVIRVQY
19	GKVVLPENLEYTIVITPH	52	VTEKDSPVNIEAEPPFGD
20	NLEYTIVITPHSGEEHAV	62	VFTSIGKALHQVFGAIYG
29	VLLQMEEKAWLVHRQWFL	63	ALHQVFGAIYGAAFSGVS
30	KAWLVHRQWFLDLPLPWL	64	AIYGAAFSGVSWTMKILI
32	LPWLPGADTQGSNWIQKE	66	KILIGVIITWIGMNSRST
33	DTQGSNWIQKETLVNFKN	67	ITWIGMNSRSTSLSVSLV
37	LGSQEGAMHTALTGATEI		

Fig. 4.3. Linear epitope map of peptides identified through extraction. The sequence of E protein is indicated with the relative positions of 21 epitopes that were recognized by immune sera in epitope extraction shown as short grey bars. The peptide sequences for each of the epitopes are shown in the corresponding table.

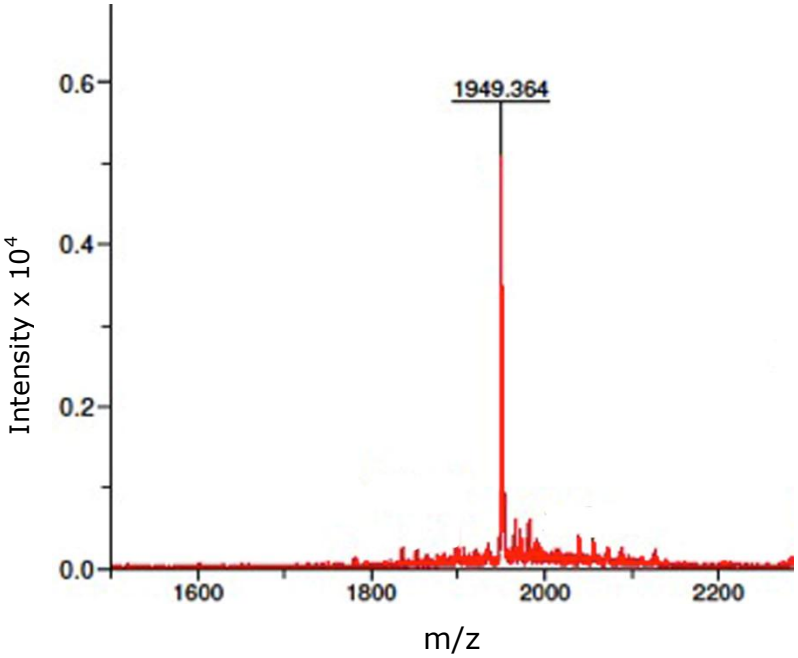


Fig. 4.4. Mass spectrum of peptide 2 identified through MALDI-ToF mass spectrometry corresponding to m/z 1949. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 1949 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 1949 unequivocally defines it as deriving from peptide 2 sequence (NRDFVEGVSGGSWVDIVL) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
N	-	-	1948.96	975.48
R	-	-	1834.93	917.97
D	386.17	-	1678.83	-
F	533.24	-	1563.80	-
V	632.31	-	-	-
E	761.34	-	-	659.32
G	818.36	-	-	594.80
V	917.44	459.21	1131.61	-
S	1004.48	502.73	1032.53	-
G	1118.52	531.24	945.50	-
G	-	559.75	888.47	444.74
S	1205.54	603.27	831.45	416.23
W	1391.64	696.32	744.41	372.70
V	1490.71	-	558.34	279.68
D	1605.72	-	459.28	-
I	1718.80	-	344.25	-
V	-	-	231.17	-
L	-	-	132.10	-

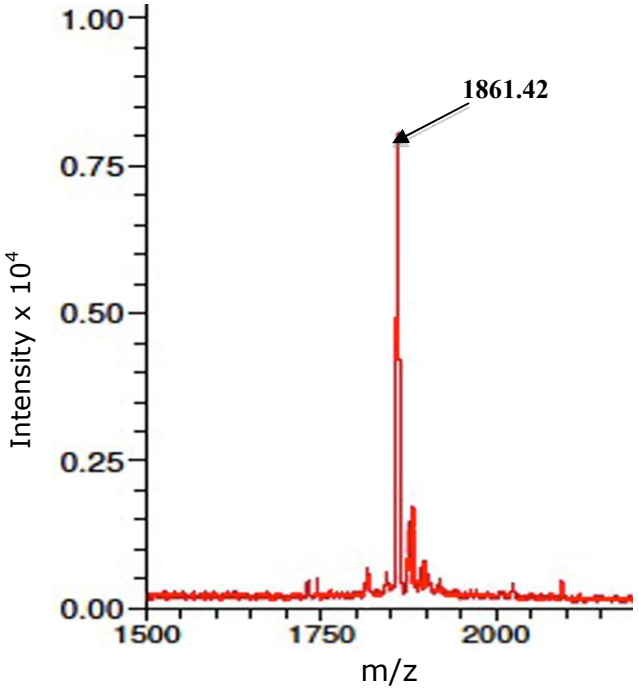


Fig. 4.5. Mass spectrum of peptide 16 identified through MALDI-ToF mass spectrometry corresponding to m/z 1861. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 1861 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 1861 unequivocally defines it as deriving from peptide 16 sequence (GLFGKGGIVTCAMFTCKK) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
G	-	-	1860.95	-
L	-	-	1803.93	-
F	318.18	-	-	-
G	375.29	-	1543.78	
K	503.29	-	1486.76	
G	560.31	-	1358.66	
G	617.34	-	1301.64	-
I	730.42	-	1244.62	-
V	829.49	-	1131.53	-
T	-	-	1032.46	-
C	-	-	931.42	-
A	-	-	-	414.71
M	1235.61	-	-	379.19
F	1382.68	691.34	-	-
T	1483.75	-	-	-
C	1586.76	-	-	-
K	1714.48	-	-	-
K	-	921.97	-	-

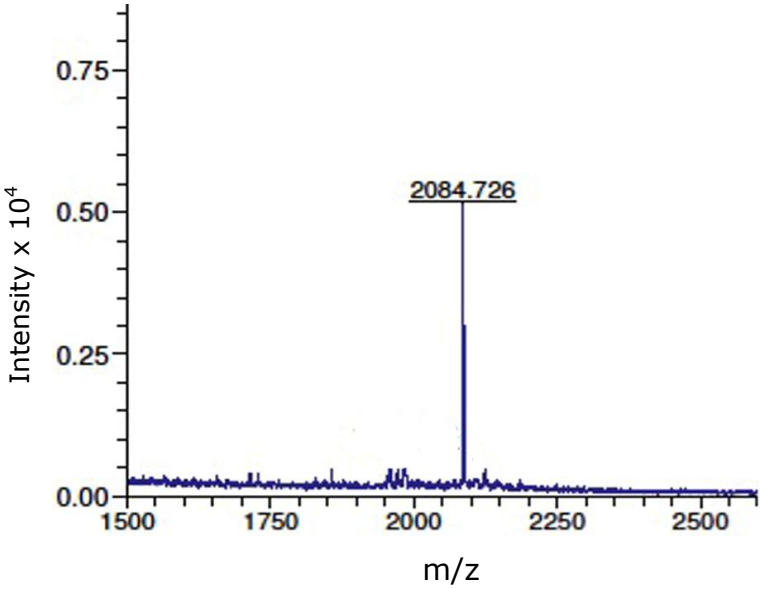


Fig. 4.6. Mass spectrum of peptide 45 identified through MALDI-ToF mass spectrometry corresponding to m/z 2084. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 2084 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 2084 unequivocally defines it as deriving from peptide 45 sequence (VKEIAETQHGTIVIRVQY) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
V	-	-	2084.14	-
K	-	-	1985.08	-
E	357.21	-	-	-
I	470.29	-	1727.94	864.47
A	541.33	-	1614.85	807.93
E	670.37	-	1543.82	772.41
T	771.42	-	1414.78	-
Q	-	-	1313.73	-
H	-	-	1185.67	-
G	1093.56	-	1048.61	-
T	1194.61	-	991.59	-
I	1307.69	-	-	-
V	1406.76	-	777.46	-
I	1519.84	-	678.39	339.70
R	-	838.47	565.30	-
V	-	888.01	-	-
Q	-	952.04	-	-
Y	-	1033.57	-	-

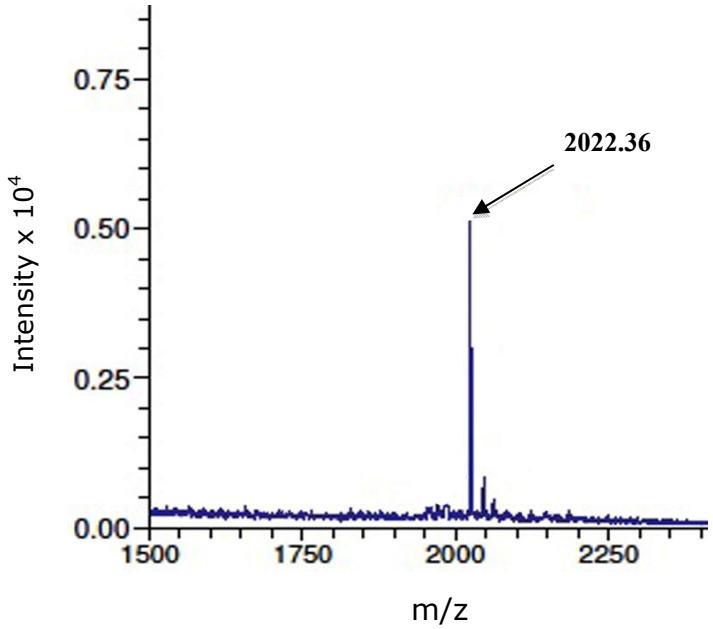


Fig. 4.7. Mass spectrum of peptide 19 identified through MALDI-ToF mass spectrometry corresponding to m/z 2022. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 2022 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 2022 unequivocally defines it as deriving from peptide 19 sequence (GKVVLPENLEYTIVITPH) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
G	-	-	2022.12	-
K	-	-	1965.10	-
V	285.19	-	-	-
V	384.26	-	1737.94	-
L	497.34	-	1638.87	-
P	594.39	-	1525.78	-
E	723.44	-	1428.73	-
N	-	-	1299.69	-
L	950.56	-	-	-
E	1079.60	-	1072.56	-
Y	1242.67	-	-	-
T	1343.72	-	780.46	-
I	1456.80	-	679.41	-
V	1555.07	-	566.32	-
I	1668.95	-	467.26	-
T	1770.00	-	354.17	-
P	-	934.03	253.12	-
H	-	-	-	-

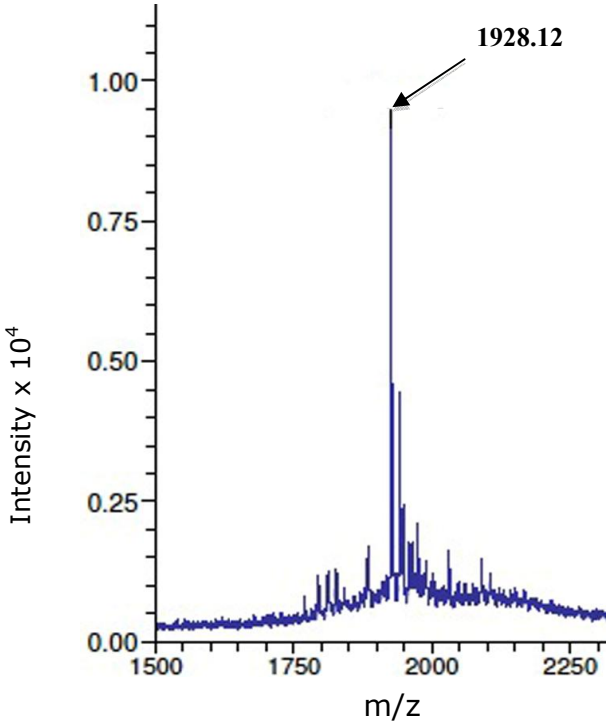


Fig. 4.8. Mass spectrum of peptide 64 identified through MALDI-ToF mass spectrometry corresponding to m/z 1928. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 1928 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 1928 unequivocally defines it as deriving from peptide 64 sequence (AIYGAAFSGVSWTMKILI) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
A	-	-	1928.03	-
I	-	-	1856.99	929.00
Y	348.19	-	1743.91	-
G	405.21	-	1580.85	-
A	476.25	-	1523.82	-
A	547.28	274.14	-	726.90
F	694.35	347.68	-	-
S	781.38	-	1234.68	-
G	838.40	-	1147.65	-
V	937.47	469.24	-	-
S	1024.50	512.75	991.56	-
W	1210.58	-	904.53	-
T	1311.63	-	718.45	-
M	1442.66	-	-	-
K	1570.77	-	-	-
I	-	842.43	-	-
L	-	898.97	-	123.09
I	-	955.51	-	-

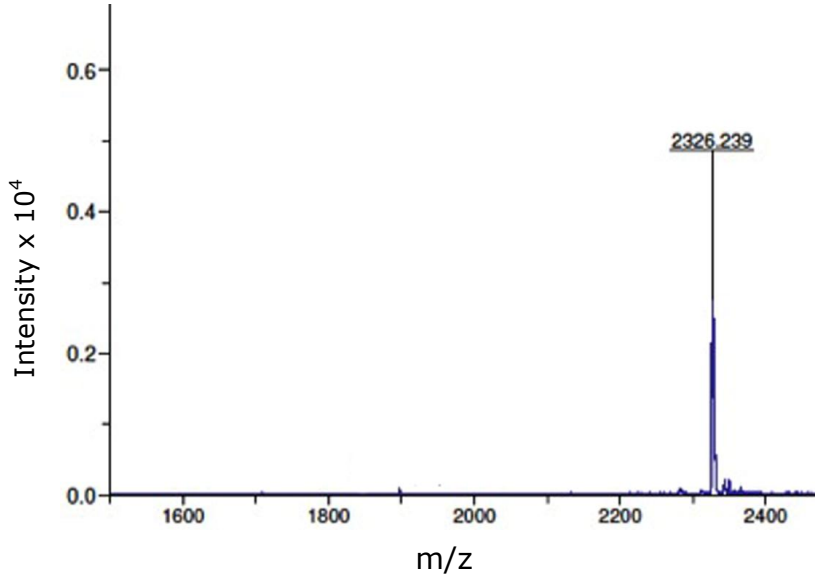


Fig. 4.9. Mass spectrum of peptide 29 identified through MALDI-ToF mass spectrometry corresponding to m/z 2326. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 2326 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 2326 unequivocally defines it as deriving from peptide 29 sequence (VLLQMEEKAWLVHRQWFL) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
V	-	-	-	1163.63
L	-	-	-	1114.09
L	-	-	2114.10	1057.55
Q	454.30	227.65	-	1001.01
M	584.34	293.17	1872.95	936.98
E	714.38	-	1741.91	-
E	843.38	-	-	-
K	971.52	-	1483.83	-
A	1042.56	-	1355.73	-
W	1228.63	-	1284.70	-
L	1341.72	-	1098.62	-
V	1440.79	-	985.53	-
H	1577.85	-	886.46	-
R	1733.95	867.47	749.40	-
Q	-	931.50	-	-
W	-	1024.54	-	-
F	-	-	279.17	-
L	-	-	132.10	-

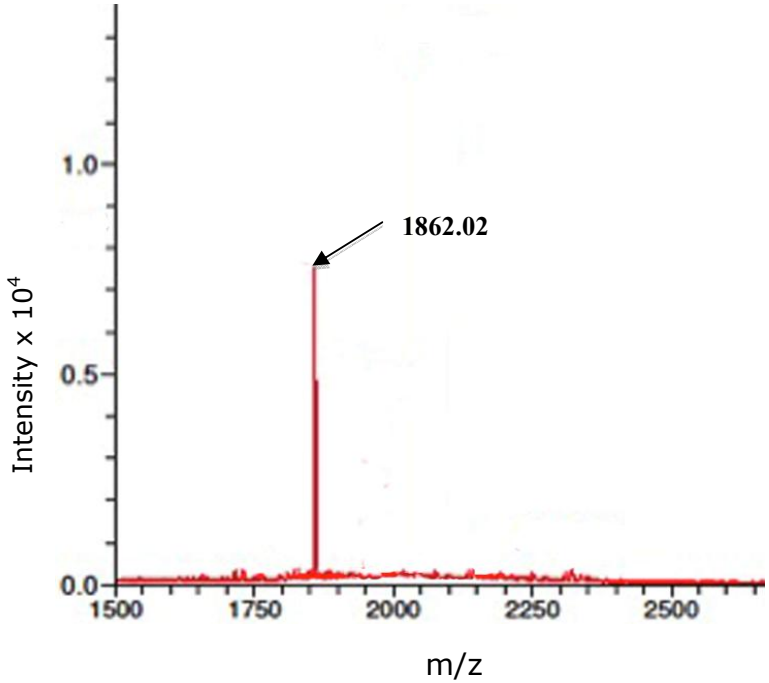


Fig. 4.10. Mass spectrum of peptide 38 identified through MALDI-ToF mass spectrometry corresponding to m/z 1862. Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, one peptide with an m/z value of 1862 was identified through MALDI-ToF mass spectrometry. MS/MS sequencing of peptide ion mass 1862 unequivocally defines it as deriving from peptide 38 sequence (MHTALTGATEIQMSSGNL) with the b and y ion coverage shown in the corresponding table.

Residue	b	b+2	y	y+2
M	-	-	1861.87	-
H	-	-	1730.83	-
T	370.15	-	1593.77	-
A	441.19	-	-	-
L	554.27	-	-	-
T	655.32	-	1308.61	-
G	-	-	1207.56	604.28
A	783.38	-	-	575.77
T	884.42	-	1079.50	-
E	1013.47	-	978.45	-
I	1126.55	-	-	-
Q	1254.61	-	736.32	-
M	1385.65	-	608.27	-
S	1472.68	736.84	477.23	-
S	-	-	390.19	195.60
G	-	808.87	-	152.08
N	-	-	246.14	123.57
L	-	922.43	-	-

4.2.4 *in silico* B-cell epitope prediction

A multi-step computational approach was used to identify the linear antibody epitopes from DENV E protein based on surface accessibility of residues, hydrophobicity and the spatial distance orientation relationship (Fig. 4.11). The potential linear epitopes identified through the computational approach were shown as 14-mer epitope sequences. A score of > 0.65 was considered as the cut off level for selecting positive epitopes. There were 12 epitopes identified on DENV-1 (Table 4.7), 16 on DENV-2 (Table 4.8), 13 on DENV-3 (Table 4.9) and 12 on DENV-4 (Table 4.10). The 16 epitopes identified on DENV-2 E protein were categorized into 6 distinct antigenic regions (Fig. 4.12). Epitopes found in greater spatial proximities were discounted as being too distant in proximity when unfolded and potentially limiting exposure with the antigen. The conservation of these epitope sequences across the E protein of all 4 DENV serotypes was carried out to predict the amino acid differences between the E proteins of different DENV serotypes. All epitope sequences were screened to determine if they shared any sequence similarity with human proteins and none were found homologous with human proteome, thus confirming there would be no occurrence of auto immune reactions. Areas that are shaded in red indicate the most conserved, and most likely immunogenic based on physiochemical properties of amino acids and their corresponding stereochemistry. Exposed and likely immunogenic regions are clustered around the underside of the canyon of this predicted model and shaded in light pink. Regions that are not considered immunogenic are shown in blue.

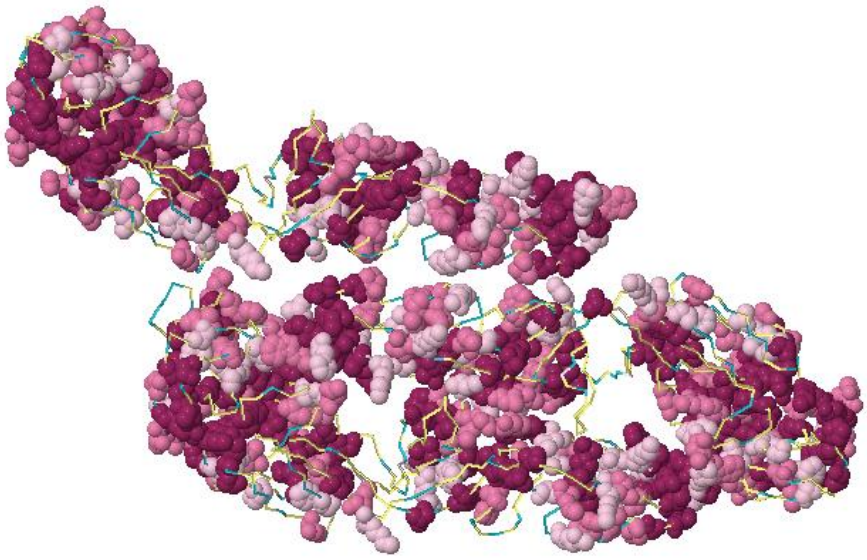


Fig. 4.11. Immunogenicity of DENV E protein (PDB id: 1k4r) super imposed with Bayesian Predictive Algorithm. Top view of the homology model of DENV E protein monomer, PDB id- 1k4r is shown with amino acid residues indicated in space filling model. Colour coded regions correspond to epitopes predicted to have neutralizing ability. Areas shaded in red are indicative of most conserved, surface exposed residues and most likely immunogenic. Areas shaded in light pink are exposed and likely immunogenic, where as those in blue are considered least conserved and not immunogenic.

Table 4.7 Epitopes identified on DENV-1 E protein

Epitope sequence	Position on E protein	Score
RCVGIGSRDFVEGL	2-15	0.86
CRRTFVDRGWGNGC	92-105	0.87
VDRGWGNGCGLFGK	97-110	0.70
GLFGKGSLITCAKF	106-119	0.67
NLKYSVIVTVHTGD	134-147	0.79
DCSPRTGLDFNEMV	184-197	0.67
LDFNEMVLLTMKEK	191-204	0.82
LDLPLPWTSGASTT	214-227	0.67
NRQDLLVTFKTAHA	232-245	0.92
KMDKLTLKGMSYVM	288-301	0.72
MSYVMCTGSFKLEK	297-310	0.90
TQHGTVLVQIKYEG	315-328	0.69

Table 4.8 Epitopes identified on DENV-2 E protein

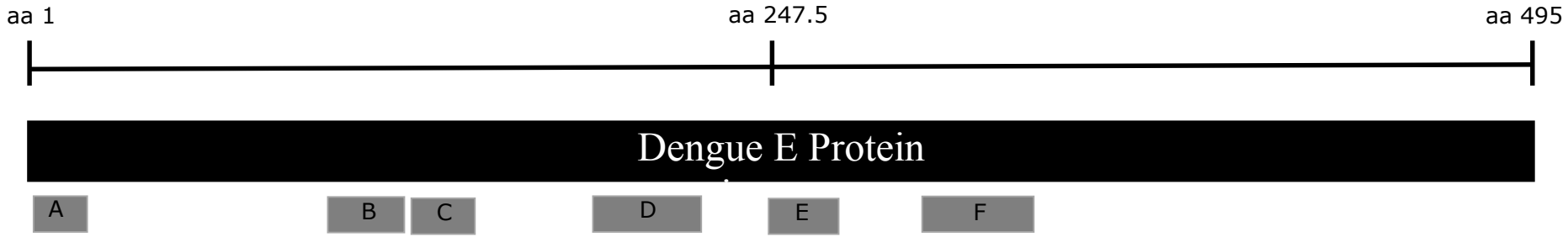
Epitope sequence	Position on E protein	Score
RCIGISNRDFVEGV	2-15	0.89
GISNRDFVEGVSGG	5-18	0.85
DRGWGNGCGLFGKG	98-111	0.80
GLFGKGGIVTCAMF	106-119	0.75
KGGIVTCAMFTCKK	110-123	0.69
EGKIVQPENLEYTI	126-139	0.82
PENLEYTIVITPHS	132-145	0.81
FNEMVLLQMENKAW	193-206	0.84
LQMENKAWLVHRQW	199-213	0.70
LDLPLPWLPGADTQ	214-227	0.70
GSQEGAMHTALTGA	254-267	0.68
TALTGATEIQMSSG	262-275	0.73
SYSMCTGKFKVVKE	298-311	0.81
KVVKEIAETQHGTI	307-320	0.78
AETQHGTIVVRVQY	313-326	0.77
IVVRVQYEGDGSPC	320-333	0.80

Table 4.9 Epitopes identified on DENV-3 E protein

Table 4.10 Epitopes identified on DENV-4 E protein

Epitope sequence	Position on E protein	Score
KPTLDIELQKTEAT	48-61	0.89
NYVCKHTYVDRGWG	89-102	0.88
RGWGNGCGLFGKGS	99-112	0.73
KFQCLESIEGKVVQ	118-131	0.89
EGKVVQPENLKYTV	126-139	0.82
VQPENLKYTVIITV	130-143	0.81
KYTVIITVHTGDQH	136-149	0.85
ITSQASTAEVILPE	162-175	0.93
DFNEMILLTMKNKA	190-203	0.87
LLTMKNKAWMVHRQ	196-209	0.91
NKAWMVHRQWFFDL	201-214	0.77
LKLKGMSYAMCLNT	290-303	0.82
KKEVSETQHGTILI	307-320	0.71

Epitope sequence	Position on E protein	Score
RCVGVGNRDFVEGF	2-15	0.82
RRDMVDRGWGNGCG	93-106	0.87
GNGCGLFGKGGVVT	102-115	0.76
LVQIENLEYTVVVT	129-142	0.88
NLEYTVVVTVHNGD	134-147	0.92
FNEMILMKMKTKTW	193-206	0.82
KQWFLDLPLPWTAG	210-223	0.67
SALAGATEVDSGDG	262-275	0.76
RMEKLRIKGMSYTM	288-301	0.70
MSYTMCSGKFSIDK	297-310	0.86
IDKEMAETQHGTTV	308-321	0.80
SVTNIELEPPFGDS	363-376	0.80



Antigenic region	Predicted linear epitope sequences	Amino acid position
A	GISNRDFVEGVSGG	5-18
B	DRGWGNGCGLFGKGGIVTCAMFTCKK	98-123
C	EGKVVLPENLEYTIVITPHS	126-145
D	FNEMVLLQMEEKAWLVHRQWFLDLPLPWLPGADTQ	193-227
E	GSQEGAMHTALTGATEIQMSSG	254-275
F	SYSMCTGKFKIVKEIAETQHGTIVIRVQYEGDGSPC	307-333

Fig. 4.12. Antigenic regions identified through *in silico* computational analysis. The sequence of E protein is indicated with the relative positions of 6 antigenic peptide regions that were identified through *in silico* analysis shown as short grey bars. The amino acid sequences for each of the antigenic regions are shown in the corresponding table.

The epitopes identified on the DENV-2 E protein through all three mapping strategies are illustrated in Fig. 4.13. There were 29 linear epitopes identified using ELISA and epitope extraction. Among them, 9 epitopes were identified by both ELISA and epitope extraction, of which 8 (peptide 2, 16, 19, 29, 33, 38, 40 and 45) were located within the sE protein and one (peptide 64) epitope located at the stem region on the ectodomain of the E protein. The computational analysis revealed 6 antigenic regions on the DENV-2 E protein representing at least 6 epitopes (peptide 2, 16, 19, 29, 38, and 45) identified by both ELISA and epitope extraction. The location of these epitopes has been displayed on the 3-dimensional structure of the DENV-2 E protein (PDB ID- LOAN) (Fig. 4.14 and 4.15). On the other hand, 8 epitopes were positive in ELISA only and 12 epitopes were identified in epitope extraction only. Hence, a combination of these three strategies might be useful for fine epitope mapping of DENV using polyclonal immune sera.

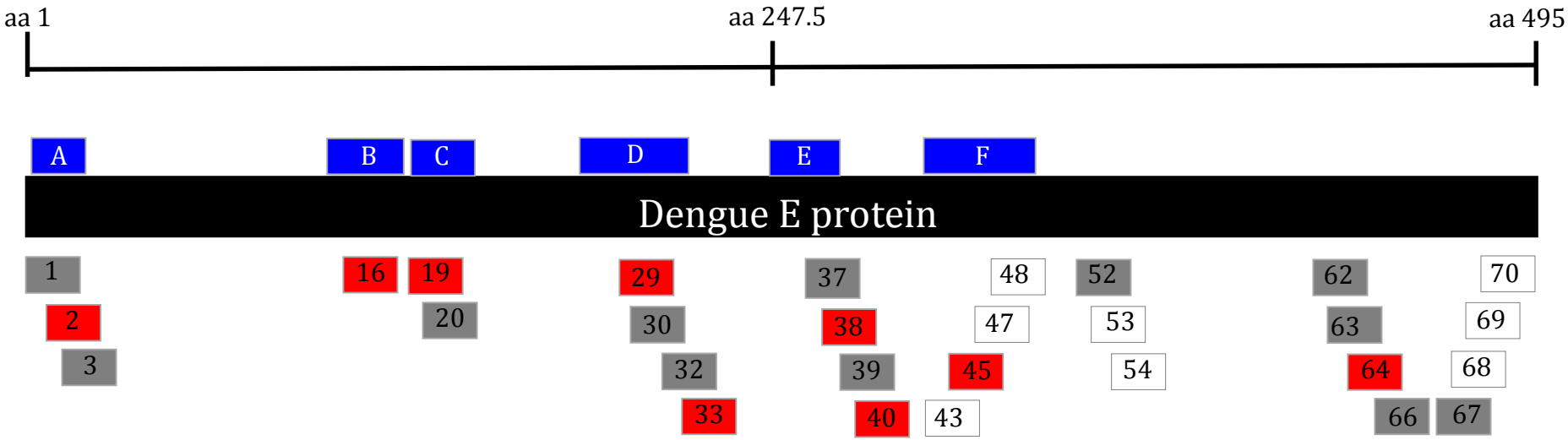
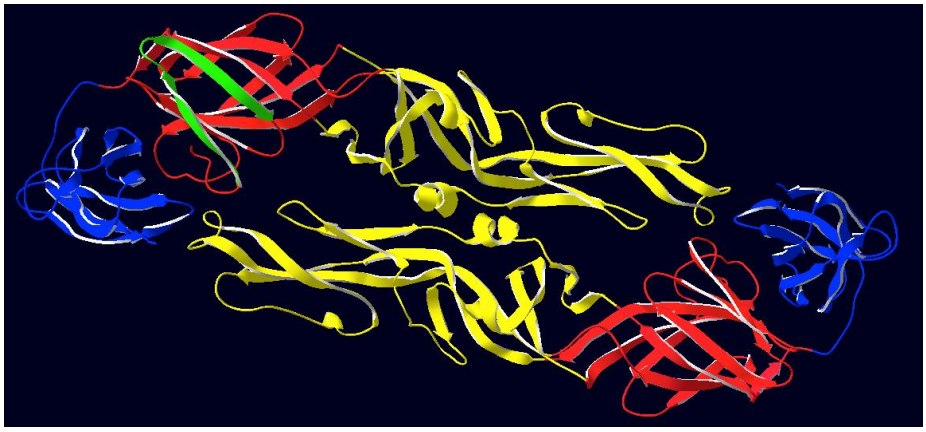


Fig. 4.13. Epitopes identified on the E protein through a combination of ELISA, extraction and *in silico* techniques. The sequence of E protein is indicated with the relative positions of epitopes that were recognized by immune sera shown as short bars. Red bars indicate those epitopes identified both by epitope extraction and ELISA; grey bars indicate epitopes defined by epitope extraction only; white bars indicate those epitopes that were identified by ELISA alone and blue bars indicate the 6 antigenic regions (A, B, C, D, E and F) identified by *in silico* computational analysis.

A



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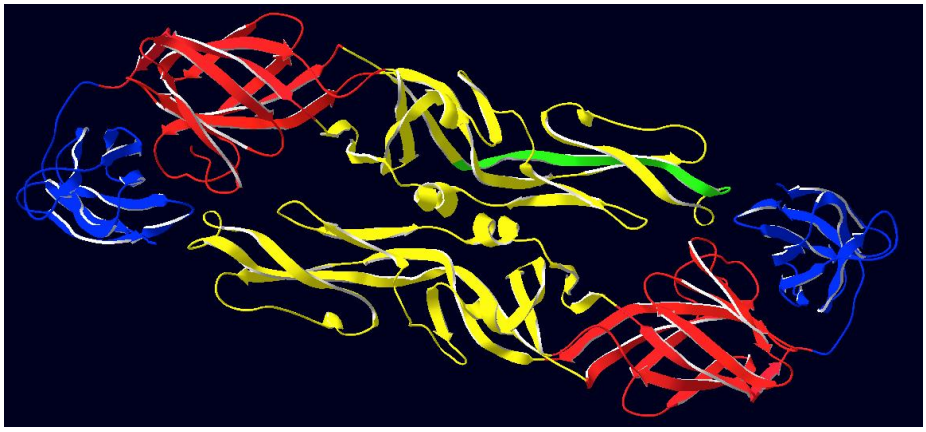

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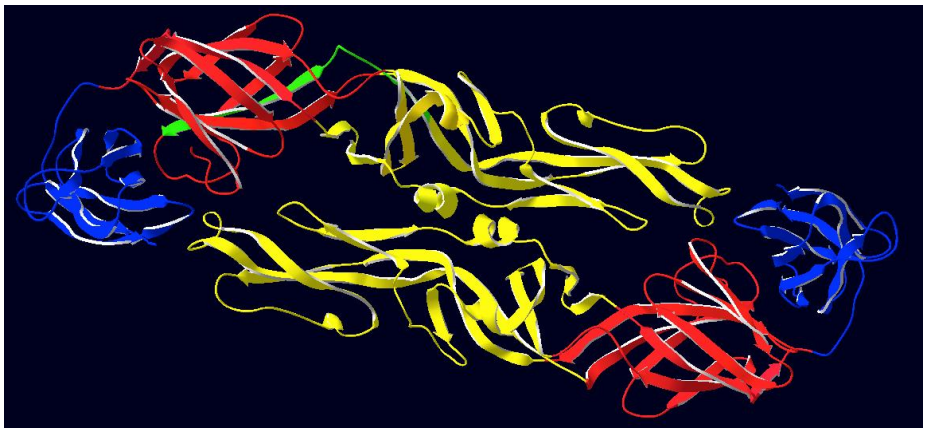
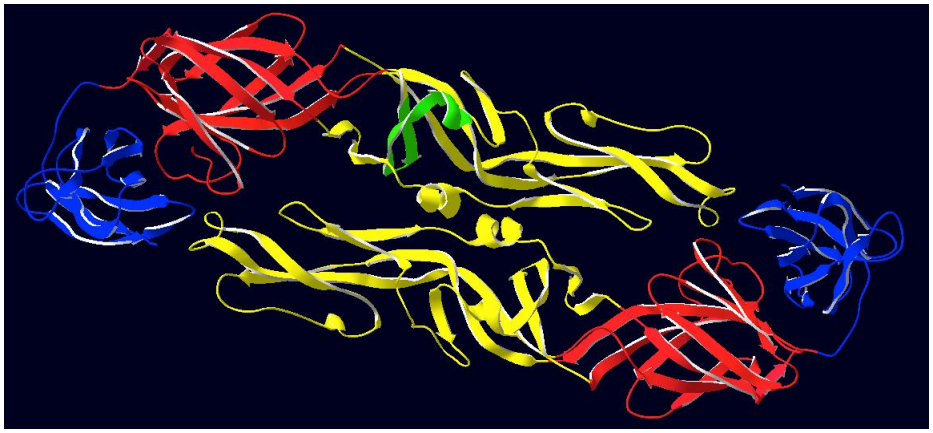


Fig. 4.14. Location of epitopes 2, 16 and 19 on the DENV-E. Top view of the dimeric form of the DENV-2 E protein residues 1–395 (PDB ID-LOAN). The domains I, II and III are coloured red, yellow and blue, respectively. The epitopes identified from 3 different epitope mapping strategies were coloured in green. A) Location of epitope 2, B) Location of epitope 16, C) Location of epitope 19.

A



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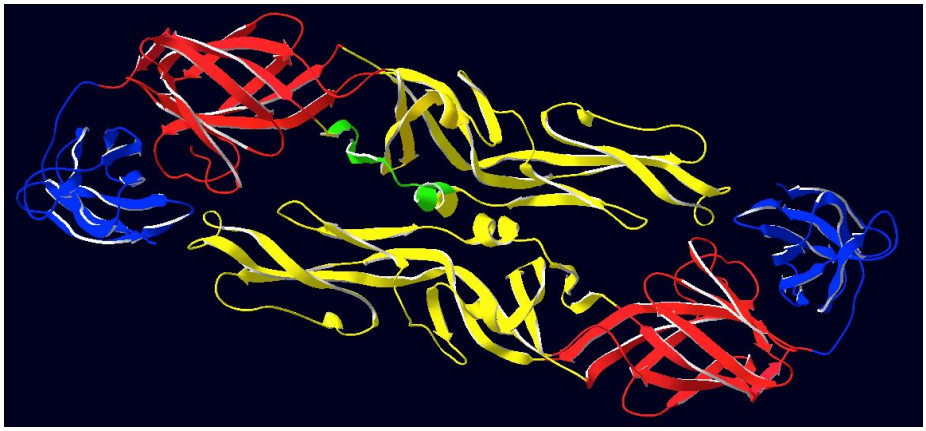

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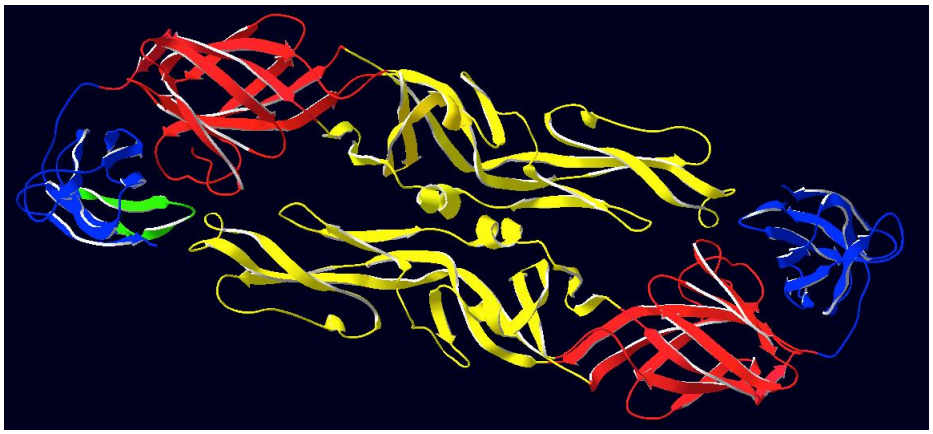


Fig. 4.15. Location of epitopes 29, 38 and 45 on the DENV-E. This schematic depicts the dimeric form of the DENV-2 E protein residues 1–395 (PDB ID-LOAN). The domains I, II and III are coloured red, yellow and blue, respectively. The epitopes identified from 3 different epitope mapping strategies were coloured in green. A) Location of epitope 29, B) Location of epitope 38, C) Location of epitope 45.

4.3 Discussion

In the present study, three different approaches were used to map the B-cell epitopes of the DENV E protein recognized by serum samples of dengue patients from recent outbreaks in far North Queensland, using an overlapping synthetic peptide library as the antigen. The positive polyclonal anti-DENV antibodies, with different types of viral infections, and normal individual sera were tested against the peptides, in order to evaluate their ability to discriminate between DENV and non-dengue infections. The solid phase ELISA results showed that the dengue immune IgG from patients sera reacted with 17 peptides spanning 10 continuous regions along the entire E protein at amino acids 8-25, 106-123, 127-144, 190-207, 225-242, 260-291, 295-347, 365-389, 442-459 and 470-495. The soluble E-glycoprotein has three structural domains: EDI (residues 1-52, 132–182 and 280-295), EDII (residues 53–131 and 193–279) and EDIII (residues 303–395) (Rey et al., 1995). From the 17 peptides mapped through ELISA, those included at positions 106-123 (peptide 16), 197-214 (peptide 29), 225-242 (peptide 33) and 260-277 (peptide 38) are located on EDII and those included on positions 295-312 (peptide 43), 309-326 (peptide 45), 323-340 (peptide 47), 330-347 (peptide 48), 365-382 (peptide 53) and 372-389 (peptide 54) are located on EDIII.

Earlier studies showed that the EDII contains many cross-reactive epitopes eliciting neutralizing and non-neutralizing monoclonal antibodies that bind to fusion peptides, while EDIII contains multiple type- and subtype-specific epitopes and several conformational virus neutralizing epitopes (Roehrig *et al.,* 1998; Crill and Roehrig, 2001; Oliphant *et al.,* 2006; Gromowski *et al.,* 2008). Apart from epitopes on EDII and EDIII, an epitope region at aa 8-25 (peptide 2) of the EDI was identified in the present study. This domain has been shown to represent predominately type-specific non-neutralizing epitopes with varying antigenic specificity as targeted by MAbs (Roehrig *et al.,* 1998). To note, earlier studies with murine MAb 4G2 showed a strong reaction of 4G2 against the amino acid sequences found within the “hinge” region between EDI/EDIII (Innis *et al.,* 1989). The results of the present study using the polyclonal DENV human sera confirms and extends the above finding with epitopes identified on the hinge regions at positions 127-144 (peptide 19), between EDI/EDII and 274-291 (peptide 40) and between EDI/EDIII. In addition to the epitopes identified on the soluble E protein, epitopes on the “stem” region outside the domains located at 442-459

(peptide 64), 470-487 (peptide 68) and 477-495 (peptide 69 and 70) were also identified. The majority of the sequences in the stem region have been shown to be conserved across all DENV serotypes and other flaviviruses (Schmidt *et al.*, 2010).

The solution phase epitope extraction revealed 21 peptides were capable of being bound by the immune IgG, of which, 9 were also identified positive in ELISA. These in-solution reactions were useful to maintain the conformational integrity required for some of the peptides to be able to bind to the antibody. For example, peptides 29 and 38 showed mild reactivity against the IgG samples in ELISA whereas; in-solution epitope extraction revealed that these peptides were able to bind well to several IgG samples. On the other hand, peptides 68, 69 and 70 showed positive reactions in ELISA, but did not bind to the antibody in solution. Similar binding reactions in ELISA were seen in DENV-2 transmembrane domain using murine MAbs (Falconar, 1999).

A multi-step *in silico* epitope prediction method was also used to identify the B-cell epitopes based on surface accessibility and hydrophobicity of the residues on the E protein of all four serotypes of DENV. Earlier computational approaches to identify the epitopes on DENV mostly used a single algorithm to predict the epitopes (Amexis and Young, 2007; Tambunan *et al.,* 2009; Sanchez-Burgos *et al.,* 2010; Li *et al.,* 2011). Furthermore, most of the predictive epitope data training sets contain data entry ambiguities or error prone alignments with too high a degree of similarity with other peptide sequences in the same data set. Hence, a sequence-based approach was used in combination with a structural-based approach to identify and compare the potential epitope candidates on the E protein. This improved multi-step approach revealed several epitopes on all four serotypes of DENV, of which the epitopes identified on the DENV-2 were organized in to six distinct antigenic regions. No peptides were included that shared sequence homology with human proteins which could induce a deleterious response.

Overall, the ELISA and epitope extraction approaches revealed 29 epitopes on the E protein of DENV-2, of which nine were identified by both methods. These epitopes span all three domains of the sE protein (peptide 2, 16, 19, 29, 33, 38, 40 and 45) and the ectodomain of the native E protein (peptide 64). Six antigenic regions on the DENV-2 E protein were identified through the computational analysis and these regions anchor six epitopes (peptide 2, 16, 19, 29, 38, and 45) identified by both wet-lab

methods. In addition, eight epitopes were identified in ELISA only and 12 peptides were recognized in epitope extraction only.

The partial antigenic regions of peptide 16 (aa 106-111) have been shown to be part of a fusion peptide, which showed higher cross-activity against all DENV antibodies and is reported to be an immunogenic antigenic region (Crill and Chang, 2004). The importance of the EDII fusion peptide as an immunodominant antigenic region containing a series of overlapping epitopes stimulating broadly cross-reactive antibodies has been well established in mice (Crill and Chang, 2004; Stiasny *et al.,* 2006; Oliphant *et al.,* 2007). The results presented in this thesis and other earlier studies confirm the extension of this observation to humans (Stiasny *et al.,* 2006; Oliphant *et al.,* 2007; Lai *et al.,* 2008). In an earlier study, Aaskov *et al.* 1989 showed that the partial sequence of peptide 19 (aa 127-134) and peptide 64 (aa 448-455) was recognized by mouse MAb (1B7) and sera from DENV-2 patients respectively. Although few epitopes identified in our study overlap with antigenic regions described previously (Aaskov *et al.,* 1989; Crill and Chang, 2004; Stiasny *et al.,* 2006; Amexis and Young, 2007; Oliphant *et al.,* 2007; Lai *et al.,* 2008), the epitope sequences identified through a combination of different strategies have not been reported previously.

Here, a novel strategy for epitope identification is proposed using a combination of ELISA, epitope extraction and computer-based bioinformatics approaches. The ELISA and epitope extraction methods are high throughput, cost effective and less time consuming such that the antibody binding potential of synthetic peptides can be rapidly screened. However, when compared to ELISA, the epitope extraction technique requires additional sample preparation steps and technical expertise to perform the peptide identification through mass spectrometry. On the other hand, computational epitope prediction is quick with epitopes identified using a combination of several predictive algorithms. Hence, these three strategies might be useful for fine epitope mapping of DENV and other related flaviviruses. The epitopes identified in this multi-disciplinary approach in conjunction with other well-documented epitopes of DENV together have implications for future development of epitope-specific diagnostics and epitope-based dengue vaccines.

Chapter 5

Humoral immune response of the epitopes identified on the DENV-2 E glycoprotein

5.1 Introduction

Humoral immunity is an essential part of host protection against dengue virus (DENV) infection (Murphy and Whitehead, 2011). Antibodies, in particular, have emerged as key effector molecules responsible for protective and pathogenic immune responses to DENV. The majority of DENV-neutralizing antibodies recognize the major envelope (E) protein and the E protein mediates the low pH fusion of the viral and host cell membrane (Lindenbach and Rice, 2001; Kuhn *et al.,* 2002). Currently, there is no vaccine against DENV and this disease represents a particularly challenging problem for vaccine development. The possibility of developing dengue heamorrhagic fever (DHF) or dengue shock syndrome (DSS) as a result of antibody-dependent enhancement (ADE) with monotypic DENV antibodies and the lack of cross-protection against the different DENV serotypes mean that a successful DENV vaccine will need to be tetravalent with protection against all four DENV serotypes (DENV-1, -2, -3, and -4) (Whitehead *et al.,* 2007; Murrell *et al.,* 2011).

The E-glycoprotein of DENV is a good target to test novel approaches for vaccine development, since it contains several neutralizing epitopes and binding motifs for virus attachment (Aaskov *et al.,* 1989; Roehrig *et al.,* 1998; Beasley and Aaskov, 2001; Crill and Roehrig, 2001; Crill and Chang, 2004; Gromowski, 2007). Epitopes involved in neutralization of DENV are predicted to be surface accessible on intact virion and these epitopes exist on all three domains of the E protein (Wu *et al.*, 2003; Roehrig *et al.,* 2004). However, recent studies have shown that DENV-immune humans have low levels of serum EDIII-specific antibodies, and these sera retained potently neutralizing activity even after depletion of EDIII-binding antibodies suggesting that humans produce neutralizing antibodies that bind to epitopes other than those on EDIII (Wahala *et al.,* 2009; de Alwis *et al.,* 2012; Wahala *et al.,* 2012). Thus, the identity of DENV epitopes recognized by the human antibody repertoire responsible for potent and long-term neutralization remains unknown, which complicates the current global effort to develop dengue vaccines.

Chapter 5

Efforts to develop a DENV vaccine have focused mainly on live attenuated virus vaccines, chimeric vaccines, inactivated virus vaccines and subunit virus vaccines (Whitehead *et al.,* 2007; Webster *et al.,* 2009; Murrell *et al.,* 2011). Even though there has been a large increase in potential DENV vaccine candidates in recent years, there are still challenges that need to be addressed. Live attenuated vaccine candidates had success as monovalent formulations but the tetravalent formulations were unable to elicit a balanced immune response against all four serotypes with weak immunity against at least one of the four DENV serotypes (Sun *et al.,* 2003). In addition, live attenuated and chimeric vaccine formulations developed so far contain the native viral precursor membrane (prM) sequence, which has been reported to potentially induce ADE following secondary infections (Dejnirattisai *et al.,* 2010). These limitations emphasize the need for better understanding of epitopes that elicit the most protective immune response against each of the four DENV serotypes.

Previous animal studies have shown that synthetic peptides of the E-glycoprotein of DENV elicit humoral immune responses and confer protection in mice (Roehrig *et al.,* 1994; Putnak *et al.,* 2003; Amexis and Young, 2007; da Silva *et al.,* 2009; Li *et al.,* 2011). Two of the major difficulties in working with different DENV's are the lack of a reliable animal model that mimics human disease and the apparent involvement of the host immune system in disease pathogenesis leading to ADE. These factors have hampered the development of a vaccine against dengue. While it is well recognized that the E protein is one of the major proteins responsible for the pathogenicity and immunogenic properties of flaviviruses, there is still work to be done to identify the neutralizing epitope regions within E protein. Previously, six B-cell epitopes (peptide 2, 16, 19, 29, 38 and 45) were identified within sE protein region of DENV-2 through a combination of ELISA, epitope extraction and computational approach (Chapter 4). An additional epitope (peptide 64) was identified through ELISA and epitope extraction on the ectodomain of the full length E protein.

The aim of the work described in this chapter is to determine whether these seven candidate epitopes were capable of eliciting a humoral immune response in mice with the potential to bind to and neutralize DENV. Since there is a lack of appropriate T-cell help, short synthetic peptides usually do not induce good immune responses on their own. T-cell help can be provided through co-synthesizing linear helper T-cell epitopes along with the B-cell epitopes (Ghosh and Jackson, 1999). In addition,

adjuvants are commonly used to boost the immunogenicity of a synthetic peptide antigen (Olive *et al.*, 2001). Using an alternative approach, the potential epitope candidates were co-synthesized with a previously published T_H–cell epitope (Roehrig *et al.,* 1992). The resulting seven vaccine constructs (B2, B16, B29, B38, B45, B64 and B19) were emulsified in Freund's complete adjuvant and used to immunize mice. Five novel epitopes with potential DENV binding and neutralizing activity were identified as determined by ELISA and *in vitro* virus neutralization assays. The results suggest that these peptides, together with other epitope regions reported in literature, could be targeted for the development of peptide-based vaccines against DENV.

5.2 Results

5.2.1 Synthesis of peptide vaccine constructs

Seven vaccine constructs were designed consisting of B cell epitopes identified in chapter 4 co-synthesized with a previously identified helper T-cell epitope (Roehrig *et al.,* 1992). The seven B cell epitopes consisted of linear 18-mer synthetic peptides denoted B2, B16, B29, B38, B45, B64 and B19. Amino acid sequence comparisons of these peptides among the four DENV serotypes are shown in Table 5.1. Individual linear peptides were co-synthesized with a helper T-cell epitope representing the amino acids 352- LITVNPIVTEKDSPVNIE-368 of the DENV-2 E protein (Jamaica strain). This T_H–cell epitope has previously been reported to enhance the antibody response to flavivirus B-cell epitopes in BALB/c mice (Roehrig *et al.,* 1992).

5.2.2 Immunogenicity of B-cell epitopes

In order to see whether the identified epitope regions were able to elicit an immune response in an animal model, groups of Balb/c mice (n=5) received 2 doses of 50 μg of peptide immunogen in adjuvant (CFA/IFA) on days 0 and 28. Mice were bled on days 0, 10 and 38, and the anti-peptide antibody titres in sera determined by ELISA. The antibody response of individual mice within a group and the mean antibody titre of each vaccine construct are shown in Fig. 5.1. After a single dose, several animals across all groups produced low levels of anti-peptide antibodies. Following the secondary dose, the peptides B16, B29, B45, B64 and B19 elicited significant anti-peptide antibody titres ($P < 0.05$) when compared to the primary dose. Though the antibody titres between these five peptides following secondary dose were not significantly different, the highest mean titre was observed in B19 (2.918) followed by B16 (2.697). In contrast, the antibody response between primary and secondary dose in peptides B2 and B38 did not vary significantly ($P > 0.05$) though a few animals showed lower titres. However, following the secondary dose, the mean antibody titres of B2 and B38 were significantly different ($P < 0.05$) when compared to the mean titres of B16, B29, B45, B64 and B19.

Table 5.1. Amino acid sequence comparison of peptides B2, B16, B29, B38, B45, B64 and B19 between four DENV serotypes.

Serotype	NCBI accession number	Peptides			
		B2	B16	B29	B38
		(NRDFVEGVSGGSWVDIVL)	(GLFGKGGIVTCAMFTCKK)	(VLLQMEEKAWLVHRQWFL)	(MHTALTGATEIQMSSGNL)
DENV-1	AAZ43213.1	NRDFVEGLSGATWVDVVL	GLFGKGSLITCAKFKCVT	VLLTMKEKSWLVHKQWFL	MHTALTGATEIQTSGTTT
DENV-2	AAA17500.1	NRDFVEGVSGGSWVDIVL	GLFGKGGIVTCAMFTCKK	VLLQMENKAWLVHRQWFL	MHTALTGATEIQMSSGNL
DENV-3	ABY82134.1	NRDFVEGLSGATWVDVVL	GLFGKGSLVTCAKFQCLE	ILLTMKNKAWMVHRQWFF	MHTALTGATEIQTSGGTS
DENV-4	AEX97810.1	NRDFVEGVSGGAWVDLVL	GLFGKGGVVTCAKFLCSG	ILMKMKKKTWLVHKQWFL	MHSALAGATEVDSGDGNH

Serotype	NCBI accession number	Peptides		
		B45	B64	B19
		(VKEIAETQHGTIVIRVQY)	(AIYGAAFSGVSWTMKILI)	(GKVVLPENLEYTIVITPH)
DENV-1	AAZ43213.1	EKEVAETQHGTVLVQVKY	TAYGVLFSGVSWTMKIGI	GKIVQYENLKYSVIVTVH
DENV-2	AAA17500.1	VKEIAETQHGTIVVRVQY	AIYGAAFSGVSWTMKILI	GKVVQPENLEYTIVITPH
DENV-3	ABY82134.1	KKEVSETQHGTILIKVEY	SAYTALFSGVSWIMKIGI	GKAVQHENLKYTVIITVH
DENV-4	AEX97810.1	DKEMAETQHGTTVVKVKY	SVYTTMFGGVSWMIRILI	GNLVQIENLEYTVVVTVH

The table shows sequence alignments of multiple viruses. Sequence variations observed between four DENV serotypes are indicated in grey shaded residues.

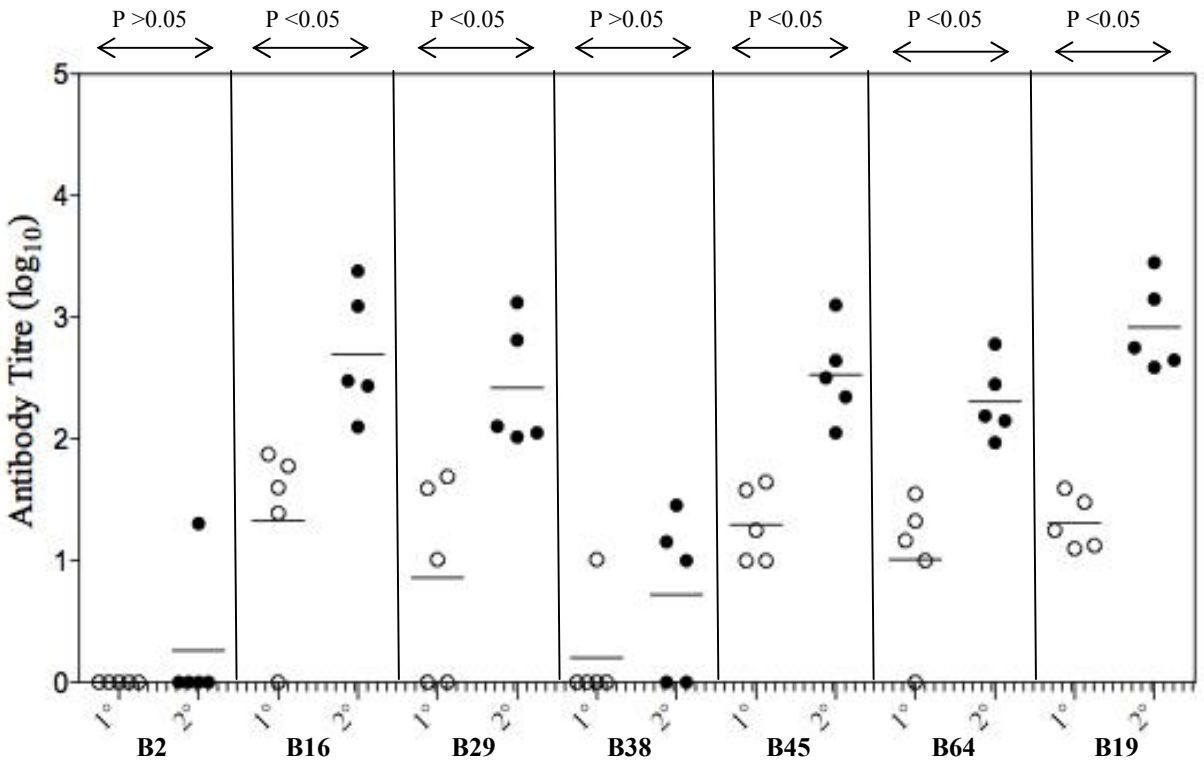


Fig. 5.1. Immunogenicity of peptide vaccines B2, B16, B29, B38, B45, B64 and B19 coupled to a helper T-cell epitope. For both primary (open circles) and secondary (closed circles) inoculations, groups of BALB/c mice (n=5) 50 μg of peptide immunogen were administered sub-cutaneously on days 0 and 28, respectively. Negative control animal groups received CFA and saline. Mice were bled on days 0, 10 (1^o) and 38 (2^o), and sera obtained. Antibody levels were determined by ELISA. Antibody titres are expressed as the reciprocal of the logarithm of that dilution of serum that gave an optical density four times above that obtained in wells with preimmune control sera. Individual animal titres are presented with the mean value represented by the horizontal bar and p values are indicated between the primary and secondary dose.

5.2.3 Cross-reactive antibody response against the E recombinant protein of DENV

Sera from all five mice within a group were pooled and the resulting immune sera were used to test the cross-reactive antibody response in ELISA against soluble E (sE) recombinant protein of DENV-1 (395 aa), DENV-2 (395 aa) and DENV-3 (393 aa). The $\log_{10}$ antibody titre of six vaccine constructs, B16, B29, B38, B45, B64 and B19, are shown in Fig. 5.2. The construct B2 did not elicit an antibody response to any of the recombinant proteins. All six sera panels were reacted with DENV-2; however, the anti-peptide antibodies differed widely in their cross-reactivity. The sera representing the vaccine constructs, B16, B29 and B45, showed cross-reactivity against all three DENV recombinant proteins. In particularly, the immunoglobulins elicited against the conserved E protein fusion loop (peptide B16) were broadly cross-reactive against DENV-1, DENV-2 and DENV-3 recombinant proteins with $\log_{10}$ titres of 4.000, 4.215 and 4.05, respectively. In contrast, three peptides elicited only a homologous antibody response against DENV-2; these were anti-peptide B19 (antibody titre $\log_{10}$ 3.691), anti-peptide B64 (antibody titre $\log_{10}$ 2.975) and anti-peptide B38 (antibody titre $\log_{10}$ 2.025).

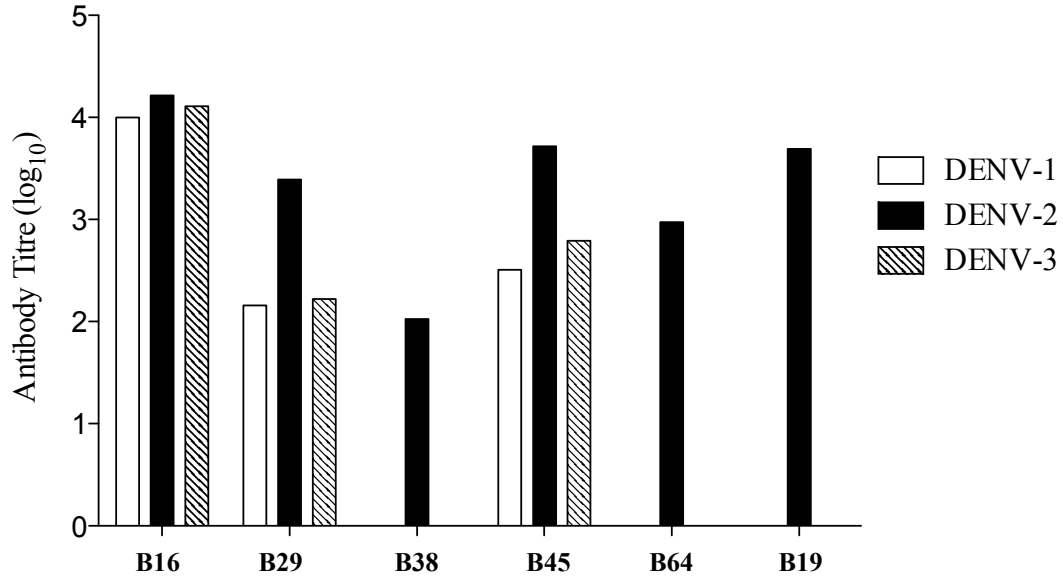


Fig. 5.2. Cross-reactive response of anti-peptide antibody against the sE recombinant protein of DENV-1, DENV-2 and DENV-3. All sera within a peptide group were pooled and the resulting immune sera pools were used to test the cross-reactive antibody response in ELISA. The log_{10} antibody titres of each vaccine construct (B16, B29, B38, B45, B64 and B19) against the E protein of DENV-1 (clear bar), DENV-2 (dark bar) and DENV-3 (shaded bar) are shown.

5.2.4 Neutralizing ability of anti-peptide antibodies *in vitro*

Pooled immune sera at various dilutions were used to test the neutralizing ability against DENV-1, DENV-2, DENV-3 and DENV-4 in an *in vitro* focus reduction neutralization assay (FRNT) using BHK-21 cells. The sera dilution resulting in a 50% reduction of focus, when compared to the pre-immune serum/saline-adjuvant serum control, was considered to be the end-point titre ($FRNT_{50}$). The results of each neutralization curve were expressed as an average (±S.E.M) of at least two independent experiments (Fig. 5.3 and 5.4) and the neutralizing antibody titres of each vaccine constructs are shown in Table 5.2. Homologous neutralizing antibody response against DENV-2 was observed in vaccine constructs B16 (Fig. 5.3A), B38 (Fig. 5.3C) and B19 (Fig. 5.4F) with neutralizing antibody titres ($FRNT_{50}$) of 1:80, 1:10 and 1:40 respectively. The vaccine constructs B29 (Fig. 5.3B) and B45 (Fig. 5.4D) showed a heterotypic neutralizing antibody response against both DENV-2 and DENV-3. The 50% neutralizing antibody titres of B29 were 1:80 (DENV-2) and 1:10 (DENV-3), whereas the titres for B45 were 1:80 (DENV-2) and 1:20 (DENV-3). The pooled serum from construct B2 did not elicit a neutralizing antibody response against any of the four DENV serotypes. In contrast, the vaccine construct representing the E ectodomain region (B64) elicited a neutralizing antibody response against both DENV-1 and DENV-2, with $FRNT_{50}$ titres of 1:10 and 1:80, respectively.

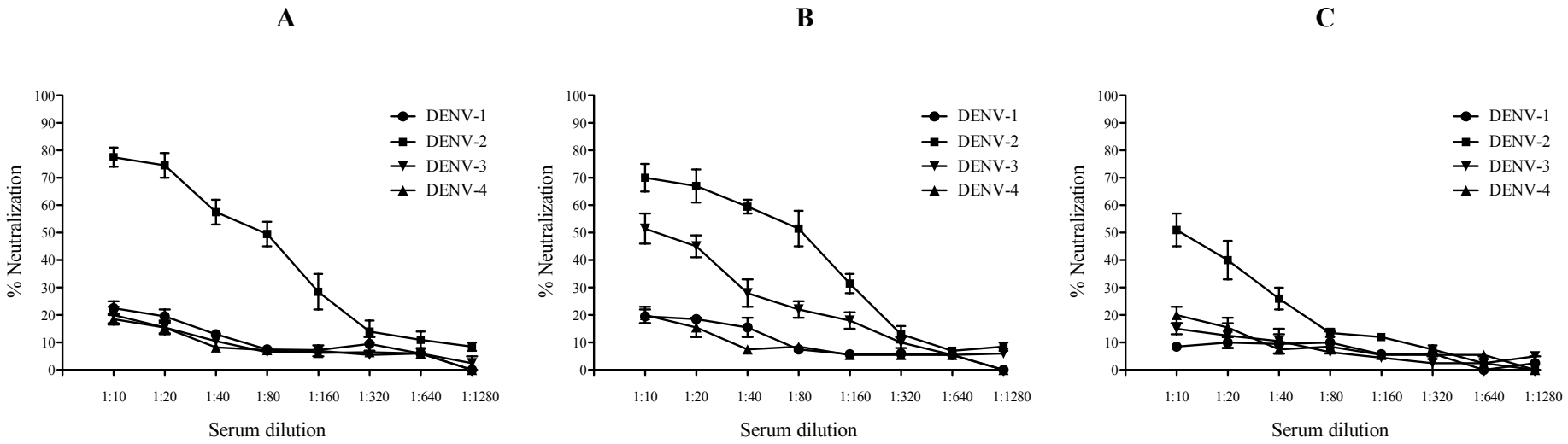


Fig. 5.3. Neutralizing ability of anti-peptide antibodies B16, B29 and B38 against four DENV serotypes. Serially diluted pooled immune sera were used to test the virus neutralizing ability against DENV-1, DENV-2, DENV-3 and DENV-4 in an *in vitro* focus reduction neutralization assay (FRNT) employing BHK-21 cells. A dilution resulting in 50% reduction of focus when compared to the pre-immune serum was considered as the end-point titre ($FRNT_{50}$). Each neutralization curve was an average (±S.E.M) of two independent neutralization experiments with a pooled serum group. A) FRNT titre of anti-peptide antibody B16, B) FRNT titre of anti-peptide antibody B29, C) FRNT titre of anti-peptide antibody B38.

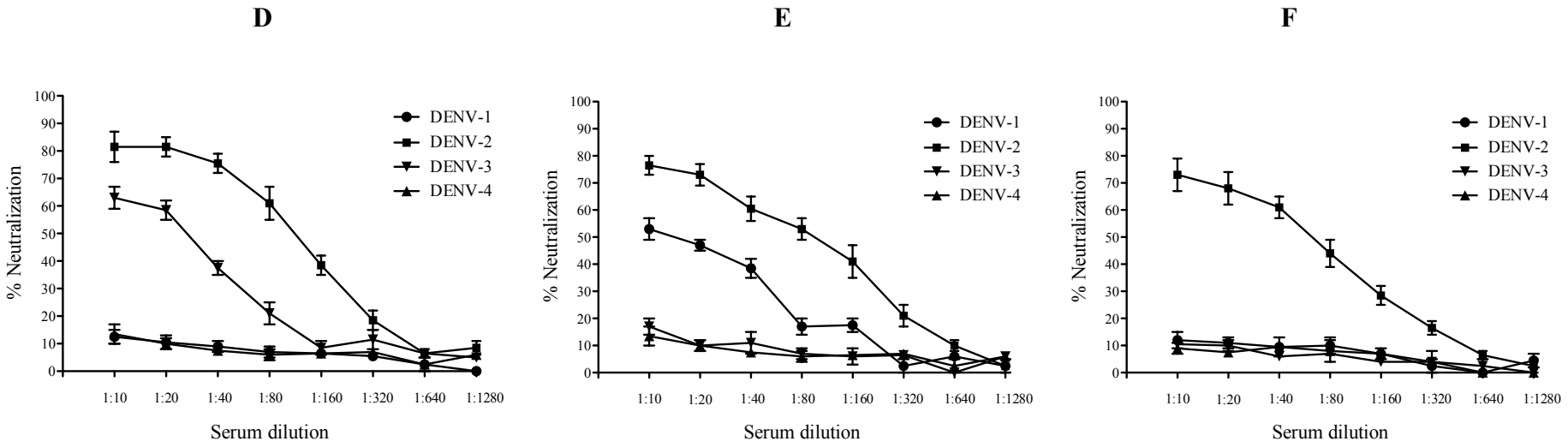


Fig. 5.4. Neutralizing ability of anti-peptide antibodies B45, B64 and B19 against four DENV serotypes. Serially diluted pooled immune sera were used to test the virus neutralizing ability against DENV-1, DENV-2, DENV-3 and DENV-4 in an *in vitro* focus reduction neutralization assay (FRNT) employing BHK-21 cells. A dilution resulting in 50% reduction of focus when compared to the pre-immune serum was considered as the end-point titre ($FRNT_{50}$). Each neutralization curve was an average (±S.E.M) of two independent neutralization experiments with a pooled serum group. D) FRNT titre of anti-peptide antibody B45, E) FRNT titre of anti-peptide antibody B64, F) FRNT titre of anti-peptide antibody B19.

Table 5.2. Neutralizing antibody titres of six vaccine constructs against DENV-1, DENV-2, DENV-3 and DENV-4 measured in an *in vitro* focus reduction neutralization assay (FRNT).

Vaccine constructs	50% neutralizing antibody titres ($FRNT_{50}$)*			
	DENV-1	DENV-2	DENV-3	DENV-4
B16	<10	80	<10	<10
B29	<10	80	10	<10
B38	<10	10	<10	<10
B45	<10	80	20	<10
B64	10	80	<10	<10
B19	<10	40	<10	<10

* Antibody titre ≥10 was considered as positive neutralization

5.3 Discussion

Dengue is the most important arthropod-borne viral disease in humans in terms of morbidity and mortality. Vaccine development against dengue remains a considerable scientific challenge due to the four antigenically distinct serotypes (Whitehead *et al.,* 2007; Murrell *et al.,* 2011). Earlier studies have shown that the majority of DENV neutralizing epitopes are located within the E glycoprotein, which is the major structural protein on the surface of viral particles (Guirakhoo *et al.,* 1989; Rey *et al.,* 1995; Roehrig *et al.,* 1998; Serafin and Aaskov, 2001). One possible strategy to design a successful vaccine might be to use a pool of synthetic peptides as a selected set of B-cell epitopes from all four of the DENV serotypes, which can elicit neutralizing antibody responses (da Silva *et al.,* 2009). In addition, using synthetic peptides to induce mature B-cell memory responses often requires cytokines from T helper (T_H) cells. In Chapter 4, several peptides on the E glycoprotein of DENV were identified through a combination of ELISA and epitope extraction using DENV immune human sera, and these epitope regions were further analysed for their surface accessibility and sequence conservation among multiple serotypes in an *in silico* epitope prediction approach.

The main focus in this chapter was to evaluate the vaccine potency of selected linear B-cell epitopes, which might be potentially useful in vaccine design and immunological studies. Seven potential B-cell epitopes were synthesized along with a T_H–cell epitope and these vaccine constructs were used to immunize inbred BALB/c mice. Synthetic peptide-based ELISA has been used in the development of serological assays for several viruses in addition to recombinant or inactivated antigens to determine antibody titers. However, the presence of neutralizing anti-DENV antibodies in post-immune sera can only be determined by virus neutralization assays performed with live DENV (Russell *et al.,* 1967; Morens *et al.,* 1985).

The results presented in this chapter indicated that six synthetic vaccine constructs elicited humoral immune responses after two vaccine doses as evidenced by both antibody binding and neutralization studies against multiple virus serotypes. The constructs B29 and B45 elicited cross-neutralizing antibody responses against DENV-2 and DENV-3, and were cross-reactive against the recombinant proteins of DENV-1, -2, and -3. The peptide B29 is located within the EDII domain and nine amino acids were conserved between position 204-KAWLVHRQWF-213 in both DENV-2 and -3. The

computational analysis of this region has revealed the highly accessible nature on the surface of the E protein. This is encouraging as many epitopes reported so far within EDII have been cross-reactive but poorly neutralizing or non-neutralizing (Roehrig *et al.,* 1998). The peptide B45 is located on the EDIII domain that has been reported to elicit powerful neutralizing antibodies, and be more virus type-specific (Crill and Roehrig, 2001; Gromowski, 2007; Gromowski *et al.,* 2008). However, the results obtained from this study contrasted to the earlier findings, as the antibodies were able to cross-neutralize both DENV-2 and DENV-3, and both serotypes exhibit 10 conserved amino acids between positions 314-ETQHGTIVIRVQY-326. This is a novel finding as the EDIII neutralizing epitopes reported in literature were mainly serotype specific (Oliphant *et al.,* 2007; Sukupolvi-Petty *et al.,* 2007; Wahala *et al.,* 2009).

Another critical finding from the current studies was the neutralizing epitope B64 representing the "stem" anchor region located at aa 442-459. It has been shown that the majority of sequences in "stem" region were conserved across all DENV serotypes and other flaviviruses (Schmidt *et al.,* 2010). The peptide B64 had 11 conserved amino acids in positions 448-FSGVSWTMKILI-459 between DENV-1 and DENV-2, and computational analyses has revealed this epitope region to be moderately accessible on the surface of the virion particle. The antibodies elicited by B64 were able to neutralize DENV-2 with $FRNT_{50}$ titre 1:80 and DENV-1 at 1:10. Surprisingly, these antibodies reacted against the 395 aa DENV-2 soluble recombinant protein but did not cross-react with DENV-1. Since the linear amino acid sequence of peptide B64 does not represent any epitope region on the sE protein, the affinity binding might be due to recognition of a conformational epitopte(s) on DENV-2 but not in other serotypes. This is yet another interesting finding because our current knowledge on the DENV neutralizing epitopes is mainly limited to sE protein (Murphy and Whitehead, 2011; de Alwis *et al.,* 2012). Clearly, further work is needed to characterize the neutralizing epitopes found within the "stem" anchor region.

In addition to the peptides eliciting heterologous antibody responses against different serotypes, three epitopes (B16, B38 and B19) eliciting homologous neutralizing response against DENV-2 were also identified. The peptide B16 (aa 106-123) representing the fusion peptide region on the EDII was able to neutralize DENV-2 ($FRNT_{50}$ titre 1:80), however it was capable of cross-reacting against all three DENV recombinant E proteins. The fusion peptide is a hydrophobic sequence conserved

among all flaviviruses and hidden on the dimer interface that becomes exposed during the conformational change at low pH (Zhang *et al.,* 2003; Zhang *et al.,* 2004). This antigenic region elicits highly cross-reactive antibodies but exhibit serotype-specific neutralizing antibody response as reported with studies both in mice and humans (Crill and Chang, 2004; Stiasny *et al.,* 2006; Oliphant *et al.,* 2007; Lai *et al.,* 2008).

The peptide B38 elicited detectable neutralizing antibody response ($FRNT_{50}$ titre 1:10) against DENV-2 and was able to bind to the DENV-2 sE protein. A similar binding pattern was seen in B19 with $FRNT_{50}$ titre 1:140. The amino acid sequence of B19 is highly variable in DENV-1, -2 and -3 but highly accessible on the virion surface. This epitope is found within the hinge region between EDI/EDII and the region corresponding to 127-GKVVLPEN-134 has been shown to elicit serotype-specific neutralizing antibodies (Aaskov *et al.,* 1989; Roehrig *et al.,* 1998; Falconar, 1999; Crill and Roehrig, 2001; Oliphant *et al.,* 2006; Gromowski *et al.,* 2008). The current study has also revealed similar results with homologous antibody binding and neutralizing response against DENV-2 suggesting that peptide 38 and 19 can be used in vaccine formulation specifically against DENV-2. Our preliminary vaccine potency testing study has limitations. Due to limited availability of experimental animals, we have not tested all the epitopes identified through our epitope mapping strategies; however, the preliminary results encourages us to design experiments for further screening of all the potential epitope candidates identified in our study. Also, a detailed study with different T-helper epitopes is necessary to see how the peptides react in an endemic population with dengue antibodies presented in the sera already. In Addition, though these epitopes reacted with the dengue immune sera of volunteers from diverse ethnic group with diverse HLA polymorphism, clearly further studies are needed in an out bred mice population.

In this study, five novel neutralizing epitopes (B16, B29, B38, B45, B64) eliciting humoral immune responses against different DENV serotypes were identified. These neutralizing epitopes are located at distinct regions within the E protein of DENV. It is crucial to develop a tetravalent dengue vaccine that elicits specific neutralizing antibodies against different DENV serotypes to overcome the phenomenon of antibody- dependent enhancement associated with DSS and DHF (Dejnirattisai *et al.,* 2010; Murrell *et al.,* 2011; de Alwis *et al.,* 2012). Since vaccination with a tetravalent vaccine appears to be a sustainable strategy for dengue disease prevention, the

synthetic-peptide based approach could be used to test the vaccine candidates against all four DENV serotypes. These epitope-based vaccines may provide some important information for the development of a tetravalent multi-epitope peptide vaccine to improve protective long-lasting immune responses against DENV infection.

Chapter 6

Summary

Dengue is one of the major arboviral diseases affecting humans and it is crucial to generate effective countermeasures against this disease in the form of vaccines, antivirals, and other therapeutics (Whitehead *et al.*, 2007). Neutralizing antibodies are a critical component of immune-mediated protection from dengue and the E protein has been shown to be the primary target (Roehrig *et al.*, 2004). Earlier studies have suggested that neutralization potency correlates with the epitopes located within particular E protein domain recognized by neutralizing antibodies (Oliphant *et al.*, 2006; Throsby *et al.*, 2006; Lai *et al.*, 2008; Beltramello *et al.*, 2010; Murphy and Whitehead, 2011; de Alwis *et al.*, 2012; Lin, 2012; Wahala *et al.*, 2012). These observations led to the objective of this dissertation, which was to use novel strategies to map the epitopes of DENV E protein recognized by human antibody repertoire. The results of this dissertation confirm and expand previous observations of DENV E protein antigenicity. The results also provide new insights into the dengue virus humoral immune response, and suggest new strategies for B-cell epitope identification.

6.1 Analysis of humoral immune responses of DENV infected individuals

Investigation of polyclonal human immune responses following dengue outbreaks is important to understand the interactions between DENV and neutralizing antibody levels (de Alwis *et al.,* 2012). Despite the large body of work with mouse MAbs, remarkably little work has been done to characterize the relationship between human antibody binding and neutralization of human DENV immune sera (Wahala *et al.*, 2009). This study presents results from experiments that begin to dissect the complexities of the human polyclonal immune response to DENV infection from previously unstudied cohort of dengue-infected individuals from Queensland, Australia. Our study reveals that the predominant neutralizing antibodies found among dengue volunteers were against DENV-2 and DENV-3. An interesting observation was that the primary infection with DENV-3 led to one DHF case; which is not a common phenomenon in dengue infection.

Sera from volunteers who had recovered from both primary and secondary DENV infections were investigated and IgG titres against primary infection were found to be greater than secondary infections. However, the neutralizing antibody levels of sera from secondary infections (Sera no. 3 and 20) were higher against secondary infecting virus. These epidemiological data admits and suggests the fact that the pre-existing antibody levels in adult human populations must be considered, as candidate DENV vaccines must eventually be evaluated in adult populations in DENV-endemic regions. Our study demonstrates the ability to define the incidence of DENV infection and neutralizing antibody levels in an adult cohort living in an area where all four virus serotypes circulate. The study also resulted in the collection of valuable reagents that can be used to study the immunopathogenesis of DENV and to further define correlates of human antibody repertoire following DENV infection.

6.2 Strategies for epitope mapping of DENV E protein

Several methods for the identification of linear and discontinuous epitopes in DENV have been used such as, competition assays, phage display, neutralization escape mutants, peptide scan and computer based epitope prediction (Aaskov *et al.*, 1989) (Roehrig *et al.*, 1998; Falconar, 1999; Amexis and Young, 2007; da Silva *et al.*, 2009; Sanchez-Burgos *et al.*, 2010; Li *et al.*, 2011; Lin, 2012). The results obtained in this dissertation significantly expand on these previous studies and focuses on the mapping of cross-reactive epitopes of the DENV E protein using a combination of 3 different strategies. Twenty-nine epitopes have been identified, described and putatively mapped on the E protein of DENV-2 using ELISA and a novel epitope extraction approach. These epitopes are spanning all three domains of the sE protein and the ectodomain of the native E protein. Nine epitopes were identified common in both methods. Eight epitopes were identified in ELISA only and 12 peptides were recognized in epitope extraction only. Six antigenic regions on the DENV-2 E protein were identified through the computational analysis and these regions harbour 6 epitopes identified by both wet-lab methods.

The presence of these cross-reactive antibody epitopes is presumably long-lived as we used convalescent sera from dengue-infected individuals to exploit the information inherent in the binding sites of DENV E protein. We explored, for the first

time, the possibilities of using a solution phase epitope extraction strategy with the help of sensitive mass spectrometry in dengue to map the epitopes of E protein. In addition, our multi-step computational approach revealed several potential epitope candidates on the E protein of all four serotypes of DENV. The *in silico* approach employed in our study is a useful tool that streamlines the process of vaccine design, and the peptides identified through this approach were thought to contain potential antigenic sites based on antigenicity profiles and algorithmic measures of binding. These three epitope mapping strategies might be useful for fine epitope mapping of DENV and other related flaviviruses.

6.3 Vaccine potency of the epitopes identified on the DENV-2 E

The binding of the dengue-derived peptides to antibody has important biomedical applications in drug discovery and vaccine design. Our main focus in this study was to examine the immunogenicity of individual B-cell epitopes identified through our epitope mapping strategies. A successful synthetic peptide vaccine should incorporate both a B-cell and T-Helper cell epitope in order to induce a strong protective response. Because immunization with short peptides often yields antibody preparation with poor titre and specificity, we coupled these peptides with a known T-helper epitope and used in our immunization protocol. Synthetic peptide-based vaccines allow for a simplified approach to vaccine design, whereby deleterious sequences can be eliminated and a vaccine can consist of the minimal proportion of a pathogen required to induce an effective and efficient immune response.

The data presented in our study demonstrates that 5 novel synthetic vaccine constructs elicited humoral immune responses and neutralizing one or more DENV serotypes *in vitro*, and are cross-reactive towards soluble recombinant E protein. The findings from our study also confirm and extend previous reports on the EDII fusion peptide as an immunodominant region made of a series of overlapping epitopes stimulating broadly cross-reactive antibodies (Stiasny *et al.*, 2006; Oliphant *et al.*, 2007; Lai *et al.,* 2008). However, our results contrast with the study by Oliphant *et al.* 2007, who showed that all of their cross-reactive neutralizing mouse MAbs recognizing domains I and II of the E protein recognized the fusion loop. It seems highly unlikely that all of these immunoglobulins could bind to the fusion loop. Our results presented in

this dissertation may underscore a significant difference between the human and murine immune responses. Furthermore, our study would add new insight into our understanding of epitopes related to protective humoral immune responses and provide another piece of critical information for rationale design of peptide-based vaccine against DENV.

6.4 Conclusion and future directions

No dengue vaccine is currently licensed for human use. Developing a vaccine for dengue has been an elusive goal because of the need to confer solid and long-lasting tetravalent protection. In this study we have identified B-cell epitopes from the DENV E protein, that when incorporated into a synthetic peptide-based vaccine construct elicit antibodies with the ability to neutralize one or more DENV serotypes. These peptide epitopes represent potential new vaccine candidates not only for a single serotype, but multiple DENV serotypes. The epitopes identified in our multi-disciplinary approach in conjunction with other well-documented epitopes of DENV together have implications for designing future epitope-specific diagnostics and epitope based dengue vaccine.

There is a significant amount of research that still needs to be done in order to understand the epitopes of E protein as it relates to virus neutralization. This dissertation, however, provides some valuable insights into the physical and biological interactions between DENV-2 neutralizing antibodies and epitopes on the E protein. Future studies should focus on doing similar analyses of epitopes identified in E protein against DENV-1, -3, and -4. This will offer a more complete picture of the antigenic surface of E protein for the DENVs. Synthetic mono and/or polyvalent vaccine constructs could be used for presentation of antigenic B-cell epitopes to evaluate individual or combined antigens in experimental DENV vaccines (Amexis and Young, 2007). In addition, promiscuous B- and T-helper epitopes could be incorporated during vaccine design to create synthetic vaccine constructs, which could enhance immune responses. Immunogenicity of synthetic vaccine constructs could be further augmented by covalent attachment of “built-in adjuvants” such as lipids (Zeng *et al.*, 2005). Since vaccination with a tetravalent vaccine formulation appears to be the sustainable strategy for disease prevention, the synthetic peptide based vaccine platform could be used to test DENV vaccine candidates against all four DEN serotypes.

Appendices

Appendix I

Evidence of human ethics approval for collection and usage of sera from dengue infected volunteers

Queensland Government

Office of the Human Research Ethics Committee
P O Box 902 Cairns Q 4870

29 March 2010

Enquiries to: Margaret Grasso
Phone: 07 40 50 8012
Fax: 07 40 50 6331
Queensland Health
Our Ref: HREC/10/QCH/17 – 646
NB:mg 58.03
E-mail Margaret_Grasso@health.qld.gov.au

Dr John McBride
JCU School of Medicine
Cairns Base Hospital

Dear Dr McBride

HREC Reference number: HREC/10/QCH/17 - 646
Project title: Using the information inherent in binding sites of dengue-virus-specific antibodies from patients who have successfully cleared infection to identify neutralizing antibody epitopes with the potential to be vaccine candidates
Protocol number: Protocol Ref N/A

Thank you for submitting the above project for ethical and scientific review. This project was first considered by the Cairns & Hinterland Health Service District Human Research Ethics Committee (HREC) held on 11th March 2010. I have reviewed your correspondence of 25th March 2010 in which you provided amendments requested by the HREC in my letter of 17th March 2010 and grant final approval.

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007)* and the *CPMP/ICH Note for Guidance on Good Clinical Practice*. Attached is the HREC Composition with specialty and affiliation with the Hospital (Attachment I).

I am pleased to advise that the Human Research Ethics Committee has now granted final approval of this research project. The documents reviewed and approved include:

Document	*Version*	*Date*
Covering Letter		16 February 2010
Patient Information Sheet/Consent Form	3.0	25 March 2010
ATSI Guidelines		16 February 2010
Application: NEAF		
Response to Request for Further Information		25 March 2010

Please note the following conditions of approval:

1. If relevant to your project, your attention is drawn to standards for clinical trials reporting as enunciated in the CONSORT statement *(hhpt://www.consort-statement.ort/?o=1001)* and a requirement by many journals for certain categories of clinical trials to be registered (see: *hhpt://www.actr.org.au*)

2. The Principal Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including:
    a. Unforeseen events that might affect continued ethical acceptability of the project. Serious Adverse Events must be notified to the Committee as soon as possible. In addition the Investigator must provide a summary of the adverse events, in the specified format, including a comment as to suspected causality and whether changes are required to the Patient Information and Consent Form. In the case of Serious Adverse Events occurring at the local site, a full report is required from the Principal Investigator, including duration of treatment and outcome of event.

3. Amendments to the research project which may affect the ongoing ethical acceptability of a project must be submitted to the HREC for review. Major amendments should be reflected in a revised online NEAF (accompanied by all relevant updated documentation and a cover letter from the principal investigator, providing a brief description of the changes, the rationale for the changes, and their implications for the ongoing conduct of the study). Hard copies of the revised NEAF, the cover letter and all relevant updated documents with tracked changes must also be submitted to the HREC coordinator as per standard HREC SOP. Further advice on submitting amendments is available from http://www.health.qld.gov.au/cpic/documents/ethics/researcher_userguide.pdf

4. Amendments to the research project which only affect the ongoing site acceptability of the project are not required to be submitted to the HREC for review. These amendment requests should be submitted directly to the Research Governance Office/r (by-passing the HREC).

5. Proposed amendments to the research project which may affect both the ethical acceptability and site suitability of the project must be submitted firstly to the HREC for review and, once HREC approval has been granted, then submitted to the RGO.

6. Amendments which do not affect either the ethical acceptability or site acceptability of the project(e.g. typographical errors) should be submitted in hard copy to the HREC coordinator. These should include a cover letter from the principal investigator providing a brief description of the changes and the rationale for the changes, and accompanied by all relevant updated documents with tracked changes.

7. The HREC will be notified, giving reasons, if the project is discontinued at a site before the expected date of completion.

8. The Principal Investigator will provide an annual report to the HREC and at completion of the study in the specified format.

9. The District administration and the Human Research Ethics Committee may inquire into the conduct of any research or purported research, whether approved or not and regardless of the source of funding, being conducted on hospital premises or claiming any association with the Hospital; or which the Committee has approved if conducted outside Cairns & Hinterland Health Service District.

You are reminded that this letter constitutes ethical approval only. You must not commence this research project at a Queensland Health site until separate authorisation from the District CEO or Delegate of that site has been obtained.

A copy of this approval must be submitted to the District Research Governance Officer/Delegated Personnel with a completed Site Specific Assessment (SSA) Form for authorisation from the District CEO or Delegate to conduct this research at the Cairns & Hinterland Health Service District. Andrea Willets is contactable on 40 40 7492.

HREC approval is valid from the date of this letter until 31st January 2015.

Should you have any queries about the HREC's consideration of your project please contact Margaret Grasso, HREC Administrator on 07 40 50 8012. The HREC terms of Reference, Standard Operating Procedures, membership and standard forms are available from http://www.health.qld.gov.au/cpic/ethics/reagu_homepage.asp

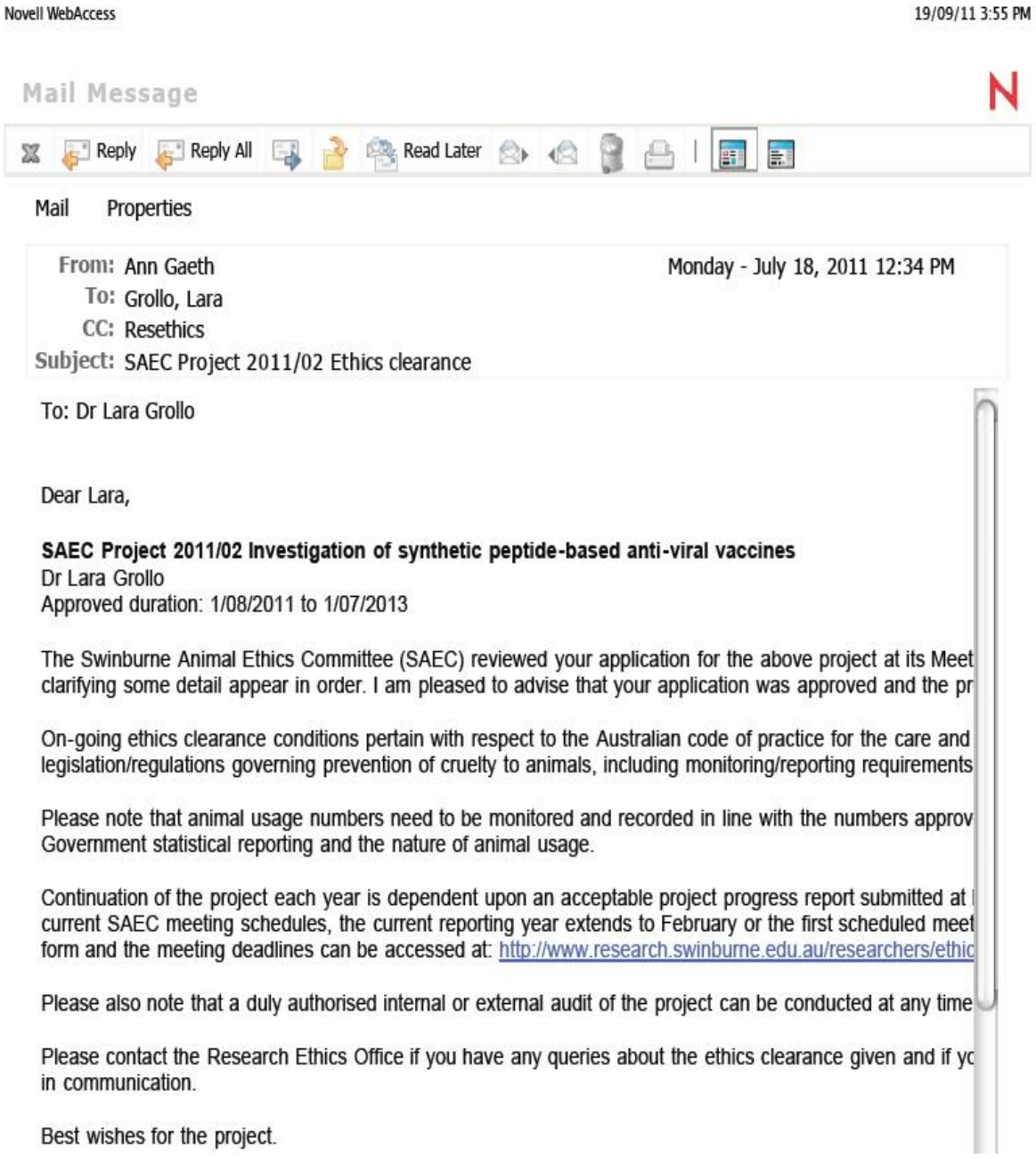
Once authorisation to conduct the research has been granted, please complete the Commencement Form (Attachment II) and return to the office of the Human Research Ethics Committee.

The HREC wishes you every success in your research.

Yours faithfully

A/Prof. Neil Beaton
A/CHAIR
HUMAN RESEARCH ETHICS COMMITTEE
CAIRNS & HINTERLAND HEALTH SERVICE DISTRICT

Evidence of animal ethics approval for using mice in vaccine potency testing



Novell WebAccess 19/09/11 3:55 PM

Mail Message

Reply Reply All Read Later

Mail Properties

From: Ann Gaeth Monday - July 18, 2011 12:34 PM
To: Grollo, Lara
CC: Resethics
Subject: SAEC Project 2011/02 Ethics clearance

To: Dr Lara Grollo

Dear Lara,

SAEC Project 2011/02 Investigation of synthetic peptide-based anti-viral vaccines
Dr Lara Grollo
Approved duration: 1/08/2011 to 1/07/2013

The Swinburne Animal Ethics Committee (SAEC) reviewed your application for the above project at its Meet
clarifying some detail appear in order. I am pleased to advise that your application was approved and the pr

On-going ethics clearance conditions pertain with respect to the Australian code of practice for the care and
legislation/regulations governing prevention of cruelty to animals, including monitoring/reporting requirements

Please note that animal usage numbers need to be monitored and recorded in line with the numbers approv
Government statistical reporting and the nature of animal usage.

Continuation of the project each year is dependent upon an acceptable project progress report submitted at
current SAEC meeting schedules, the current reporting year extends to February or the first scheduled meet
form and the meeting deadlines can be accessed at: http://www.research.swinburne.edu.au/researchers/ethic

Please also note that a duly authorised internal or external audit of the project can be conducted at any time

Please contact the Research Ethics Office if you have any queries about the ethics clearance given and if yc
in communication.

Best wishes for the project.

Ethics statement:

The Cairns Base Hospital Ethics Committee approved the human ethics application and trained professionals at an approved performed the collection of all human blood samples at Cairns Base Hospital. The collected samples were then used at Swinburne University of Technology, Hawthorn Campus, with the approval of Swinburne University Human Research Ethics Committee. All conditions stated in the ethics approval were met.

Swinburne Animal Ethics Committee approved the animal ethics. All scientific procedures using animals were carried out in accordance with the conditions stated in the ethics approval.

Appendix II

ELISA data of peptides reacted against anti-dengue human IgG

Peptide number	Sera number and corresponding OD* values								
	15	**19**	**33**	**1**	**4**	**8**	**9**	**12**	**13**
2	0.854	0.253	0.551	0.381	0.216	0.177	0.417	0.592	0.075
16	1.390	1.132	0.944	0.834	1.055	0.611	0.852	1.254	0.382
19	0.618	0.406	0.288	0.516	0.406	0.325	0.367	0.512	0.064
28	0.054	0.095	0.055	0.326	0.352	0.191	0.107	0.292	0.384
29	0.199	0.299	0.069	0.036	0.066	0.093	0.049	0.123	0.046
33	0.083	0.250	0.112	0.094	0.082	0.121	0.076	0.042	0.061
38	0.251	0.228	0.048	0.081	0.058	0.116	0.072	0.056	0.068
40	0.816	0.854	0.548	0.635	0.858	0.369	0.403	0.589	0.062
43	0.343	0.434	0.211	0.087	0.055	0.203	0.097	0.354	0.109
45	0.992	0.634	0.555	1.181	0.913	0.740	0.475	0.755	0.239
46	0.049	0.056	0.103	0.360	0.240	0.176	0.062	0.304	0.089
47	0.200	0.103	0.117	0.057	0.430	0.420	0.070	0.058	0.062
48	0.187	0.043	0.431	0.107	0.246	0.169	0.083	0.071	0.067
53	0.048	0.361	0.070	0.412	0.065	0.270	0.088	0.337	0.093
54	0.550	0.410	0.271	0.436	0.378	0.221	0.262	0.428	0.055
64	0.598	0.498	0.394	0.628	0.554	0.340	0.352	0.590	0.047
68	0.270	0.208	0.107	0.054	0.517	0.499	0.120	0.083	0.067
69	0.615	0.421	0.155	0.572	0.482	0.434	0.292	0.490	0.039
70	0.904	0.616	0.045	0.917	0.706	0.597	0.442	0.669	0.096

* OD- Optical density

Samples of IgG at a concentration of 20 µg/ml were used in direct binding ELISA to test the cross reactivity against peptides and the results are presented as the mean optical density of triplicates. IgG from 4 non-infected individuals were used as a negative control and the cut-off value to select the positive peptides was OD-0.128. The positive peptides were selected based on the antibody reaction above the cut-off value.

ELISA data continued.,

Peptide number	Sera number and corresponding OD* values								
	36	**40**	**38**	**2**	**7**	**18**	**21**	**22**	**24**
2	0.404	0.378	0.375	0.522	0.367	0.070	0.457	0.268	1.131
16	0.611	0.389	0.942	0.608	1.339	0.413	0.803	0.800	1.203
19	0.292	0.202	0.089	0.916	0.668	0.235	0.192	0.190	0.521
28	0.152	0.254	0.105	0.782	0.465	0.094	0.080	0.090	0.481
29	0.061	0.107	0.071	0.086	0.076	0.113	0.088	0.082	0.046
33	0.046	0.306	0.057	0.042	0.069	0.119	0.095	0.093	0.840
38	0.223	0.109	0.090	0.340	0.076	0.101	0.072	0.062	0.291
40	0.603	0.987	0.571	0.484	0.920	0.305	0.323	0.581	0.629
43	0.253	0.224	0.062	0.065	0.086	0.110	0.071	0.118	0.118
45	0.491	0.372	0.325	1.279	1.154	0.693	0.163	0.050	0.048
46	0.097	0.087	0.091	0.648	0.381	0.093	0.074	0.119	0.110
47	0.062	0.062	0.055	0.863	0.858	0.461	0.270	0.235	0.051
48	0.037	0.543	0.048	0.605	0.263	0.219	0.112	0.068	0.051
53	0.282	0.085	0.069	0.539	0.423	0.361	0.068	0.085	0.540
54	0.046	0.053	0.038	0.752	0.397	0.256	0.227	0.303	0.568
64	0.306	0.297	0.262	0.562	0.552	0.460	0.285	0.360	0.702
68	0.103	0.059	0.090	0.609	0.497	0.483	0.093	0.070	0.448
69	0.274	0.080	0.086	0.551	0.565	0.416	0.111	0.083	0.498
70	0.245	0.069	0.098	0.757	0.843	0.641	0.098	0.076	0.501

* OD- Optical density

Samples of IgG at a concentration of 20 µg/ml were used in direct binding ELISA to test the cross reactivity against peptides and the results are presented as the mean optical density of triplicates. IgG from 4 non-infected individuals were used as a negative control and the cut-off value to select the positive peptides was OD-0.128. The positive peptides were selected based on the antibody reaction above the cut-off value.

ELISA data continued.,

Peptide number	Sera number and corresponding OD* values								
	27	**28**	**29**	**31**	**35**	**37**	**3**	**16**	**6**
2	0.074	0.596	0.578	0.572	0.998	0.611	0.798	0.647	0.060
16	0.548	0.682	1.023	1.048	0.838	1.069	1.547	0.690	0.647
19	0.752	0.495	0.318	0.352	0.305	0.327	0.899	0.425	0.341
28	0.042	0.361	0.089	0.237	0.239	0.290	0.041	0.342	0.275
29	0.074	0.093	0.071	0.108	0.110	0.075	0.205	0.216	0.170
33	0.100	0.088	0.094	0.089	0.075	0.117	0.189	0.074	0.101
38	0.064	0.090	0.084	0.091	0.124	0.072	0.191	0.145	0.117
40	0.048	0.372	0.497	0.418	0.641	0.679	0.720	0.417	0.578
43	0.056	0.086	0.055	0.082	0.105	0.092	0.344	0.056	0.443
45	0.493	0.544	0.456	0.412	0.501	0.508	0.840	0.596	0.934
46	0.100	0.084	0.060	0.121	0.056	0.071	0.055	0.228	0.240
47	0.070	0.125	0.085	0.080	0.077	0.089	0.135	0.210	0.468
48	0.046	0.111	0.072	0.056	0.068	0.080	0.145	0.154	0.265
53	0.065	0.387	0.215	0.046	0.287	0.302	0.695	0.305	0.443
54	0.259	0.406	0.284	0.242	0.260	0.278	0.836	0.357	0.347
64	0.392	0.423	0.286	0.322	0.336	0.458	0.838	0.381	0.506
68	0.046	0.060	0.096	0.110	0.091	0.270	0.336	0.171	0.524
69	0.056	0.083	0.052	0.115	0.113	0.265	0.742	0.352	0.477
70	0.045	0.094	0.117	0.118	0.109	0.283	1.068	0.482	0.701

* OD- Optical density

Samples of IgG at a concentration of 20 µg/ml were used in direct binding ELISA to test the cross reactivity against peptides and the results are presented as the mean optical density of triplicates. IgG from 4 non-infected individuals were used as a negative control and the cut-off value to select the positive peptides was OD-0.128. The positive peptides were selected based on the antibody reaction above the cut-off value.

ELISA data continued.,

Peptide number	Sera number and corresponding OD* values						
	14	**25**	**26**	**30**	**32**	**34**	**39**
2	0.287	0.450	0.206	0.698	0.478	0.562	0.473
16	0.605	0.779	0.725	0.908	0.858	0.490	0.693
19	0.322	0.226	0.464	0.413	0.389	0.195	0.232
28	0.271	0.075	0.114	0.106	0.320	0.086	0.193
29	0.115	0.089	0.058	0.077	0.060	0.044	0.057
33	0.150	0.072	0.264	0.098	0.120	0.190	0.052
38	0.087	0.082	0.098	0.089	0.111	0.069	0.090
40	0.384	0.372	0.347	0.359	0.417	0.385	0.512
43	0.093	0.123	0.242	0.161	0.178	0.223	0.187
45	1.107	0.349	0.695	0.511	0.476	0.342	0.299
46	0.089	0.101	0.095	0.170	0.168	0.210	0.189
47	0.063	0.069	0.113	0.183	0.154	0.062	0.147
48	0.052	0.155	0.119	0.068	0.169	0.050	0.047
53	0.757	0.178	0.392	0.341	0.330	0.094	0.076
54	0.343	0.245	0.456	0.316	0.292	0.050	0.061
64	0.468	0.206	0.310	0.318	0.419	0.235	0.267
68	0.074	0.112	0.078	0.111	0.222	0.081	0.067
69	0.534	0.120	0.248	0.144	0.236	0.184	0.095
70	0.853	0.118	0.262	0.131	0.205	0.188	0.088

* OD- Optical density

Samples of IgG at a concentration of 20 µg/ml were used in direct binding ELISA to test the cross reactivity against peptides and the results are presented as the mean optical density of triplicates. IgG from 4 non-infected individuals were used as a negative control and the cut-off value to select the positive peptides was OD-0.128. The positive peptides were selected based on the antibody reaction above the cut-off value.

Appendix III

Epitope extraction results of peptides reacted against anti-dengue human IgG

Peptides	Sera number and peptides reacted														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	p	p	p			p	p	p			p				
2	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
3	p	p	p	p	p	p	p	p	p	p	p	p		p	p
16	p	p	p	p	p		p	p	p		p	p	p	p	p
19	p	p	p	p	p	p	p			p	p	p			p
20	p	p	p	p	p	p	p			p	p	p			p
29	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
30	p		p	p	p	p	p	p	p	p	p	p	p		p
32	p	p	p	p			p			p	p	p			p
33				p		p	p			p	p	p			
37	p		p	p	p	p	p		p	p	p				p
38	p		p	p	p	p	p	p	p	p	p	p		p	
39	p		p	p	p	p	p	p	p	p	p	p		p	
40	p		p	p	p	p	p	p	p	p	p			p	
45	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
52	p		p	p	p	p	p			p	p	p			p
62	p	p	p	p			p			p	p	p			p
63	p	p	p	p		p	p	p		p	p	p		p	p
64	p	p	p		p		p	p		p		p		p	p
65															
66	p	p	p	p		p	p	p	p	p	p	p		p	p
67	p	p	p	p		p			p	p	p	p		p	

Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, the peptides were identified through mass spectrometry. Letter "p" indicates the peptides reacted positively.

Epitope extraction results continued.,

Peptides	Sera number and peptides reacted														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	p			p		p		p		p	p				
2	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
3	p	p		p	p	p	p		p	p	p	p		p	p
16	p	p	p	p	p		p	p	p	p	p		p	p	p
19	p	p		p		p	p			p	p	p			p
20	p	p		p		p	p			p	p	p			p
29	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
30	p	p		p	p	p	p	p	p	p	p	p	p		p
32	p			p			p			p	p	p			p
33	p			p		p	p			p	p	p			
37	p	p			p	p	p		p	p	p				p
38	p	p	p	p	p	p	p	p	p	p	p	p		p	
39	p	p	p	p	p	p	p	p	p	p	p	p		p	
40	p	p	p	p	p	p	p	p	p	p	p	p			
45	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
52	p	p		p		p	p			p	p	p			p
62	p			p			p			p	p	p			p
63	p		p	p	p	p	p	p	p	p	p	p	p	p	p
64	p	p	p	p			p	p		p		p		p	p
66	p		p	p	p	p	p	p	p	p	p	p		p	p
67	p		p	p	p	p			p	p	p	p		p	

Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, the peptides were identified through mass spectrometry. Letter "p" indicates the peptides reacted positively.

Epitope extraction results continued.,

Peptides	Sera number and peptides reacted									
	31	32	33	34	35	36	37	38	39	40
1	p	p	p			p	p	p		
2	p	p	p	p	p	p	p	p	p	p
3	p	p	p	p	p	p	p		p	p
16	p	p	p	p	p		p	p	p	
19	p	p	p	p	p	p	p			p
20	p	p	p	p	p	p	p			p
29	p	p	p	p	p	p	p	p	p	p
30	p		p	p	p	p	p	p	p	p
32	p	p	p	p			p			p
33				p		p	p			p
37	p		p	p	p	p	p		p	p
38	p		p	p	p	p	p	p	p	p
39	p		p	p	p	p	p	p	p	p
40	p		p	p	p	p	p	p	p	p
45	p	p	p	p	p	p	p	p	p	p
52	p		p	p	p	p	p			p
62	p	p	p				p	p		p
63	p	p	p	p	p	p	p	p		p
64	p	p	p		p		p	p		p
66	p	p	p	p		p	p		p	p
67	p		p	p		p				p

Pools of overlapping peptides spanning the E protein of DENV-2 were assembled such that no pool contained peptides of similar mass. These peptide pools were allowed to react with IgG purified from DENV infected patient's sera and the antibody-bound peptides were extracted in acidic conditions. Following extraction, the peptides were identified through mass spectrometry. Letter "p" indicates the peptides reacted positively.

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