ANTIGENIC EVOLUTION OF DENGUE VIRUS IN MALAYSIA

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ABSTRACT

Neutralization escape mutant has played an important role in antigenic evolution of various virus-causing diseases. DENV, the causative agent of dengue has been shown to escape antibody neutralization, thereby causing an epidemic outbreak. The pattern through which DENV escape neutralization that leads to antigenic evolution, however, has not been thoroughly investigated in hyper endemic countries. It is hypothesized that, the highly susceptible DENV demonstrates a clear pattern of antigenic evolution from being minor strains (less frequent during an outbreak) to dominant strains (very frequent during an outbreak). This suggests that the minor strains may act as a springboard for the emergence of more virulent dominant strains by acquiring some characteristics of the minor strains. Hence, the overall objective of the present study is to provide an insight into the antigenic evolution of DENV-1 and DENV-2 in Malaysia. To investigate this possibility, the present study used truncated DENV-1 and DENV-2 recombinant E, cloned and expressed on phage M13 g3p attachment protein. Using site-directed mutagenesis, six single mutations were engineered separately onto the respective recombinant E gene. Effects of these mutations on binding to insects and mammalian cells were determined *in vitro.* The polyclonal antibodies generated against these recombinant proteins in BALB/c mice were used in microneutralization and Foci Reduction Neutralization Test to evaluate neutralizing antibody determinant associated with antigenic evolution. Serial propagation *in vitro* was adopted to handpick the neutralization escape mutant in the sub-neutralizing antibody concentration. To evaluate potential host where the selection occurs, the escape mutant were adapted to mimic mosquito and human cycle *in vitro*. Finding from the present study shows that two mutations (E272 and E390) on DENV-1 E were demonstrated to affect binding of the recombinant proteins to C6/36 mosquito and Vero cells. Polyclonal antibodies generated against recombinant E272 and E390 proteins exhibited decreased binding to

DENV-1 virion. The polyclonal antibodies reduced the number of DENV-1 foci formed up to four-folds for the five endemic DENV-1 strains (GIa, GIb, GIc, GII and GIII) tested. Two DENV-1 isolates likely to escape neutralization were identified; D1.10245 (GII) and D1.11177 (GIb). In DENV-2, mutations on E52, E164 and E393 were demonstrated to affect binding of the recombinant proteins to C6/36 mosquito and Vero cells. Polyclonal antibodies generated in mice against recombinant E52, E164 and E176 exhibited decreased binding to DENV-2 (Asian 1 strain) and E164 and E335 on one of the Cosmopolitan strain. The polyclonal antibodies against E52, E71, E335 and E393 reduced the number of foci formed up to four-folds for Cosmopolitan DENV-2 strains tested. The E52 and E71 substitutions select isolates likely to escape neutralization in both Cosmopolitan strain (D2.14245 and D2.1877347) and Asian 1 strain (D2.16041). Evidence from the present study show that the neutralization escape strains from DENV-1 and DENV-2 alternating between two different hosts adapts differently. The observed difference in DENV-1 and DENV-2 on C6/36 and Vero cells was evident in the variation in amino acid residues on E. Of these 6 mutations engineered on DENV-1 and DENV-2 recombinant E, substitution on EDI/EDII junction are likely to facilitates antigenic evolution of DENV-1 (E272) and DENV-2 (E52 and E71) in Malaysia. Overall, the study identified dominant DENV-1 and DENV-2 strains expected to evolve in Malaysian natural population in the future. The antigenic determinants involved were identified. The result of the study directly underlines the effects of these mutations on naturally emerging DENV-1 and DENV-2 in Malaysian population and its implications on dengue affliction and vaccines design.

ABSTRAK

Peneutralan melarikan diri mutan telah memainkan peranan penting dalam evolusi antigen pelbagai penyakit virus penyebab. DENV, agen penyebab denggi telah terbukti untuk melarikan diri antibodi peneutralan, sekali gus menyebabkan wabak wabak. Corak di mana DENV melarikan diri peneutralan yang membawa kepada evolusi antigen, bagaimanapun, telah tidak dikaji secara menyeluruh di negara-negara endemik hyper. Ia hipotesis bahawa, DENV yang sangat mudah terdedah menunjukkan corak yang jelas evolusi antigen daripada menjadi strain kecil (kurang kerap semasa wabak) untuk strain dominan (sangat kerap semasa wabak). Ini menunjukkan bahawa strain kecil boleh bertindak sebagai batu loncatan untuk kemunculan strain dominan lebih getir dengan memperoleh beberapa ciri-ciri strain kecil. Oleh itu, objektif keseluruhan kajian ini adalah untuk memberi kefahaman tentang evolusi antigen daripada DENV-1 dan DENV-2 di Malaysia. Untuk menyiasat kemungkinan ini, kajian ini digunakan dipenggal DENV-1 dan DENV-2 rekombinan E, klon dan menyatakan pada faj M13 g3p lampiran protein. Menggunakan mutagenesis diarahkan tapak , enam mutasi tunggal telah kejuruteraan secara berasingan ke gen E rekombinan masing-masing. Kesan mutasi pada mengikat kepada serangga dan sel-sel mamalia telah ditentukan dalam vitro. Antibodi polyclonal dijana terhadap ini protein rekombinan dalam BALB/c tikus telah digunakan dalam microneutralization dan tumpuan Pengurangan Peneutralan Ujian untuk menilai meneutralkan penentu antibodi yang berkaitan dengan evolusi antigen. Pembiakan bersiri dalam vitro telah diguna pakai untuk memilih dgn rapi peneutralan melarikan diri mutan dalam antibodi penumpuan sub- meneutralkan . Untuk menilai tuan rumah yang berpotensi di mana pemilihan berlaku, mutan melarikan diri telah disesuaikan untuk meniru nyamuk dan kitaran manusia dalam vitro. Dapatan kajian ini menunjukkan bahawa dua mutasi (E272 dan E390) pada DENV-1 E telah ditunjukkan untuk memberi kesan mengikat satu protein rekombinan untuk C6/36

nyamuk dan sel-sel Vero. Antibodi polyclonal dijana terhadap E272 rekombinan dan protein E390 dipamerkan menurun mengikat untuk DENV-1 virion . Antibodi polyclonal mengurangkan bilangan DENV-1 tumpuan ditubuhkan sehingga empat kali ganda dalam tempoh lima endemik DENV-1 strain (GIA, GIB, GIC, GII dan GIII) diuji. Dua DENV-1 diasingkan mungkin untuk melarikan diri peneutralan telah dikenal pasti; D1.10245 (GII) dan D1.11177 (GIB). Dalam DENV-2, mutasi pada E52, E164 dan E393 telah ditunjukkan untuk memberi kesan mengikat satu protein rekombinan untuk C6/36 nyamuk dan sel-sel Vero. Antibodi polyclonal dijana dalam tikus terhadap rekombinan E52, E164 dan E176 dipamerkan menurun mengikat DENV-2 (Asia satu ketegangan) dan E164 dan E335 pada salah satu tekanan yang Cosmopolitan. Antibodi polyclonal terhadap E52, E71, E335 dan E393 mengurangkan bilangan tumpuan ditubuhkan sehingga empat kali ganda untuk Cosmopolitan DENV-2 strain diuji. E52 dan E71 penggantian pilih mengasingkan mungkin untuk melarikan diri peneutralan dalam kedua-dua strain Cosmopolitan (D2.14245 dan D2.1877347) dan satu ketegangan Asia (D2.1604). Bukti-bukti dari kajian menunjukkan hadir bahawa strain peneutralan melarikan diri dari DENV-1 dan DENV-2 seli antara kedua-dua kumpulan yang berbeza menyesuaikan diri berbeza. Perbezaan diperhatikan dalam DENV -1 dan DENV-2 pada C6/36 dan sel-sel Vero terbukti dalam variasi dalam sisa-sisa asid amino di E. Daripada 6 mutasi kejuruteraan pada DENV-1 dan DENV-2 E rekombinan , penggantian di EDI/EDII simpang mungkin memudahkan evolusi antigen daripada DENV-1 (E272) dan DENV-2 (E52 dan E71) di Malaysia. Secara keseluruhannya, kajian yang dikenal pasti dominan DENV-1 dan DENV-2 strain dijangka akan berkembang penduduk asli Malaysia pada masa akan datang. Penentu antigen yang terlibat telah dikenal pasti. Hasil kajian secara langsung menggariskan kesan mutasi ini pada DENV-1 secara semula jadi yang baru muncul dan DENV-2 dalam populasi Malaysia dan implikasinya terhadap penderitaan denggi dan reka bentuk vaksin.

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ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

INTRODUCTION

Dengue virus (DENV) infection is an example of infectious disease that presents antigenically variable strains that are capable of evading immune response. Mutant DENV strains could evolve under selection pressure by altering their antigenic determinants to reduce their immunogenicity. Competition between co-circulating strains facilitates the selection and adaptation of the fittest genotype, leading to the rise of new genotype from the pre-existing genotypes. The new strains become established; transmit its lineage to the host population thereby causing an epidemic outbreak. Antigenic evolution of DENV is seen as a process in which the new virulent strains emerge by evading herd immunity in a population. The new strain evolves as a result of variation in antigenic proteins due to the response to antibody selection. In DENV, these variations comprise of minor or major changes in antigenic proteins, leading to new antigenic properties such that antibodies in the population no longer recognize the virus. The acquisition of new antigenic properties changes the ability of the existing antibodies to neutralize the virus by interfering with antibody binding or receptor binding. Consequently, the antibodies produced in response to existing virus have decreased effectiveness against the virus strains with new antigenic properties. This leads to immune escape and the rise of a new dominant virus strain. Although considerable information is available on DENV evolution, however, very little is known on how exactly changes in the virus antigenic properties could have contributed to the antigenic evolution of DENV in hyper endemic population.

In recurring dengue outbreak, attention has been focused on selective and adaptive evolution of DENV. Interest in the area was instigated by evolutionary pattern suggesting that much of the lineage turnover is driven by selection on the viral genotype (Halstead 2008b). Subsequently, other groups reported that majority of antigenic

variations that arise within each host are likely to give rise to diverse viruses able to adapt to selective pressures (Twiddy et al. 2003; Twiddy et al. 2002b). Since then, genetic changes in DENV which circulated at irregular intervals causing disease in short periods were reported (Bennett et al. 2003). Most of these studies have hypothesized that an increase in disease severity might be the result of multiple infection of DENV having been selected to escape neutralization antibodies (Guzman et al. 2000a). These studies collectively look for changes in E that might signify emergence of neutralization escape mutants (Rico-Hesse 2003; Rico-Hesse 2010). Over twenty publications on genetic evolution of DENV found no changes in E that might signify emergence of neutralization escape mutant (Guzman et al. 2000a; Halstead 2008a). However, a clear pattern of virus evolution during epidemic outbreaks was observed (Guzman et al. 2000a; Halstead 2008a; Rodriguez-Roche et al. 2011). These changes require further studies and research.

In the last two decades, DENV-1 and DENV-2 are the more persistent genotype causing major outbreaks in Malaysia (Abubakar and Shafee 2002; AbuBakar et al. 2002b; Teoh et al. 2010). Genetic analysis of E shows that the highly susceptible DENV-1 and DENV-2 demonstrates a clear pattern of evolution from being minor strains (strains less frequent during an outbreak) to dominant strains (frequent throughout an outbreak) (Danlami et al. 2012; Teoh et al. 2012). This suggests that the minor strains may act as a springboard for the emergence of dominant strains by acquiring some characteristics of the minor strains. Thus, assessing the functional importance of the major variable antigenic sites on E of DENV-1 and DENV-2 genotypic strains in Malaysia would provide new insights into their antigenic evolution. In this study, we investigate the effects of dominant strains acquiring the characteristics of minor strains in recurring dengue outbreaks in Malaysia.

1.0 LITERATURE REVIEW

1.1 DENGUE

1.1.1 DISEASE

Dengue fever is caused by the arthropod-borne flavivirus. It is characterized by biphasic fever, myalgia or arthralgia, rash, leukopenia and lymphadenopathy (Halstead 1980; Halstead 2008b). Dengue haemorrhagic fever (DHF) is a severe, potentially foetal, febrile disease caused by DENV characterized by capillary permeability that leads, in severe cases, to a dengue shock syndrome (DSS) (De Madrid and Porterfield 1974). There are four antigenically distinct members of the dengue subgroup of the genus flavivirus. These are named dengue types 1 through 4 (DENV-1, DENV-2, DENV-3 and DENV-4) (Halstead 1980; Halstead 2008b).

Many genera of subhuman primates are susceptible to infection by DENV. Species belonging to *Macacus, Pongidae, Certhopicicus, Cercocebus, Papio, Hylobates* and *Pan* can be infected by bites of virus-infected mosquitoes or by injection of infectious virus preparations (Halstead 2008b; Rudnick 1965). Infection is essentially asymptomatic but viremia occurs at levels sufficient to infect mosquitoes (Rudnick 1966; Wallace et al. 1980). In the early 1980's, an extensive epizootic outbreak of DENV-2 involving subhuman primates was recognized over wide area of West Africa (Chungue et al. 1993). During successive outbreaks, DENV-2 strains were recovered from humans after being transmitted by mosquitoes that also feed on subhuman primates (Diallo et al. 2005; Fernandez et al. 2004; Moncayo et al. 2004).

In humans, primary clinical features of dengue depend on susceptible host. The infection in infants and young children may appear as undifferentiated febrile diseases associated with maculopapular rash (Burke et al. 1988; Kliks et al. 1988). In children

and adults, symptoms like high fever, headache, myalgia, joint pains, nausea, vomiting and rash may be observed. In most cases leucopoenia and thrombocytopenia are also visible. DHF/DSS are commonly observed in older children experiencing secondary dengue infection (Halstead 1970). Dengue symptoms in primary and secondary infections are undifferentiated. However, on day three through seven the haemorrhagic manifestations could begin to appear. DHF is characterized by the occurrence of high fever, hepatomegaly and circulatory collapse (Srichaikul and Nimmannitya 2000; Vaughn et al. 2000). Thrombocytopenia with concurrent hemoconcentration, a sign of plasma leakage is a common clinical symptom. In some cases, DHF patients may deteriorate rapidly leading to shock syndrome (DSS) generally occur after defervescence- a fall in temperature between third and seventh day of fever (Burke et al. 1988).

There is no specific treatment for dengue; hence, management of the disease has always been supportive therapy. Patients with high fever may be given antipyretics or acetaminophen (Kliks et al. 1988). Patients that show signs of plasma loss would require intravenous fluid therapy for fluid replacement. Transfusion is only required when there is substantial bleeding accompanied by hematemesis and melena (Kliks et al. 1988). Most patients with DF, DHF or DSS usually have a good prognosis. The foetal cases are usually because of failure of recognizing and managing occult internal bleeding with prolonged shock (Boonpucknavig et al. 1979; Kliks et al. 1988).

1.1.2 TRANSMISSION

To date, mosquito-borne DENV have two discrete transmission cycles: a sylvatic cycle between non-human primates and sylvatic *Aedes* mosquitoes, and an endemic cycle between humans and peridomestic *Aedes* mosquitoes (primarily *Ae. aegypti* and *Ae.*

albopictus) (Joshi et al. 1996; Rosen 1987). The sylvatic cycle occurs in the jungle between the non-human primates and sylvatic mosquitoes (Monath 1994; Uzcategui et al. 2001; Wang et al. 2000). The non-primates serve as the host and reservoir. The sylvatic cycle involve several *Aedes* mosquito species found in the forest of most tropical countries in Africa and Malaysia in the Southeast Asia (Cardosa et al. 2009; Monath 1994; Vasilakis et al. 2011). The human endemic cycle is associated with the peridomestic mosquitoes *Ae. aegypti* and *Ae. albopictus* and a susceptible human host (Figure 1.1). In the human endemic cycle, human individual serves as the reservoir and the amplifying host. The mosquito dwells in human habitats and this contribute to transmission into multiple hosts. The viremic period in a dengue-infected person is usually two to seven days (Chaturvedi et al. 1992; Kalayanarooj et al. 1997; Luz et al. 2011; Rico-Hesse 2010). The incubation period in an infected mosquito usually lasts for three to ten days, during which the virus continues to replicate in the mosquitoes midgut through body tissues and then into the salivary gland (Cox et al. 2011). The virus can be transmitted to a susceptible host through its saliva. Once the virus is inside the human body, it migrates to other organs such as liver and spleen. The virus replicates in these organs before it is released into the bloodstream. Dengue fever symptoms appear after an incubation period of 3 to 10 days, and the mosquito remains infectious for days or a week (Gubler 1998b; Gubler 2002; Gubler et al. 1986; Smith 1956).

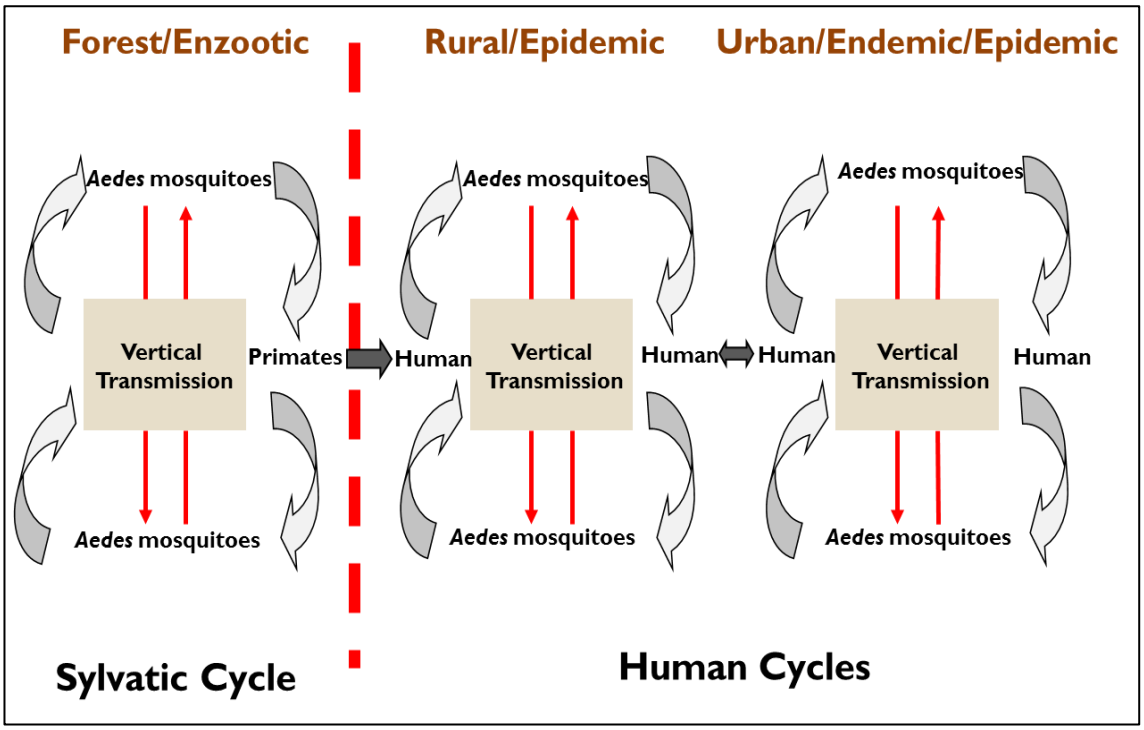


Figure 1.1. Modified diagram showing the dengue transmission cycle (Gubler 1998a).

In exceptional cases, dengue can be transmitted during blood transfusions from infected donors onto a healthy individual (Khin and Than 1983; Perret et al. 2005). There are also reports suggesting infected pregnant mother transmitting the virus to her neonate (vertical transmission) (Basurko et al. 2009; Phongsamart et al. 2008). In many dengue endemic areas, dengue occurs every year usually when there is optimal condition and availability of breeding sites for the *Aedes* mosquito (Monath 1994; Wang et al. 2000). These areas are at risk of an epidemic, especially when a large number of vector mosquitos' presence coincides with a large number of people with no prior immunity to the circulating DENV serotypes. The lack of DENV transmission in some endemic areas could be attributed to sporadic contact between vector and the host (Halstead et al. 2001; Lambrechts et al. 2010; Lin et al. 2004; Luz et al. 2011).

1.1.3 EPIDEMIOLOGY AND HISTORY

Dengue is prevalent in over 125 countries in Asia, Pacific, the Americas, Africa, and the Caribbean (Guzman et al. 2000a; Monath 1994). An estimated 2.5 billion people, 35% of the world's populace, live in areas that are at a greater risk of dengue transmission, (Figure 1.2). There is an estimated 100 million cases of DF annually, with 500,000 DHF cases and 22,000 deaths, commonly within school-going children (Guzman et al. 2002; Simasathien et al. 2008).

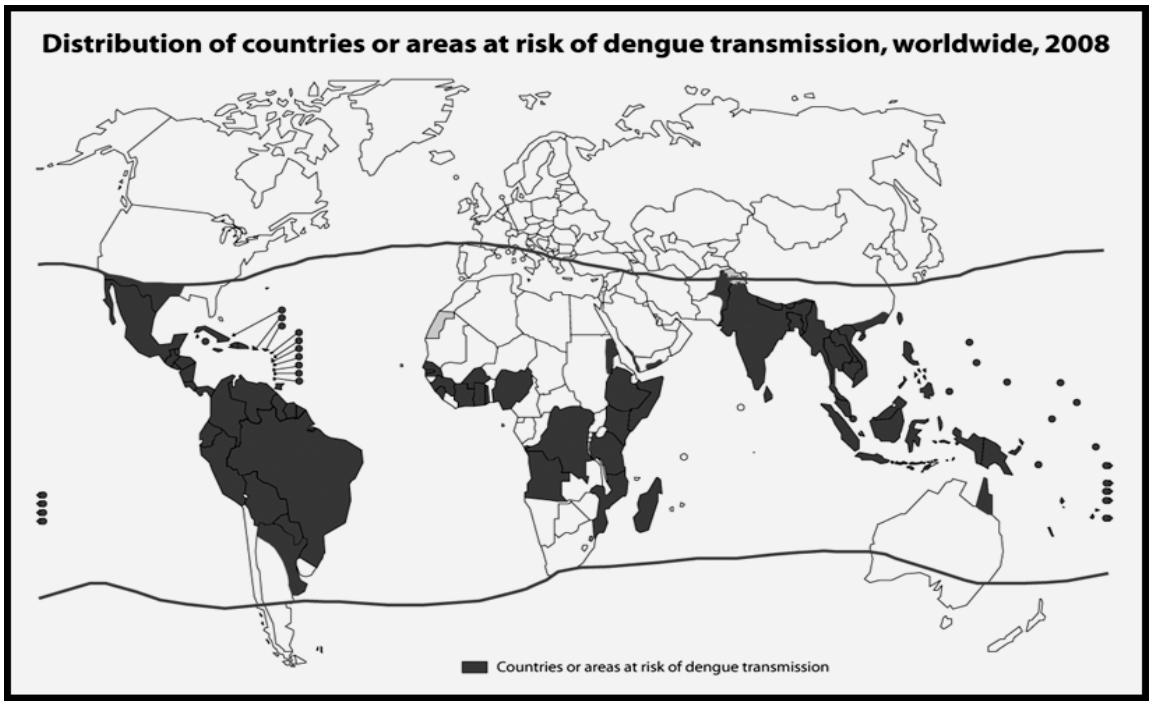


Figure 1.2. Global distribution of dengue virus (WHO 2010).

The first dengue-like infection was reported in China during the Chin Dynasty (McSherry 1982; Siler 1926). Dengue-like infections were also reported in French West Indies in 1635 and Panama in 1699 (McSherry 1982). Epidemics of dengue infections were reported in 1779 in Jakarta and Cairo (Siler 1926). Dengue was first reported in Malaysia over a century ago(Skae 1902). In Southeast Asia, the first epidemic outbreak was reported in Manila Philippines in 1954 just after the Second World War. This could

be due to influx of traders, tourists and immigrants (Halstead 2008b). Historically this was followed by record of dengue outbreaks in Malaysia (Rudnick 1965; Smith 1957). By early 1960s, dengue has become well entrenched in Malaysia (Abubakar and Shafee 2002). The first confirmed case of DHF was reported in 1962 (Rudnick 1965)and it coincided with similar reports in neighbouring countries; the Philippines in 1956 (Hammon 1966), Thailand 1958 (Rudnick 1966) and Singapore 1960 (Rudnick 1966; Wallace et al. 1980) in that order. DENV-1 was first isolated in Malaysia (Abubakar and Shafee 2002; Smith 1957). Since then other serotypes of DENV were isolated and all have caused DF/DHF outbreaks in Malaysia (Abubakar and Shafee 2002; AbuBakar et al. 2002b). Each outbreak is usually associated with at least one dominant serotype of DENV-1, DENV-2 or DENV-3. Outbreaks involving DENV-4 are rare. The last DENV-4 major outbreak was in the 1960's (Abubakar and Shafee 2002; AbuBakar et al. 2002b).

1.1.4 PATHOGENESIS OF DENGUE INFECTION

There are two independent mechanisms, which have been hypothesized to describe the disease progression in dengue infection. The first mechanism is the antibody dependant enhancement. There is evidence suggesting that individuals with pre-existing antibody from late infection with one of the DENV serotypes are predisposed to more severe dengue if subsequently infected by a different DENV serotype (Falconar 2008; Gubler 1998a; Halstead and O'Rourke 1977; McBride and Bielefeldt-Ohmann 2000; Stephenson 2005; Whitehorn and Simmons 2011). This implies that there is an association between DENV-antibody complexes with secondary heterotypic DENV infection. This occurs when there is an increase in the virus uptake during entry into the FcγR (fragment, crystallisable gamma receptor) receptor-bearing cells through the Fc (fragment, crystallisable) portion of a non-neutralizing antibody. This mechanism in

turn increases disease severity and viral load in dengue patients (Halstead 1970; Halstead 2003). Consequently, the virus incubation period is reduced. Host mononuclear phagocytes are destroyed and lead to the release of vascular permeability factors and complement activating factors. The increase of viral load because of enhanced entry of viruses would result in sufficient formation of virus- immunoglobulin gamma complexes that would increase activation of the complement pathway resulting in increased complement consumption. Complement (C) reduction would release C3a and C5a anaphylatoxins. This promotes vascular permeability that may lead to DHF/DSS (Cardosa 2000; Halstead et al. 2005; Halstead and O'Rourke 1977; McBride and Bielefeldt-Ohmann 2000; Rothman 2010; Whitehorn and Simmons 2011). The enhancement of DENV infection was observed in monkeys infected with DENV-1 and DENV-3 *in vivo* and subsequently boosted with DENV-2. The monkeys developed high levels of viremia when compared with the non-immune monkeys given the same dose of DENV-2 (Halstead 1979; Halstead et al. 1973). Similar observations were made when monkeys infected with DENV-1 cord blood were boosted with DENV-2 (Halstead 1979).

Alternatively, evidence of severe dengue in individuals with no detectable anti-dengue antibodies has been reported to correlate with the more virulent strains of dengue (Guzman et al. 2002; Guzman and Vazquez 2010; Halstead et al. 1983). This suggested that severe dengue could be associated with DENV virulence. This argument is reinforced with cases of severe DENV-2 DHF epidemics in Cuba in 1981 following DENV-1 epidemic in 1977 (Guzman and Kouri 2003; Guzman et al. 2000b). Similar observations were made with the introduction of Asian DENV-3 into the Americas (Gubler and Clark 1995).The lack of severe cases of dengue virus in endemic regions

with prior existing antibody to another dengue virus serotypes could be due to also population difference (Watts et al. 1999).

In addition, thymus (T) lymphocytes have also been implicated in contributing to dengue severity. Serotype-cross-reactive memory T-cells, tend to reactivate during a secondary infection. An abnormal T-cell activation cascades and apoptosis will occur (the original antigenic sin) (Halstead et al. 1983; Kuno et al. 1993; Midgley et al. 2011b; Rothman 2011). This abnormal response increases the T-cell population of the lower avidity memory T-cells during secondary DENV infections. The response favours the former over the naïve T-cell following a new infection (Chaturvedi et al. 2000; Halstead and O'Rourke 1977; Midgley et al. 2011a; Rothman 2010).

Genome structural differences between the virus strains also correlate with severe dengue infections and pathogenesis. Leitmeyer (1999) identified that the primary determinants of DHF reside in (i) amino acid 390 of the E, which purportedly alters virion binding to host cells; (ii) in the downstream loop (nucleotides 68 to 80) of the 5′ non translated region, which may be involved in translation initiation; and (iii) in the upstream 300 nucleotides of the 3′ non translated region, which may regulate viral replication via the formation of replicative intermediates (Leitmeyer et al. 1999; Rico-Hesse 2003). Effect of these mutations using DENV-2 was evaluated in human primary cell lines. They account for cell tropism and virulence in primary human cells (Armstrong and Rico-Hesse 2003; Cologna and Rico-Hesse 2003; Rico-Hesse 2003).

1.2 DENGUE VIRUS

1.2.1 TAXONOMY OF FLAVIVIRUSES

Flavivirus is from the Latin word *flavus*-meaning yellow- characterized by the jaundice caused by Yellow Fever virus (YFV). The genus flavivirus includes two other genera namely, Pestivirus and Hepacivirus, primarily transmitted by arthropods (Mukhopadhyay et al. 2005). The *flaviviridae* family includes 73 viruses divided into non-vector transmitted, tick-borne and mosquito-borne Flavivirus(Mackenzie et al. 2004). DENV belongs to the mosquito-borne Flavivirus group. A number of other Flavivirus comprise the Yellow Fever virus from which the group derives its name. And there is the Japanese encephalitis virus (JE). The tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) are also members of genus *Flaviviridae*. Majority of the *Flaviviridae* family are human pathogens and cause serious outbreak and epidemics around the world (Calisher and Gould 2003; Mackenzie et al. 2004; Mukhopadhyay et al. 2005).

1.2.2 CLASSIFICATION OF DENGUE FEVER VIRUS

Flaviviruses have different virion morphology and genome organization. The serological relationship, however, can been determined by (1) antibodies (monoclonal or polyclonal); (2) by using neutralization tests and hemagglutination-inhibition (HAI); and (3) identification based on complement-fixation activity associated with the non-structural 1 protein. Neutralization tests have been used to identify Flaviviruses serological subgroups. One of these serogroup is the dengue serogroup (Halstead 1980; Halstead et al. 1973).

Nucleotide sequencing of regions of the E, NS3 or NS5 of most flaviviruses has resulted in more detailed relationships than was possible by serological studies. The current

classification of the genus flavivirus is mainly based on genetic groupings (Figure 1.3 (Churdboonchart et al. 1991; Churdboonchart et al. 1990). Although, scientists have discovered a new type of the virus- DENV-5, that causes a centuries-old epidemic, dengue (Normile 2013). flavivirus classification identified four different dengue serotypes that tend to relate to the geographic origin of the virus strain, each exists as a single serotype (Halstead 2008b).

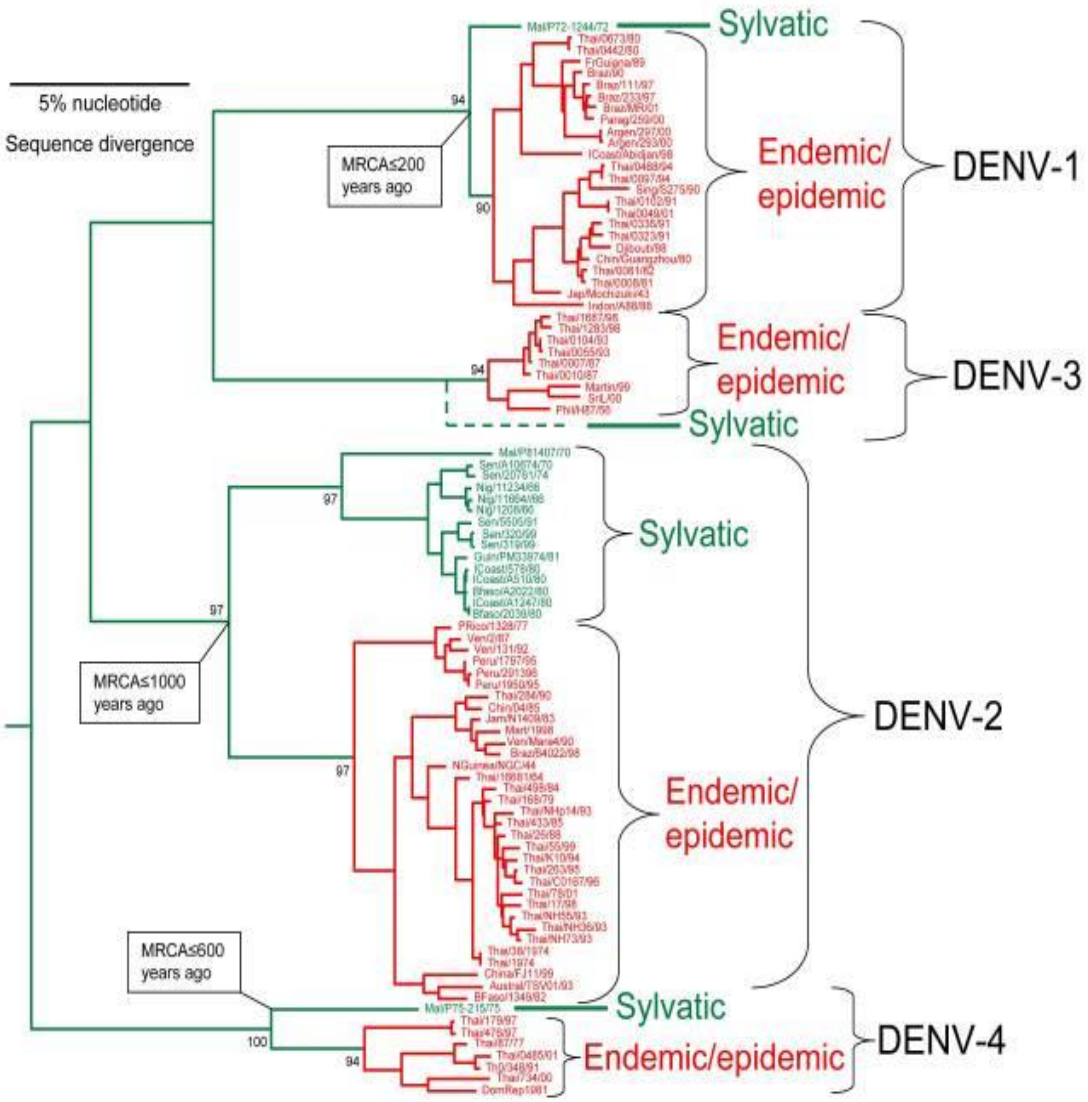


Figure 1.3. Phylogenetic tree of DENV strains from all 4 serotypes. The phylogeny was inferred using Bayesian analysis (one million reiterations) and all horizontal branches are scaled according to the number of substitutions per site. Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection. (Weaver and Vasilakis 2009).

1.2.3 STRUCTURE OF THE DENGUE VIRION

Flavivirus particles are spherical in shape, with a lipid envelope; the particles are approximately 50 nm in diameter. The lipid envelope is derived from host cell membranes and constitutes 15–20% of the total weight of the virus particle. Carbohydrates represent 9–10% of the weight of virus particles and are found as glycolipids and glycoproteins (Halstead 2008b; Kuhn et al. 2002). DENV genome is a positive sense single-stranded RNA genome. A single continuous open reading frame, representing 95% of the genome translated to a single large polyprotein (around 10 kb in length) (Gubler 1998b). The ORF is flanked by the 5` and 3`un-translated region (UTR). The genome is packaged by the host-capsid protein in a host-derived lipid bilayer. This produces three structural proteins, the capsid (C), premembrane (prM/M) and Envelope (E), which form the virus particles. The seven non-structural (NS) proteins consist of NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 that are essential for virus replication (Figure 1.4) (Kuhn et al. 2002; Markoff 1989). The DENV-1, DENV-2, DENV-3 and DENV-4 open reading frames are estimated to contained 10,188, 10,173, 10,170, and 10,158 nucleotides in length, which encode polyprotein precursors of 3396, 3391, 3390 and 3386 amino acids, respectively (Blok 1985; Kuhn et al. 2002; Weaver and Vasilakis 2009).

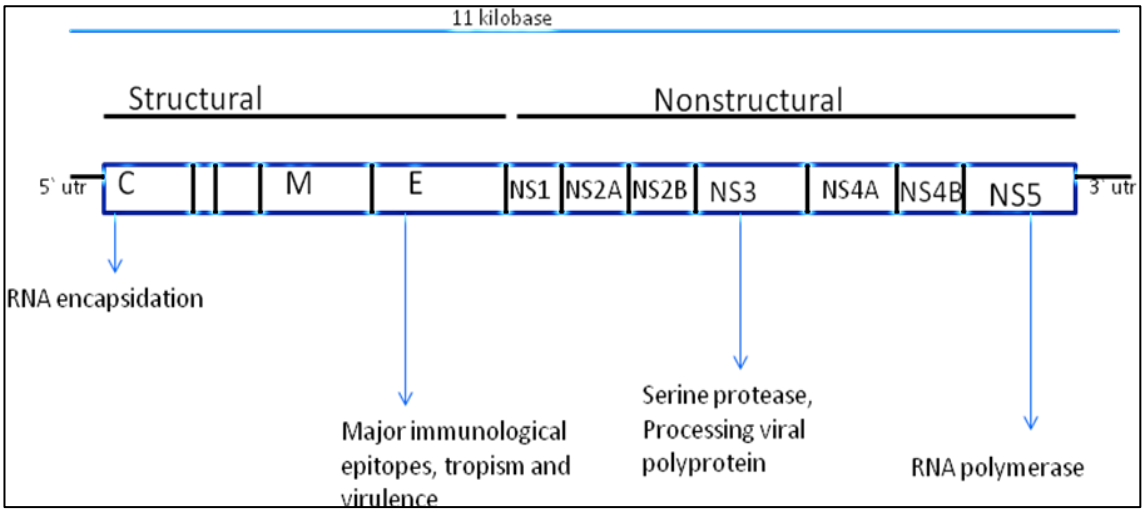


Figure 1.4. Schematic depicting the DENV genome

1.2.3.1 The capsid protein (C) is the smallest, (9-12 kDa) consisting of 112 to 127 amino acids. The C is a highly positive-charged residue. It is associated with the negatively charged viral RNA genome during virus replication to make the nucleocapsid core (Trent 1977; Yu et al. 2008).

1.2.3.2 The premembrane/membrane protein (prM/M) is the second smallest protein of 27-31 kDa. The protein exists in two forms; prM, (precursor form associated with the intracellular immature virion) and M (found in extracellular mature virion). Cleavage of prM to M by a cell encoded furin-like protease at the cleavage maturation motif is an essential and terminal event in the virus (Cardosa et al. 2002; Markoff 1989). The process changes the virus surface structure and results in an increase of virus fusogenic activity and infectivity. The prM also serves as chaperone protein for E. It protects from irreversible conformational damage in the low-pH environment. This occurs during the virus maturation process in the trans-Golgi network (Kuhn et al. 2002; Yu et al. 2008).

1.2.3.3 Envelope protein (E) is the largest DENV structural protein. The protein is approximately 55 to 60 kDa in size. It consists of 494 to 501 amino acids. The two

glycosylation sites at E67 and E155 (Bryant et al. 2007; Johnson et al. 1994; Mondotte et al. 2007) makes up the principal component of the virus surface (Modis et al. 2005). E exists as a dimer (Modis et al. 2004; Modis et al. 2005). The two monomers consist of three domains, a main structure (EDI), flanked by the dimerization domain (EDII) and an immunoglobulin (Ig)-like domain (EDIII). EDII include the conserved fusion loop at the distal end of EDI and EDIII, which is embedded within the target membrane. Rearrangements of domains I and II with respect to domain III suggest that a folding back of the E trimer occurs which would bring the virion and target membranes into close proximity, leading to lipid mixing and membrane fusion (Modis et al. 2004). Domain III is thought to contain the putative receptor binding sites (Beasley 2005). The E plays critical roles in fusion and interaction of the DENV with cellular receptors (Chen et al. 1996). It also serves as target for host humoral response, resulting in neutralization of the virus (Kuhn et al. 2002; Modis et al. 2004; Rey 2003; Weaver and Vasilakis 2009).

1.2.3.4 NS1 is the most extensively studied DENV protein. This antigenic protein is 42 to 50 kDa, is glycosylated, and exists as a dimer in its basic form in the intracellular and extracellular fluids of infected cells (Abubakar et al. 2002a; Mason et al. 1990). The NS1 plays a role in virus maturation. The immature E in the endoplasmic reticulum lumen is associated with the synthesis of the negative strand in the RNA replication cycle (Jacobs et al. 2000; Somnuke et al. 2011; Winkler et al. 1989).

1.2.3.5 The NS2A is 20 to 22 kDa protein. It is necessary for the proteolysis of the C terminus of NS1 and coordinating RNA packaging and replication (Weaver and Vasilakis 2009). A possibility of interferon antagonizing is also reported (Calvert et al.

2006). This hydrophobic protein is suggested to play a role in mature viral assembly (Blaney et al. 2003; Kuhn et al. 2002).

1.2.3.6 NS2B is a14.5-kDa protein. It is a membrane-associated protein, which functions with NS3 to serve as cofactor in the structural formation of DENV serine protease in NS3 (Leung et al. 2001; Othman et al. 2007; Weaver and Vasilakis 2009) and receptor binding (Abubakar et al. 2002a). Recently the protein was reported to play a significant role in mediating the activation of NS3 proteases (Weaver and Vasilakis 2009; Zuo et al. 2009).

1.2.3.7 NS3 is believed to play distinct roles in DENV replication (Falgout et al. 1991). The NS3 is a 70-kDa multifunctional protein, which served as both a serine protease and helicase together with NS2B. It cleaves the dibasic sites of the viral polyprotein during post-translational processing (Jones et al. 2005; Li et al. 1999; Luo et al. 2008).

1.2.3.8 NS4A and NS4B are small hydrophobic proteins of 16 kDa and 27 kDa, respectively. The proteins function as RNA replication complex cofactors in the virus replication (Cahour et al. 1992). The protein preserved replication efficiency in two hosts, human and mosquitoes (Hanley et al. 2003). Mutation at amino acid residue7129 decreases replication in mosquito cells, and increases replication in simian cell lines (Cahour et al. 1992; Hanley et al. 2003). NS4B also functions as an interferon signalling inhibitor (Jones et al. 2005; Kuhn et al. 2002; Munoz-Jordan et al. 2003).

1.2.3.9 NS5 is the largest non-structural protein of about 104 to 106 kDa (Taylor and Best 2011). It is believed to be the putative RNA polymerase (residue 270 – 900), and methyltransferase for capping the viral RNA during replication (residue 1 – 269)

(Cahour et al. 1992; Kuhn et al. 2002). Most recently, the well-conserved protein is reported to function in interlukin-8 induction. The same NS5 domain could also have a guanylyltransferase activity (residue 387 – 404) (Kuhn et al. 2002; Medin et al. 2005; Polacek et al. 2009; Yap et al. 2010).

1.2.3.10 The un-translated region (UTR) of the genotypes includes approximately 100 and 400-800 nucleotides of un-translated regions at the 5` and 3` ends of the genome, respectively(Kuhn et al. 2002; Zou et al. 2009). The regions include several conserved structures that forms stem loop. They play a vital role in viral replication and translation (Chiu et al. 2005; Gebhard et al. 2011; Mason et al. 1987; Wei et al. 2009). Mutations that alter the structural conformation of the UTR were found to affect virulence and cause attenuation (Durbin et al. 2001). A single nucleotide mutation in the 5`UTR of the South East Asian strains of DENV-4 resulted in low replication efficiency in mosquito cells (Butrapet et al. 2000). The stems loop at the 3` region play a major role in determining the regulation and RNA synthesis in many flaviviruses (Holden et al. 2006).

1.3. DENGUE VIRUS E

Dengue virus E is a "class II" viral fusion protein (Schibli and Weissenhorn 2004), which initiates attachment of the virus to host cell receptors (Modis et al. 2004; Modis et al. 2005). E also mediates fusion with host cell membranes, hemagglutinates erythrocytes and stimulates humoral and cell-mediated immune responses. In neutral pH environment, E lies parallel to the surface of the viral membrane as dimeric or trimeric units. Each monomeric unit anchored to the viral membrane by a carboxyl terminal trans membrane region (Modis et al. 2004).

1.3.1 MOLECULAR STRUCTURE OF E

The three dimensional structure of E ectodomain has been resolved at 2 Armstrong (Å) (Kuhn et al. 2002; Modis et al. 2005). But it is composed of amino acid residue 1-395 only. The ectodomain, which is the soluble fragment, is anchored in to the membrane through transmembrane domain at residues 451 to 495 (Kuhn et al. 2002; Modis et al. 2004).The E ectodomain is divided into 3 domains: a main structure (EDI), flanked by the dimerization domain (EDII) and an immunoglobulin (Ig)-like domain (EDIII) (Figure 1.5). A stem region, residues 396-450, links DENV ectodomain and transmembrane domain together. The E forms homodimers with an average dimension of approximately 150Å x 55 Å x 30 Å. This dimer is oriented similar to the viral membrane and smoothly curled. Infectious virions contain 90 E dimers (180 E monomers) covering the entire viral surface and forming a smooth shell (Zhang et al. 2003). Conservation of cysteine residues and similar hydrophobic profiles suggest a common folded structure of the E for all flaviviruses (Howard 2006; Modis et al. 2004; Roehrig 2003).

1.3.1.1 EDI of DENV contains 120 amino acids (1-51, 137-189, 285-302)(Mandl et al. 1989). The domain consists of two beta (β) sheets that constitute eight stranded up and down β-barrel in a tightly packed discontinuous hydrophobic region. The domain contains a unique glycosylation site. Attached to the loop on the external surface of the protein are two disulphide bridges joining the cysteine residues at 3-30 and 186-290 (Allison et al. 2001; Howard 2006; Stiasny et al. 2002) (Figure 1.5).

1.3.1.2 EDII of DENV consists of 178 discontinuous amino acid residues (52-136 and 190-284). The domain is a finger-like structure. It consists of antiparallel β-sheets of five strands packed within two alpha (α) helices. Sandwiched between the structures are

three stranded β-sheets and one beta hairpin (Allison et al. 2001; Modis et al. 2004; Stiasny et al. 2002). Disulphide bridges link the three-β sheets together that stabilizes the tightly folded cd loop structure (98-111 amino acids)(Allison et al. 2001). The fusion peptide in the cd loop has been reported to be the primary neutralization site and the hemaagglutinin site of the virus (Stiasny et al. 2002). Mutation on domain II is reported to affect fusion activity essential for the attachment of soluble E to target membranes (Allison et al. 2001) (Figure 1.5).

1.3.1.3 EDIII consists of continuous amino acid residues from 303 – 395. EDIII is an immunoglobulin like protein withinβ-barrel structure that constitutes two β-sheets. One of the β-sheets is in contact with the cd loop and adjacent to domain I, while the second sheet forms the outer lateral surface of the dimer. EDIII is projected above domain I and EDII through a fifteen amino acids hinge residues and disulphide bridges connecting domain I and domain II (Mandl et al. 1989). The projection of EDIII above EDI and EDII from the viral surface facilitates its attachments to host cell receptors. Antibodies that bind to domain three prevent attachment to host cells (Crill and Roehrig 2001; Roehrig 2003). The domain contains Arg-Gly-Asp motif that is specific for mosquito borne flaviviruses binding (Lee and Lobigs 2002; Munoz et al. 1998). The region has been implicated for heparan sulfate, glucosamine-glycan (GAG) recognition (Howard 2006; Lee et al. 2010) (Figure 1.5).

In addition to the three antigenic domains, the E contains an amphipathic alpha helical stem anchor region that represents 20% of E (Allison et al. 2001) (Figure 1.6). The membrane stably folded E fragment with the C-terminal transmembrane anchor. The 53-residue amino acid residues have been implicated in membrane anchoring and in low pH-induced structural changes associated with virus fusion (Allison et al. 2001).

The region also plays an important role in intracellular localization of E in DENV-2 (Stiasny et al. 2002).

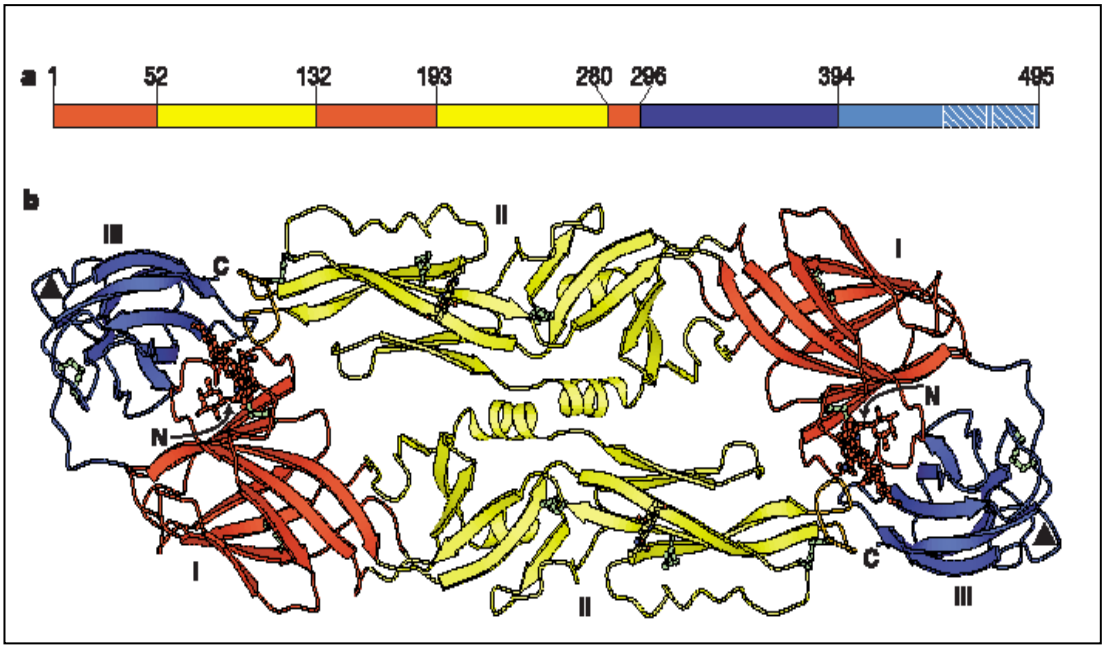


Figure 1.5. Structure of DENV E in it dimeric form. The three domains of E, EDI is in red, EDII is in yellow, and EDIII is in blue (Modis et al. 2004).

1.3.2. ANTIGENIC STRUCTURE OF DENGUE VIRUS

The E of flavivirus encodes for important immunogenic properties of the viruses (Roehrig 2003). The protein initiate infection through cell binding and these trigger the immune response against the proteins. These immunogenic properties mediate virus cell membrane fusion (Modis et al. 2004). Antigenic and immunogenic properties of DENV E have been determined and were reported to be similar with other flavivirus (Gromowski and Barrett 2007; Henchal et al. 1985; Roehrig 2003; Roehrig et al. 1990). Following characterization of E of the TBEV, using monoclonal antibodies (Klaus et al. 2011; Matveeva et al. 1995). The functional antigenic determinant of DENV were mapped against the E antibodies (Howard 2006). The regions were similar with that of TBEV with three antigenic domains A (EDII), B (EDIII) and C (EDI) (Crill and

Roehrig 2001; Roehrig et al. 1998). An antibody against domain A recognized peptides at amino acids residues 1-400 and 1-120. It is implicated for hemagglutination inhibition, blocks virus fusion to host cell receptors and neutralizes virus in vitro (Roehrig et al. 1998). Antibodies against domain B recognized peptides at amino acids 300-400 base pairs, inhibits hemagglutination and neutralization of DENV infection *in vitro.* However, it does not block virus fusion with host cell receptors (Abd-Jamil et al. 2008; Crill and Roehrig 2001; Gromowski and Barrett 2007; Imrie et al. 2007). Antibodies against domain C does not exhibit hemagglutination, fusion or neutralization attributes in contrast to domain A and B (Crill and Roehrig 2001; Gromowski and Barrett 2007).

The major antigenic determinant on E of DENV induces protective neutralizing antibodies (Aaskov et al. 1989; Gromowski and Barrett 2007; Guzman et al. 2010; Howard 2006; Lai et al. 2008; Matsui et al. 2009). This protein releases both neutralizing and non-neutralizing antibodies during dengue infection. The neutralizing antibodies could be type-specific (Gromowski and Barrett 2007; Serafin and Aaskov 2001) or cross-reactive neutralizing antibodies (Matsui et al. 2010; Matsui et al. 2009).The specific antibody neutralization sites were mapped to amino acids 35 – 55 and 352 – 368 on EDI and EDIII (Roehrig et al. 1990). EDII amino acids 60 – 135, 60 – 205 and EDIII 298 – 397 amino acid residues (Megret et al. 1992) and amino acids 386 – 397 (Trirawatanapong et al. 1992). The regions that are responsible for direct binding of E to cells and antibodies, have been mapped to amino acids 297 – 349 and 380 – 423 (Wahala et al. 2009). These two regions have been shown to bind to host cells and DENV-2 monoclonal antibody fragment with high affinity (Howard 2006; Wahala et al. 2010; Wahala et al. 2012) (Figure 1.6A).

EDIII has been identified as a core antigenic region (Fig. 1.6B). EDIII plays vital role in binding to antigenic determinant in DENV-1 (Matsui et al. 2010; Shrestha et al. 2010), DENV-2 (Gromowski and Barrett 2007; Gromowski et al. 2008; Gromowski et al. 2010) and other flaviviruses (Allison et al. 2001; Mandl et al. 1989; Roehrig 2003). The most effective neutralizing epitopes are located at the lateral ridge of the EDIII 301 -303, 327 – 330 and 381 -382 of the E (Matsui et al. 2010; Wahala et al. 2009). This enables accessibility to antibodies. The lateral ridge was reported to contain antigenic determinant that recognizes type specific antibodies (Gromowski and Barrett 2007; Gromowski et al. 2008). The EDI/EDII inter-phase (49 -52, 136, 184 – 187 and 266 - 277) was reported to influence mouse monoclonal antibody binding (Lin et al. 2012; Wahala et al. 2010). Antigenic determinants (305 - 308) on A strand of the EDIII of E recognized sub complex neutralizing antibodies (Gromowski et al. 2008; Wahala et al. 2010), (Figure 1.6B). These antigenic determinants are the focus for mapping dengue-neutralizing antibodies. They are potential targets for inducing natural immune responses, which are type specific (Abd-Jamil et al. 2008; Lin et al. 2012; Rothman 2011; Wahala et al. 2010).

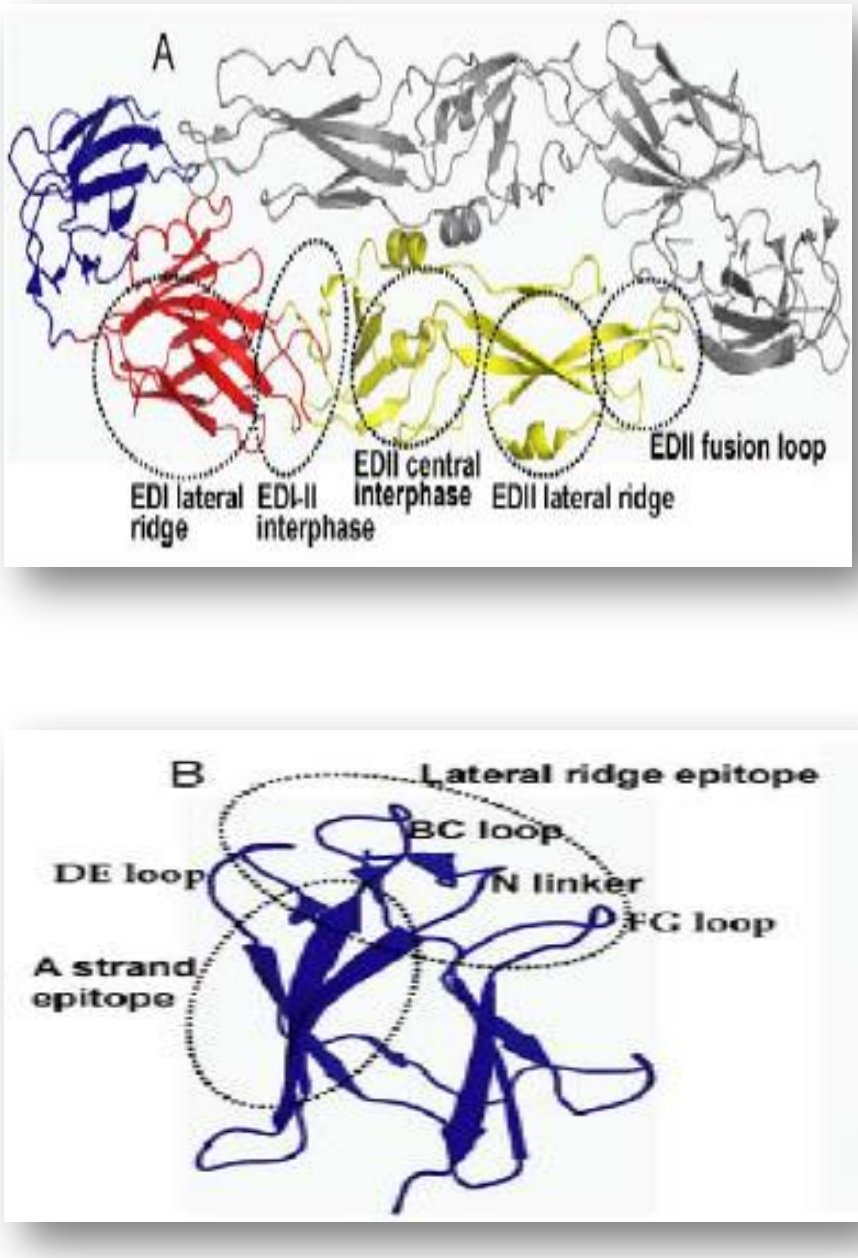


Figure 1.6. Structure of DENV antigenic regions on soluble E: (A) the image highlighted the three beta-barrel domains of flavivirus E: EDI in red, EDII in yellow and EDIII in blue. (B), a detailed image of domain III displaying its antigenic sites (Wahala et al. 2010).

1.3.3 CELLULAR RECEPTORS

The mechanisms by which DENV enter host cells has not been completely understood. However, the initial step in a DENV infection is the attachment of virus to the surface of the host cell via cell receptors (Modis et al. 2004). It has been reported that this attachment occurs in two stages.

First, attachment of DENV to the host cell and its concentration at these sites is dependent on the presence of auxiliary molecules. Molecules such as sulphate polysaccharide chains or GAGs, heparins sulfate (HS) (Chen et al. 1996; Dalrymple and Mackow 2011; Mercado-Curiel et al. 2006). Few researchers have shown how the dendritic cell-specific ICAM (intercellular adhesion molecule)-grabbing non-integrin protein (DC-SIGN) acts as cellular receptors for DENV (Navarro-Sanchez et al. 2003). The glucose-regulating protein 78 (GRP78/BiP) (Jindadamrongwech et al. 2004), the cluster of differentiation 14 (CD14) associated molecules and more recently, the heat shock proteins (HSP90) and (HSP70) (Reyes-Del Valle et al. 2005) were also been reported to act as cellular receptors. Sometimes, the binding occurs on the cell surface immunoglobulin receptors because of the existence of antibodies towards the E (Le Breton et al. 2011; Rock et al. 2007).

Moreover, it has been revealed that in the case of the DENV the entry route differs between mammalian cells and mosquito cells (Humphery-Smith and Vincendeau 1993; Mercado-Curiel et al. 2006; Munoz et al. 1998). Flaviviruses are able to infect various different cell lines suggesting that various receptors contributed for flavivirus infection and that different flaviviruses could use different receptors. The presence of an additional co-receptor has been postulated to explain the restricted tropism of flaviviruses (Chen et al. 1996; Smith 2012; Thaisomboonsuk et al. 2005).

1.3.4 BINDING AFFINITY OF DENGUE VIRUS E TO CELLULAR RECEPTORS

Biologically functional antigenic domains were identified on the E of flaviviruses using monoclonal antibodies directed against it (Howard 2006). They were described using peptides representing the regions of the E (Gromowski and Barrett 2007; Gromowski et al. 2008). The cellular receptors were also identified, by studying gene sequences of selectively modified viral variants (Chua et al. 2006; Henchal et al. 1986; Megret et al. 1992; Renneson et al. 2011). Linear epitope were mapped by overlapping peptides based on homology analysis and secondary structure prediction (Aaskov et al. 1989; Abd-Jamil et al. 2008). Regions recognized by antibodies were also mapped by fusion and expression of antigenic protein in bacteria (Mason et al. 1990; Megret et al. 1992; Thullier et al. 2001).

Using phage display technologies peptide sequences that mimic epitopes on the E of flaviviruses have been identified from infected Vero cells supernatant using anti-dengue monoclonal antibody (Cabezas et al. 2008; Cabezas et al. 2009). Competition binding studies of monoclonal antibodies on the E has been used to identify relationships between different regions competing for antigenic sites (Abd-Jamil et al. 2008; Gromowski et al. 2008; Gromowski et al. 2010). Mutant viruses have also been isolated *invitro* in the presence of neutralizing antibodies (Roehrig et al. 1990; Roehrig et al. 2004). These processes characterize (i) conformationally-dependant functional antigenic determinants.(ii) Identify amino acid changes characteristic of particular genotype(Puttikhunt et al. 2008). Inhibition of binding of one antibody by another may occur because of distant conformational changes caused by the binding of the first antibody. It might occur because of steric hindrance if the relevant epitopes are adjacent or overlapping (Wiwanitkit 2005).

1.3.5 DENGUE VIRUS FUSION WITH HOST CELL MEMBRANE

The detailed crystal structure of DENV-2 E provides an insight of interaction of fusion peptides with targeted cell membrane (Abd-Jamil et al. 2008). The crystallographic structures of the pre-fusion and post-fusion trimeric E molecules were hypothesized to be the intermediary steps involved in fusion of E of flavivirus with target cell including DENV membranes (Modis et al. 2004). Initially, the fusion loops of the three E subunits interacted to form an aromatic anchor structure. This occurs through glycan-mediated interaction at the top end of the newly formed trimer (Figure 1.7a). This binding occurs through EDIII with a relative increase in endosomal uptake. Secondly, the exposed E dimers on the virion will dissociate because of decreased pH in the endosomes. These expose the fusion loops and enables EDI and EDII to loosen together relatively (Figure 1.7b). Slackening permits EDII to deflect from the virion and put in its fusion loop into the cell membrane. Lateral protrusion of the EDII destroys interaction between the virion and the outer surface. This allows for the reorganization of E monomers, thus results in the development of a pre-fusion intermediate in which host cell and viral membranes are bridged (Figure 1.7c). Subsequently, these lead to the establishment of trimer links spreading through the fusion loops at the tip to EDI at the base (Figure 1.7d). This is followed by EDIII shifting and rotating to make trimer contacts, instigating the C terminal segment of E to fold up the fusion loop and cause the two membranes to bend towards each other. A structure called ‘hemi fusion stalks’ is formed and serves as an intermediate in the membrane fusion reaction (Figure 1.7e) (Modis et al. 2004).

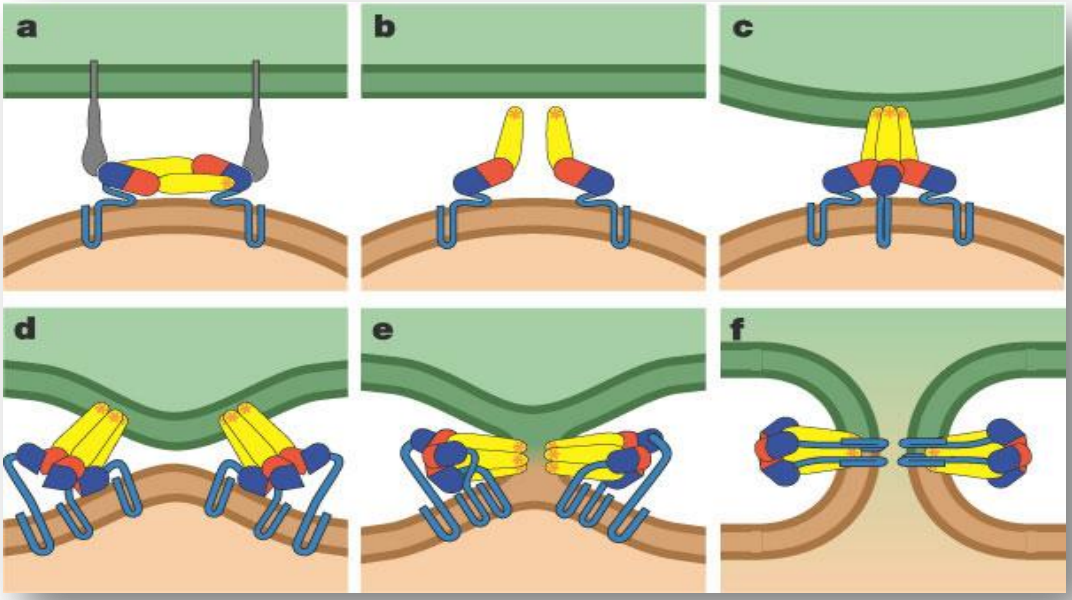


Figure 1.7. Diagram showing the proposed fusion mechanism of flaviviruses (Modis et al. 2004).

Additional trimer contacts will be created between the stem anchor and EDII projected towards the hemi fusion stack and then to the formation of a lipidic fusion pore. The C-terminus of E is projected towards the fusion peptide, and positioned because of the rearrangement of beta strands at the trimer interface folding of EDIII. Finally, the fusion loop will be extended along the inter-subunit to create enough contact with EDII (Figure 1.5f). There are 53 amino acid residues at the stem anchor region connecting the end of E with the transmembrane anchor, these residues contribute additional trimer contacts with EDII of another subunit (Modis et al. 2004).

1.3.6 ANTIGENIC DETERMINANT ON THE SURFACE OF E

The antigenic determinants of E are essential in the design of DENV vaccine capable of inducing neutralization antibodies against DENV (Lai et al. 2007; Wahala et al. 2010). In general, DENV E elicits neutralizing antibodies as well as non-neutralizing antibodies. E-specific flavivirus neutralizing antibodies can be either virus-specific or

virus cross-reactive. The virus-specific antibodies have the highest neutralizing activity *in vivo* (Mandl et al. 1989; Roehrig 2003). Previous studies identified neutralizing antigenic determinant for all the DENV serotypes by generating mutants resistant to neutralization for a defined antibody (Gromowski and Barrett 2007; Henchal et al. 1986). The specificity of E to elicit neutralizing antibodies as well as non-neutralizing cross-reactive antibodies shows neutralizing determinants are restricted to certain residues on the E (Gromowski and Barrett 2007; Gromowski et al. 2008; Wahala et al. 2010). Most of the neutralizing epitopes are located on the outer surface of the E and are thus accessible to antibodies (Gromowski and Barrett 2007; Gromowski et al. 2008; Gromowski et al. 2010; Wahala et al. 2010; Wahala et al. 2009). Table 1.1 and 1.2 below lists amino acid residues on E reported to affect neutralizing antigenic determinants for DENV.

Table 1.1: Neutralizing antigenic determinants for dengue virus

Virus	Antibody	Positions	ED	References
DENV-1	mAb(M-10)	E279	I-II	(Beasley and Aaskov 2001; Howard 2006))
DENV-1	mAb(M-17)	E293	I-III	(Beasley and Aaskov 2001)
DENV-2	MAb(10F2)	E69, E71, E112,E124,	II III	(Lok et al. 2001)
DENV-2	mAb(6B2)	E311	III	(Lok et al. 2001)
DENV-2	mAb(4G2)	E169, and E275	I-II	(Serafin and Aaskov 2001)
DENV-2	mAb(G8D11)	E307	III	(Lin et al. 1994)
DENV-2	mAb(62B)	E311	III	(Lok et al. 2001)
DENV-2	mAb(3H5)	E383,E384,E385	III	(Hiramatsu et al. 1996)
DENV-2	GTX29202, GTX77557, MDVP-55A, MA1-27093, 20-783-74014	E310, E312, E332, E389, E391	III	(Gromowski et al. 2008)
DENV-3	mAb(1H9)	E386	III	(Howard 2006; Serafin and Aaskov 2001)
DENV-3	mAb(14A4-8)	E305, E306, E309, E310, E325,E329, 381, E387	III	(Gromowski and Barrett 2007)
DENV-3	mAb(8A1) mAb(1H9)	E301, E302, E329, E380, E386	III	(Wahala et al. 2010)
DENV-4	mAb(4G2) DENV4-4	E101, E106, E107,108 E211, E215, E217	II II	(Howard 2006; Lin et al. 2012)

Table 1.2: Neutralizing antigenic determinants identified on multiple dengue virus

Virus	Antibody	Positions	ED	References
DENV-1-2	mAb(Fab 1A5)	E106 E317	II III	(Goncalvez et al. 2004; Howard 2006)
DENV-1-3	mAb(14A4-8)	E306, E308, E381, E387	III	(Howard 2006; Wahala et al. 2010)
All DENV	mAb(85A) mAb(2C1)		III	(Howard 2006; Wahala et al. 2010)
All DENV	FL0231 &FL0232 (all DENV), FL0251&DA67(DENV-1),	E83, E86, E300, E303, E307, E3310, E329, E383, E389, E391,	II III	(Howard 2006; Lin et al. 2012)

1.4 IMMUNE RESPONSE TO DENGUE VIRUS

1.4.1. INNATE AND ADAPTIVE IMMUNE RESPONSE TO DENGUE VIRUS

The humoral and cell-mediated immune response induced by DENV infection plays a major role in in vivo DENV infection in humans (Halstead 1980; Halstead and O'Rourke 1977). During DENV infection *in vitro*, fibroblasts and monocytes induce the production of interferon α andβ, respectively. Consequently, an elevated serum level of interferon α is detected (Rodriguez-Madoz et al. 2010; Sanchez et al. 2006). Interferon inhibits viral infection in monocytes. These cells are susceptible to lyses by the natural killer cells. In comparative studies between DF/DHF and in DSS patients, low level of undetectable type 1 interferon signalling genes were observed when antiviral function of interferon is blocked (Diamond et al. 2000; Gil et al. 2009; Gubler 1998a; Gubler 2002; Kurane et al. 1989; Rolph et al. 2011).

E is the major antigenic protein for inducing neutralizing antibodies (De Rivera et al. 2008) although neutralizing epitopes have been identified on the M. Genotype cross-reactive antibodies against E, prM, and NS1are also reported (Lai et al. 2008). DENV specific antibody against the E directs complement-mediated lyses and neutralization of infection in DENV-infected cells. The processes block virus attachment to cell receptors (De Rivera et al. 2008; Gil et al. 2009). The NS1 has been shown to induce antibody-mediated cellular cytotoxicity, and antibodies against NS1can mediate passive protection in mouse and primate models (Henchal et al. 1986; Walker et al. 1988). The major target of cell-mediated immunity is NS3 but T-cell epitopes have also been identified on E, M, NS1 and NS2A (Halstead 2008b). The prM-specific antibodies bind specifically to virions that are not fully matured (Bray and Lai 1991; Valdes et al. 2009; Vazquez et al. 2002). Sandwich between the E and prM-specific antibodies is the NS1 specific antibodies that neither bind to the virion nor is adept at neutralization of virus

infection. The NS1 specific antibodies are capable of directing complement-mediated lyses of infected cells (Liu et al. 2006; Mellado-Sanchez et al. 2005; Mellado-Sanchez et al. 2010; Sierra et al. 2002; Williams et al. 2009).

1.4.2. ANTIBODY

Antibodies are soluble glycoproteins whose function is identifying and elimination of immunogenic substances. They represent one of the main effectors of the humoral immunity. They composed of two basic structures, heavy chains and two light chains. Grouped into five different classes—IgA, IgD, IgE, IgG, and IgM. They are differentiated by the amino acid sequence of one of their basic structures, the heavy chain. The IgG, IgA, and IgM are normally induced after viral infection (Rabel et al. 2012; Waldman et al. 1967). They could be monoclonal or polyclonal and their function is based on a specific and physical interaction with the antigen.

1.4.2.1 Monoclonal antibodies: mAb, these are antibodies made by identical immune cells, clones from an exclusive parent cells. They are mono specific antibodies because of their ability to bind specifically to a given epitope. The monoclonal antibodies are generally produced *in vitro* using human mouse hybrid cells described by Schwaber in 1973 (Howard 2006; Schwaber and Cohen 1973; Serafin and Aaskov 2001).

1.4.2.2 Polyclonal antibodies (pAb), the polyclonal serum antibodies are antiserum derived from multiple bone marrow cells (B-cells) against specific antigens. These antibodies are immunoglobulin molecules capable of identifying and neutralizing different epitopes or antigenic determinants. Polyclonal antibodies are generally derived from susceptible animals immunized with antigen. The antigens stimulated the production of IgG immunoglobulin by B-lymphocytes for specificity against the

antigen. General objectives of polyclonal antibodies are basically for experimentation and diagnostics (Calisher et al. 1989; Lin et al. 2012; Schwaber and Cohen 1973).

1.4.3 MECHANISM OF NEUTRALIZING ANTIBODIES IN VIRAL INFECTION

Neutralizing antibodies are essential component in preventing microbial and viral infections. For antibody neutralization of virus to occur in vivo, a certain threshold level of antibodies is required. A primary antibody response occurs with first exposure to the virus. Initially IgM antibody manifests first, then, IgA on mucosal surfaces followed by the IgG in the serum. During early stages of dengue infection, the neutralizing antibodies produced at this stage could obstruct the virion binding to receptors, block uptake into cells and cause aggregation of virus particles. This strategy is adopted in response to many enveloped viruses whereby the antiviral antibodies and serum complement disrupt membranes through lyses. The non-neutralizing antibodies induced at this stage of infection also attach distinctively to virus particles, without significant effect. They may enhance infectivity because these antibodies can interact with cell receptors on macrophages (Halstead 2003; Halstead and O'Rourke 1977; Wahala et al. 2010).

In secondary dengue infection the neutralizing antibody response is more rapid than in the primary response. Because the cross-reactive but sub-neutralizing antibodies elicited during first infection binds to the second DENV and enhanced the secondary infection. This phenomenon is referred to as antibody dependant enhancement (ADE). During secondary dengue infection, higher cellular intake of the unneutralized infectious virus into the monocytes occurs rather than being eliminated (Halstead et al. 1983). ADE is of great concern in natural dengue infection. *In vitro* studies of sera from infants who develop DHF demonstrated occurrence of ADE than sera from infant who develop DF

(Guy et al. 2004; Guzman and Vazquez 2010; Kliks et al. 1989). Babies with residual maternal DENV specific antibodies were demonstrated to be more susceptible to DHF or DSS following primary infection (Guzman and Vazquez 2010; Halstead 2003; Kliks et al. 1989).

In cell-mediated immunity, the $CD4^+$ and $CD8^+$ T-cells mediate T-lymphocyte response to DENV infection (Yauch et al. 2010). The cell-mediated response consists of serotype-specific and serotype-cross-reactive responses (Beaumier and Rothman 2009). DENV-specific T-lymphocytes response produces cytokines capable of initiating the removing of DENV. Interferon gamma (γ) and tumour necrosis factor and lymphotoxin inhibit DENV infection of monocytes (Beaumier et al. 2008; Beaumier and Rothman 2009). Interferon gamma also augments the expression of immunoglobulin receptors, which can increase the ADE of infection (Beaumier and Rothman 2009; Friberg et al. 2011; Kurane et al. 1991; Smith et al. 2004). The serotype cross-reactive T-cells modify biologically functional immune response to other DENV serotypes during secondary infection (Friberg et al. 2011; Yauch et al. 2009). Specifically, DENV specific $CD8^+$ T-cells were much higher in DHF patients than in DF patients recruited after secondary infection (Beaumier et al. 2008; Yauch et al. 2010). The cytokine production will aid proliferation of elevated DENV specific T-lymphocytes much earlier in secondary dengue infections (Chaturvedi et al. 2000). Similarly, using tetramers of major histocompatibility complexes (MHC) peptides have shown elevated DENV-specific $CD8^+$ T-lymphocytes with high affinity to dengue viral serotypes other than the infecting serotype (Testa et al. 2012). A high percentage of the tetramer-positive cells were apparently primed to undergo apoptosis. Recent studies reveal that DENV-specific $CD8^+$T-cellsis not detected by MHC-peptide tetramer staining until after developing plasma leakage (Rothman 2011; Yossef et al. 2012).

Collectively, effective clearance of a persistent viral infection in our systems requires various mechanisms. The most potent and recognized mechanism is the antibody neutralization. The infectious agent is recognized and engulfed by the antibody (Halstead 2003; Wahala et al. 2009). The binding incapacitates the virus by neutralizing it capacity to infect and spread to the targeted cells. This is called neutralization. The most potent and effective neutralizing antibodies are of the IgG family (Rodrigo et al. 2009). These antibodies form complexes with the infecting virus through interaction between the Fc portion of IgG antibodies and the Fc receptors on the cell surface for phagocytic action. The infecting virus is neutralized destroyed in the process through opsonisation. The infected cell is recognized through Fc receptors on the surface of the antibody and they are eventually destroyed by the natural killer cells (NK cells). This is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) (Kurane et al. 1986; Rodrigo et al. 2009).

1.4.4. DENGUE VACCINE

An impeccable DENV vaccine must be able to induce both effective humoral and cell mediated with long-lasting protective immunity against all four DENV serotypes (Howard 2006; WHO Initiative for Vaccine Research. and World Health Organization. Dept. of Immunization Vaccines and Biologicals. 2008; Wilder-Smith et al. 2010). The ideal dengue vaccine must address the likelihood of ADE (Guy et al. 2004). Researchers have to date employed various biological approaches in designing and developing dengue vaccines. A number of the vaccines have been in various stages of clinical trials (Beckett et al. 2011; Bellanti et al. 1966; Capeding et al. 2011; Edelman et al. 2003; Sun et al. 2009; Wright et al. 2009). The most extensively trialled vaccines have been the live attenuated dengue vaccines (Capeding et al. 2011; Edelman et al. 2003). The live attenuated vaccine was developed in Thailand and licensed by Aventis Pasteur

(Kraiselburd 1987; Murrell et al. 2011). The vaccine showed remarkable seroconversion rates of 80-90% against the four DENVs following the administering two doses in school-age children. Subsequently, another live attenuated dengue vaccine was developed by Walter Reed Army Institute of Research (WRAIR) in America and was licensed by Glaxo SmithKline(Murrell et al. 2011). The vaccine had similar seroconversion rates in adults comparable to what was observed in earlier study on children (Chanthavanich et al. 2006). However, unwarranted immune response resulting in incomplete protection and potentiality of enhanced disease severity was subsequently observed (Durbin and Whitehead 2010; Murrell et al. 2011; Wilder-Smith et al. 2010).

The immunological basis of the attenuation in dengue vaccine is not clearly understood (Wilder-Smith et al. 2010). The potential of the vaccine strain to revert to a virulent strain through mutation or recombination is of great concern (Murrell et al. 2011). Another DENV vaccine that is currently under development but yet to undergo clinical trials is the inactivated whole-virion vaccine (Butrapet et al. 2000; Huang et al. 2000). Others have proposed the synthetic peptides, subunit vaccines, vector expression and recombinant live vector systems (Edelman et al. 2003; Troyer et al. 2001). The infectious cDNA clone derived vaccines and naked DNA are also yet to go on clinical trial (Costa et al. 2006; Konishi et al. 2000; Stephenson 2005). These approaches however, have proved to improve the immunogenicity and stability of these potential vaccines. Stable cDNA clones of flavivirus are now available and can be used to engineer an infectious and highly mutated region of the vaccine strain (Butrapet et al. 2006). These techniques are in addition genetically stable and are seen as more reliable in designing novel vaccine for DENV (Butrapet et al. 2006; Robert Putnak et al. 2005; Simasathien et al. 2008). An attenuated infectious clone is derived from DENV-4 by deleting regions of the 5’ and 3’ UTRs resulted in mutant viruses with low attenuation,

immunogenicity and decreased replication in cell culture (Troyer et al. 2001). Similarly attenuated DENV serotypes were generated by deleting a 30-nucleotides region of the 3`UTR of the infectious clones (Durbin and Whitehead 2010; Guzman et al. 2010; Wilder-Smith et al. 2010).These clones are still undergoing preclinical trials. For the last two decades, significant technological advances have been made but tough challenges lies ahead. As reported by Sabin, DENV-neutralizing antibodies are the only immune substance shown to prevent dengue infections (Sabin 1952; Sabin and Schlesinger 1945). While waiting for the technology to bear fruits, continuous effort in innovation and management of dengue patient is needed. A drug to prevent vascular leakage with vaccine at hand could save many lives (Avirutnan et al. 2006; Howard 2006; Trung and Wills 2010).

1.5. DENGUE VIRUS EVOLUTION

1.5.1 ANTIGENIC EVOLUTION OF DENGUE VIRUS

Evolving DENV is affected by population size and its modes of transmission. These factors are attributes of most RNA viruses. Because of selection and competition between strains that carry different beneficial mutations that assures survival of the fittest for the best possible strain (Smith et al. 2004; Twiddy et al. 2003). New lineage emerges, and it replaces old strains through this processes- emerging/reemerging, and thus is capable of evading antibody neutralization (Mackenzie et al. 2004).

High percentage of infectious disease in the world today is credited to the antigenically variable pathogens that escape from immunity induced from prior infection or vaccine. The emergence and re-emergence of antigenically distinct DENV mutants is credited to the ability of the virus to escape antibody neutralization (Chua et al. 2006; Gromowski et al. 2010; Guzman et al. 2000a). The effectiveness of antibody response induced for

an existing strain to be successful on other emerging strains is highly dependent on the difference in their antigenic determinant (Rico-Hesse 2010). Understanding this difference is very critical in vaccine strain selection and surveillance. The changing pattern of related antigenic determinant gives rise to a large number of variant strains with one or more amino acid differences that may function as alternative determinants, capable of altering DENV host immunity (Guzman et al. 2000a; Halstead 2003; Halstead 2008b; Rico-Hesse 2010).

There is little evidence of selection pressure that leads to antigenic evolution of DENVs in nature. However, selection on E resulting in multiple introductions has been reported in DENV-2 (Bennett et al. 2003; Twiddy et al. 2002a) and in DENV-3 and DENV-4 (Twiddy et al. 2002b). Bennett (2010) reported evidence of antigenic evolution because selection pressure acting on NS2A of DENV-4 viruses (Bennett et al. 2010; Bennett et al. 2003). These strains with altered antigenic determinant can enhance their immunogenicity in a variety of ways, generating different antigenic genotypes. The different genotype competes in host. The fittest strains become established and transmit it lineage. The new strains introduced to the host population in a short time interval, thereby causing an epidemic outbreak (Chen et al. 2008; Guzman et al. 2002; Rico-Hesse 2010).

Dengue infection is an example of infectious diseases that presents antigenically mutant strains that are capable of evading immune response. The virus can evolve under selection pressure (Rico-Hesse 2003; Rico-Hesse 2010; Teoh et al. 2010; Twiddy et al. 2002a). The immune pressure exerted by the virus is related to its ability to infect and transmit between mosquito and susceptible host. This process of antigenic evolution can be quantified between virus and the strain with prior exposure. The ability of the variant

strain to evolve depends on its ability to alter its antigenic determinant for binding specificity and attachment to cellular receptors during fusion (Dunham and Holmes 2007; Lin et al. 2004; Wahala et al. 2010; Wahala et al. 2009).

Recombination within each of the DENV strains has also been observed to contribute to antigenic evolution of DENV (AbuBakar et al. 2002b; Worobey et al. 1999). Convincing proof of recombination between strains has now been obtained from a patient infected with DENV-1 and DENV-2 recombinants (Lai et al. 2008; Pethel et al. 1992; Worobey et al. 1999) and in DENV4 (AbuBakar et al. 2002b). Prevalent intragenotype recombination that leads to antigenic evolution of new strains was identified among all four DENV serotypes (AbuBakar et al. 2002b; Craig et al. 2003; Tolou et al. 2001; Worobey et al. 1999).

The DENV E is one of the well-studied antigenic proteins because of its role in virus evolution (Howard 2006; Lee et al. 2006; Upanan et al. 2008). The relationship between neutralization escape and structural function of DENV antigenic protein in hyperendemic areas like Malaysia where dominant strains co-circulate with minutes or strains is imperative in understanding the pattern of evolution. Changes in the amino acid residues are related to antibody neutralization escape (Gromowski et al. 2010; Guzman et al. 2000a). Confirming the specificity and effects of these changes in endemic areas is vital in preventing future outbreaks.

1.6. PHAGE DISPLAY

Biological techniques are very powerful tools in molecular research. Phage display technology is one of the prevailing techniques commonly used in molecular research laboratories to obtain libraries of different biological compounds (Goodyear and

Silverman 2008; Smith 1985). Phage display has been effectively used to isolate proteins, study protein interactions, and binding affinity. The phage display was first developed and adopted by G. Smith in 1985 (Smith 1985). Smith showed the expressing protein of interest on the surface of filamentous bacteriophages by fusing the nucleotide sequence of the targeted protein into the genome of the phage. The targeted protein could be expressed and displayed as a fusion protein as one of the phage coat proteins. The system enables selection and screening of biological proteins with specific roles(Vithayathil et al. 2011). The most important aspect of the technology is the ability to screen quite a number of different proteins by enrichment (Smith 1985; Vithayathil et al. 2011).

The phage display system uses bacteriophages, viruses that infect a variety of gram-negative bacteria using fertility factor (F) pili as receptors. Filamentous phages are a group of bacteriophages with a tube-like shape. These phage particles are mainly composed of the major phage coat protein, gene 8 (g8p), which are helically arranged. Around 2700 copies of g8p form a wild type filamentous phage virion. Three to five copies of another phage protein, the gene 3 (g3p) proteins are found at one tip of the particle. This protein is responsible for the attachment of phage to the bacterial pili and, thus mediates the interaction to the bacteria (Kotz et al. 2004; Smith 1985; Vithayathil et al. 2011).

The phage infects bacteria through attaching the phage (g3p) protein to the bacterial F pili. This promotes the dissociation of the phage coat proteins on the bacterial surface allowing the ssDNA phage genome to enter into the cytoplasm of the bacteria. A complementary DNA strand is then synthesized by hijacking replication machinery of the host DNA. A replicative form (RF) is formed. This double-stranded DNA serves as

a template for the generation of new single-stranded DNA genomes as well as for producing phage proteins. The particles assembled in the bacterial membrane and phages are released into the extracellular compartment through an extrusion mechanism. Filamentous phages are non-lytic bacteriophages; phages are secreted continuously without killing the host (Kotz et al. 2004; Vithayathil et al. 2011). To produce and isolate recombinant phage displaying recombinant proteins onto the surface of phage, DNA sequences of targeted foreign proteins are cloned into the gene encoding the minor phage protein coat (g3p or g8p). The DNA sequence must be in frame with the minor coat proteins genes. This approach was tiresome and the recombinant phage was found to be unstable during assembly and very low yield when transformed into bacteria. The problem was addressed by using phagemid vectors to produced recombinant clones followed by rescue with a helper phage (Kotz et al. 2004; Vithayathil et al. 2011).

Phagemid were generated using phage genome with the plasmid-cloning vector. Phagemid are double-stranded DNA hybrid molecules. The phagemid pCANTAB5E used in this study is depicted in figure 1.8 (Qi et al. 2012; Weichel et al. 2008). It is composed of a plasmid backbone bearing a colicin E1 origin (colE1 ori) enabling the phagemid to replicate in the *E.coli* host cell. It contains M13 origin allowing the phagemid to be packaged into recombinant phage particles. Ampicillin resistance gene for selection of positive colonies and the β-galactosidase gene (LacZ gene) promoter, which allows the inducible expression of the fusion protein "protein of interest-g3p coat protein", were also present. The phagemid expression is inhibited by glucose and promoted by *isopropyl-beta-D-thiogalactopyranoside* (IPTG) and the gene encoding for the g3p protein of the M13 phage (Kotz et al. 2004; Qi et al. 2012). Restriction sites (RE) are also found for cloning of the protein of interest into the phagemid. An epitope

peptide (E-tag) is found on the carboxyl-terminal part of the g3p protein for detection of the fusion protein. However, the phagemid lacks all other structural and nonstructural proteins, which are required for a phage particle (Kramer et al. 2003).

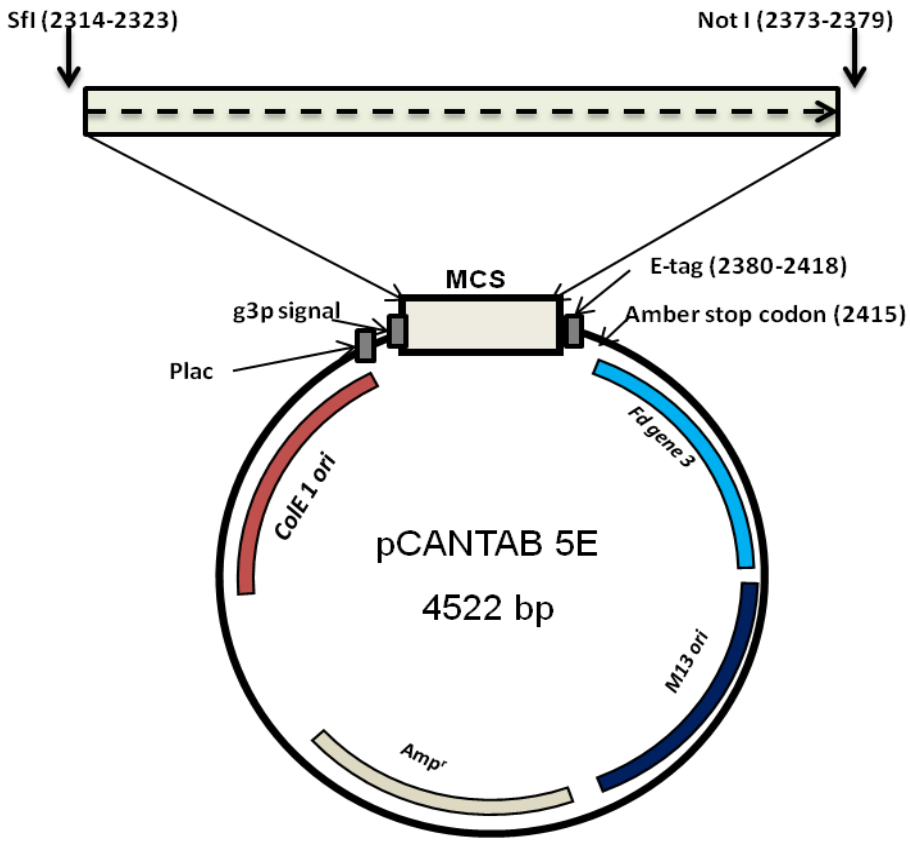


Figure 1.8. Diagram of the pCANTAB 5E phagemid vector: MCS is the multiple cloning sites on the phagemid vector.

Identical wild type phage is used in infecting the phage to complement the structural and non-structural protein component of the phage particles. Helper phage M13KO7 or VCSM13 is used to infect the phagemid transformed bacteria. This is termed “phage rescue” (Gramatikoff et al. 1994; Ontell and Nakada 1980; Vanhercke et al. 2005). The helper phage provides the missing protein components needed to package the phagemid

into virion progeny. Competition between the helper phage wild type g3p protein and the phagemid fusion protein ensures incorporation of the recombinant phagemid displaying five copies of encoded fusion protein on g3p(Abd-Jamil et al. 2008). The ability of the rescue recombinant phage in phage display system to limit the expression of the wild type g3pmakes it a worthy choice in isolating recombinant proteins against specific antigen.

1.6.1 PHAGE DISPLAY OF PROTEINS AND BINDINGS

One of the greatest potential of filamentous phage is for displaying of recombinant proteins as a prototype protein (Lucic et al. 1998; Steiner et al. 2006; Yang et al. 2000). The displayed g3p attachment proteins can be modified. Through proper selection, their binding properties or affinities may well be improved and manipulated. Researchers have used this technology to engineer recombinant proteins to study and evaluate their biological properties (Goodyear and Silverman 2008; Kramer et al. 2003). Precisely, altered proteins with mutations that increase protein function and enhanced binding properties were selected (Chakravarty et al. 2000; Kotz et al. 2004). Intracellular proteins were previously displayed as simplified library of complex mutants generated using directed mutagenesis (Fuh and Sidhu 2000). Using the technology receptor binding variant mutants were produce by altering specific role of each mutant (Abd-Jamil et al. 2008; Jolly et al. 2001; Yamamoto et al. 2009; Zhang et al. 2009).

Phage display technology can be directed to express random sequence with or without prior knowledge of their specific biological functions. This strategy enables manipulation of DNA sequences. This is done by expressing and identifying proteins with biological activities onto their specific target receptors and producing novel DNA binding proteins (Boder et al. 2000; Joyce 1992; Lin et al. 2000).A result of adaptive

evolution studies is a basis on which further diversity can be imposed. The genes coding for specific proteins as a starting point could be used to create second combinatorial library of the selected genes. A few strategies, including site directed mutagenesis could be introduced to amplify phage populations and selects for variation on the targeted proteins with alter biological properties (Boder et al. 2000; Fong et al. 2005; Lin et al. 2000).

The availability of random phage libraries has provided a powerful tool for selecting peptide sequences that mimic epitopes of infectious agents (Folgori et al. 1994; Roccasecca et al. 2001). Constructions of recombinant fusion protein libraries mimicking epitopes of DENV proteins using phage display have been reported (Donker et al. 2011; Kramer et al. 2003; Vanhercke et al. 2005). Antigen selection that mimics mutant immunologically relevant antigenic determinant of DENV was illustrated. B-cell epitopes of dengue proteins have been previously identified using mouse monoclonal antibodies (Wu et al. 2001; Yao et al. 1995). Using human pAbs against DENV, peptides capable of mimicking antigenic determinants of DENV non-structural proteins as diagnostic kit/potential antigen for vaccine production were also reported (Amin et al. 2009; Chen et al. 2003; Chen et al. 2007; Wu et al. 2003). Juraina (2008) investigated the usage of peptides derived from the hypervariable regions of monoclonal antibody fragments specific against DENV-2 E as potential inhibitors against the virus binding and infection. In the study, linear forms of two complementarily-determining regions CDR-L2 and CDR-H3 showed limited activity in inhibiting binding of E to cells proteins (Abd-Jamil et al. 2008). Similarly, specific site on DENV E that interacts with cells and a neutralizing antibody is developed using serially truncated DENV-2 E displayed on M13 phages as recombinant E-g3p fusion proteins (Abd-Jamil et al. 2008). This study highlights the power and importance of phage display technology in

competitive binding studies. Previously, a recombinant single-chain variable fragment (ScFv) of the 3H5-1 monoclonal antibody recognizing the DENV-2 E was developed using phage technology with improved binding affinity (Abd-Jamil et al. 2008; Hui 1998). Collectively, the ability of the recombinant phage particles to bind to intended target, and later estimation of binding categorized the technology as a precious approach in this study.

1.7. RESEARCH OBJECTIVES

The overall objective of the proposed study is to investigate the antigenic evolution of DENV-1 and DENV-2 in Malaysia. To achieve the overall objective the following approaches were undertaken.

1. A site directed mutagenesis approach was used to create mutations on DENV-1 and DENV-2 E gene cassettes and displayed on phage g3p minor coat protein. The mutated phage particles and their corresponding recombinant E were used in competitive binding assays to determine the effects of mutations engineered on DENV E gene cassettes on the mosquito (C6/36) and mammalian cell lines (Vero).

2. Polyclonal antibodies were generated against the phage particles displaying the recombinant E and the recombinant E of the Malaysian DENV-1 and DENV-2 in BALB/c mice. Their corresponding polyclonal antibodies were used for binding and neutralization escape studies using dominant DENV-1 and DENV-2 isolates.

3. Functional assays were employed to test the immunological properties of the polyclonal antibodies and to identify the antigenic site on E of the dominant DENV-1 and DENV-2 circulating in Malaysia that could play a significant role in neutralization escape and selection of dominant strains likely to evolve in the future. This is important for determining positions on E likely to influence future antigenic evolution of DENV-1 and DENV-2 in Malaysian natural population.

2.0 MATERIALS AND METHODS

2.1 CELL AND VIRUS

2.1.1 CELL CULTURE

The C6/36 (CRL-1660) (*Aedes albopictus*) and Vero (CRL-81) (African monkey kidney) cells used were purchased from the American Type Culture Collection (ATCC, USA). Cells were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% foetal calf serum (FCS), (Flowlab, Australia), non-essential amino acids, 2 mM L-glutamine and 50 IU penicillin/streptomycin (Flowlab, Australia) at 28°C and 37°C, respectively.

2.1.2 VIRUS PROPAGATION

DENV strains used in the study were propagated in C6/36 cell line. The strains and year of isolation were listed in the Table 2.1. Briefly, virus stocks were obtained by inoculating monolayers of C6/36 cells with MOI of 0.1 at 80% confluency. The virus was allowed to adsorb to cells for one hour at room temperature with gentle rocking. Following removal of the virus inoculums, the infected cells were cultured in EMEM supplemented with 2% FBS, 1 × NEAA and 2 mM L-glutamine until thorough cytopathic effect (CPE) was observed. The culture medium, which contained infectious virus particles, was saved following centrifugation at 40000 x g and filtered through 0.22 µm filter (Sartorius, Germany) kept at -70ºC until needed.

Table 2.1: DENV-1 and DENV-2 Malaysian strains used in this study

Isolate	Year	Genotype	Clinical status	GenBank Accession no.	Remarks
D1.036000	2005	G I a	DF	Not published	Passage 2
D1.011177*	1997	G I b	DF	Not published	Passage 2
D1.059280	1987	G I c	DF	FR666924	Passage 2
D1.010245	1997	G II a	DF	Not published	Passage 2
D1.059266	1987/1997	G III	DF	Not published	Passage 2
Isolate	**Year**	**Genotype**	**Clinical status**	**GenBank Accession no.**	**Remarks**
D2.1704378	2007	Cosmopolitan	DF	Not published	Passage 2
D2.1877347	2007	Cosmopolitan	DF	Not published	Passage 2
D2. 14245*	1997	Cosmopolitan	DF	Not published	Passage 2
D2. 14281	1997	Cosmopolitan	DF	Not published	Passage 2
D2. 16041	1998	Asian I	DF	Not published	Passage 2

*Used as backbone to construct E gene cassettes

2.1.3 VIRUS TITRATION

The foci forming assay was adapted to determine DENV infectivity titre using C6/36 cell lines. The cells, 1.5×10^5 cells/well were seeded in a 24-well plate (Costar, USA). Two hundred microliters of ten-fold serial dilution of virus stock in serum-free media was added into the wells that were previously seeded with C6/36 cells overnight. The plate was incubated at room temperature for 2 hours with gentle rocking for virus adsorption. Subsequently, following removal of the mixture, 1ml overlaid medium containing 2% FBS and carboxymethylcellulose (CMC) 1.5% (Sigma-Aldrich, USA) was added to the wells. The plate was incubated for 3 and 4 days at 28°C in 3% CO_2 atmosphere for DENV-2 and DENV-1 respectively.

2.1.4 FOCI STAINING TECHNIQUES

Six days post infection, foci of infected cells were visualized using modified peroxidase-base foci staining assay (Okuno et al. 1979). The cells were fixed with 200 µl of 4% paraformaldehyde for 30 minutes at room temperature. Subsequently, cells were permeabilized with 200 µl 1 × PBS (8 mM Na2HPO4, 1.5 mM KH2PO4, 0.14 M NaCl, 2.7 mM Kill, pH 7.4) (v/v), containing 1% Ideal detergent (Sigma-Aldrich, USA) for 15 minutes and washed three times with 1 × PBS and blocked with 300 µl of 3% skim milk for 2 hours at room temperature. The cells were rinsed three times with 1 × PBS before the addition of 200 µl of diluted rabbit hyperimmune sera (1:500) in 1% skim milk. The plate was incubated for antibody binding for one hour at 37°C followed by three times washings with 1 × PBS. Secondary antibody (200 µl), anti-rabbit IgG conjugated with horseradish peroxidase enzyme (HRP) in 1% skim milk (1:250) was added to the plates. The plate was incubated at 37°C for one hour. The cells were rinsed three times with 1 × PBS. Two hundred microliter of HRP substrate (Pierce, USA),

(metal-enhanced DAB solution diluted to 1:10 in stable peroxidase buffer) was added into each well for detection of focus of infection (foci). Foci were counted and expressed as a foci-forming unit per ml.

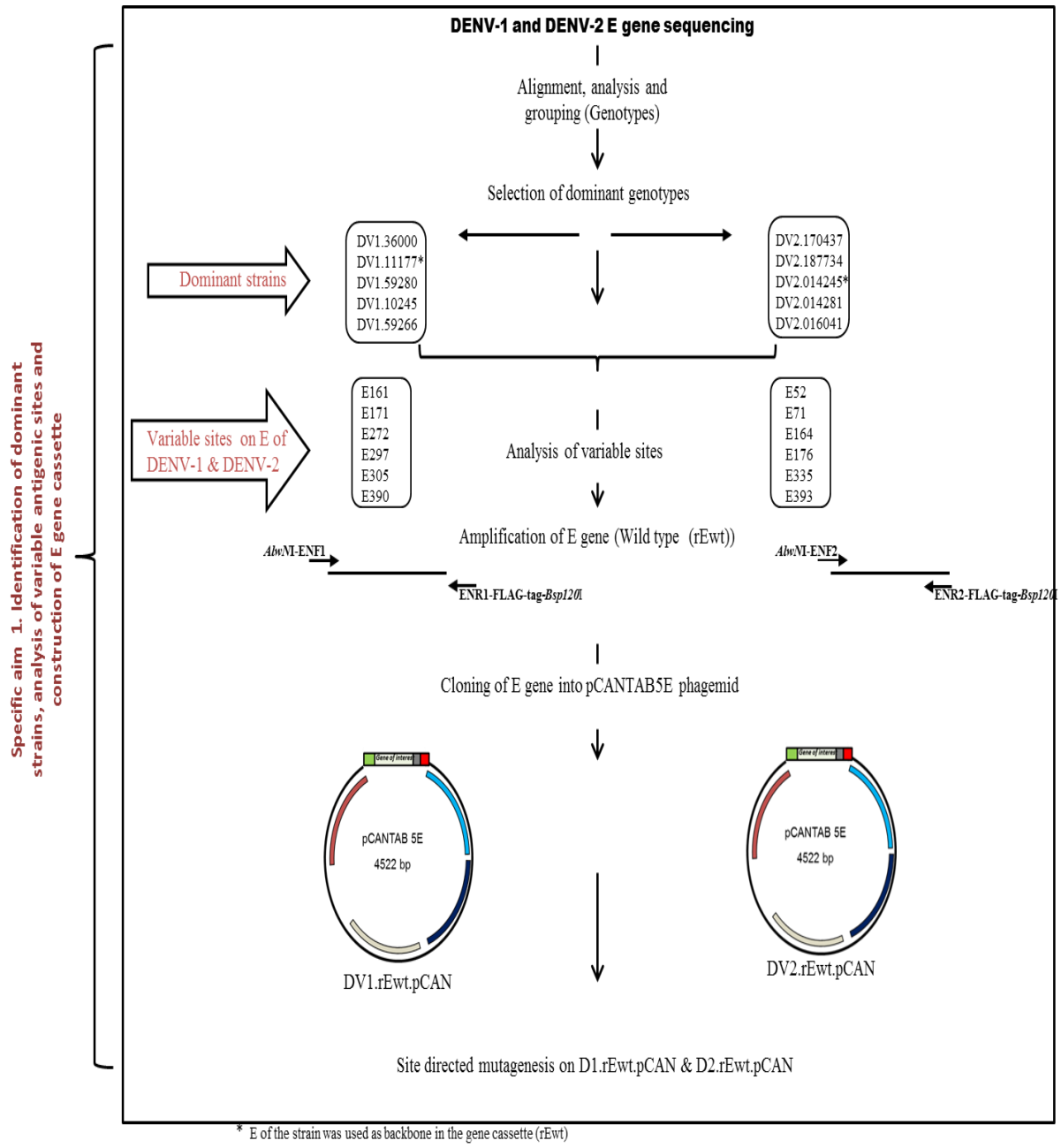


Figure 2.1a: Schematic representation and overview of the methodology

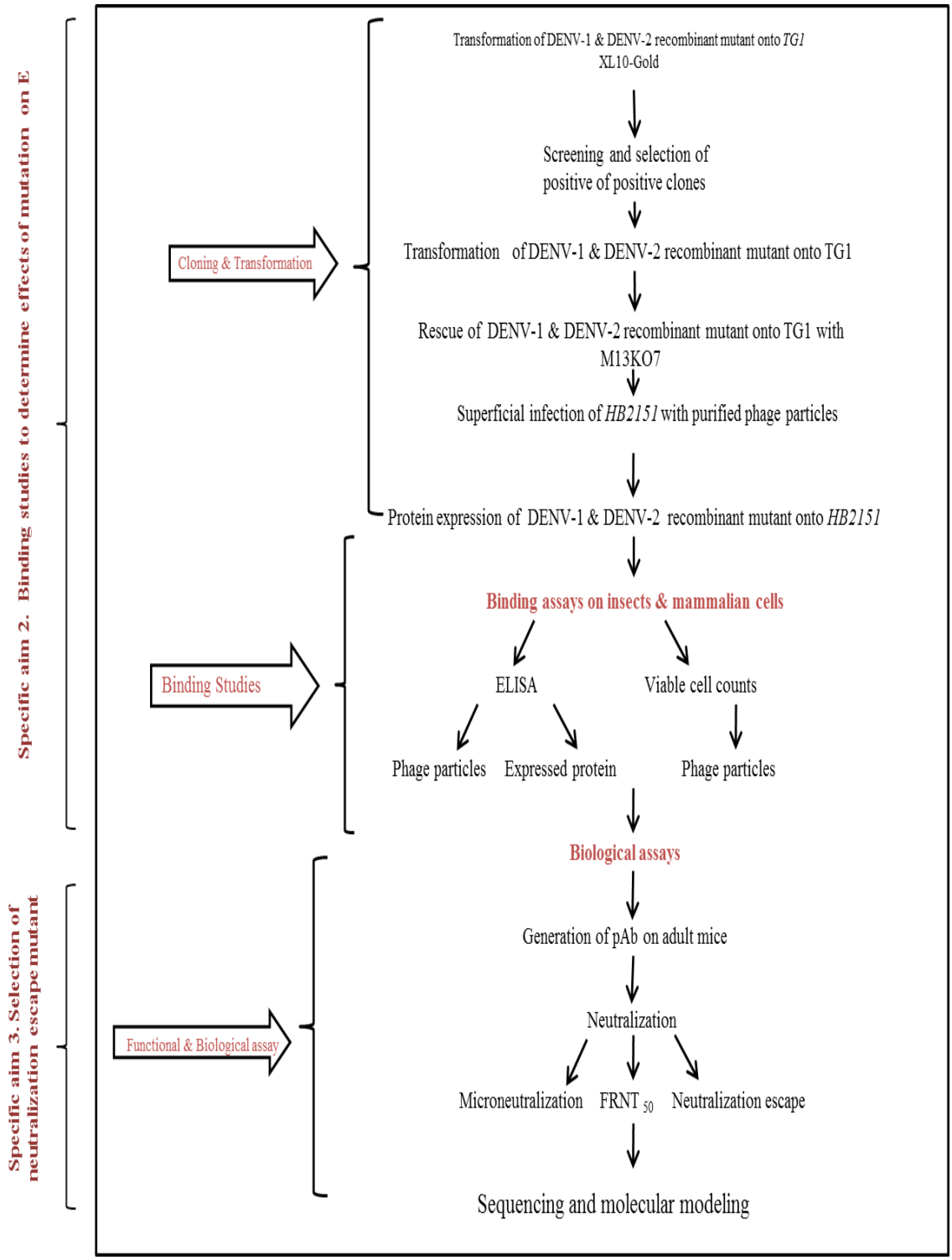


Figure 2.1b: Continuation of schematic representation and overview of the methodology

2.2.1 FIRST STRAND cDNA SYNTHESIS

Total viral RNA was extracted from culture supernatant using QiaAamp viral RNA mini kit (Qiagen, Germany) following manufacturer`s protocol. Quantitation of RNA was determined by measuring OD_{260} with Nanophotometer (Implen GmbH, Germany). The first strand of cDNA was synthesized from viral RNA lysate of a dominant prototype of DENV-1 and DENV-2 strains (Table 2.1) with gene-specific reverse primer using Superscript III One step RT-PCR kit (Invitrogen, USA) according to manufacturer`s protocol. Initially a 12 µl reaction volume consisting of reverse primers (250 ng), RNA (2 µg) and 1 µl dNTPs mix (10 mM) was prepared and heated at 65 °C for 5 minutes and followed by a quick chill on ice. The 5 × First-Strand buffer (4 µl), 0.1 M DTT (2µl) and RNasin® Ribonuclease Inhibitor 40 U/µl (Promega, USA) was added into the mixture. The mixture was then incubated at 50°C for 2 minutes prior to the addition of SuperScript™ III Reverse Transcriptase (200 U). Subsequently, the cDNA synthesis was performed at 55°C for 50 minutes and the mixture was heated at 70°C for 15 minutes to inactivate the reaction. The cDNA was quantitated using Nanophotometer and stored at –20°C for subsequent use.

2.2.2 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF E

The gene coding for complete E was amplified using the gene specific primers. The primers amplified 1485 bp of the respective DENV-1 and DENV-2 E (Table 2.2). Subsequently the gene cassettes were amplified with specific primers containing engineered FLAG tag peptide sequence and *AlwNI* and *Bsp120I* restriction sites (NEB, UK) (Table 2.2), using high fidelity *Pfu* DNA polymerase (Stratagene, USA). The following PCR conditions and protocol were used for the amplification of full-length E of DENV-1 and DENV-2 gene cassettes.

2.2.2.1 E GENE CASSETTES REACTION MIXTURE:

10 x PCR buffer	5.0 µl
10mM dNTPs mix	1.0 µl
Forward primer 10 µmol	0.5 µl
Reverse primer 10 µmol	0.5 µl
HF DNA polymerase (5U/ul)	0.2 µl
cDNA template	0.5 µl
H_2O	42.5 µl
Total	50.0 µl

2.2.2.2 AMPLIFICATION PROGRAM:

Denaturation	30 seconds	94°C	
Annealing	45 seconds	60°C	× 25 cycles
Extension	1 minute per kb	68°C	
Final extension	15 minutes	68°C	

Table 2.2: Amplifications primers used to amplified and sequence E gene cassettes

Primer	Primer sequence 5` - 3`	Position	Polarity	Amplicon size
[a]DV1EF	AT GCG ATG CGT GGG AAT AGG CA	1-22	Sense	1485
[a,d]DV1ER	T TGT CAT CGT CGT CCT TGT AGT	1463-1485	Anti-sense	
[a]DV2EF	AT GCG TTG CAT AGG AAT ATC AA	1-22	Sense	1485
[a,d]DV2ER	T TGT CAT CGT CGT CCT TGT AGT	1460-1482	Anti-sense	
[b]EN1F	GCT AGC A*GA GCC T*AT GCG ATG CGT GGG AAT AGG CA	1-22	Sense	1229
[b]ENR1	*GGG CCC C***CT TGT CAT CGT CGT CCT TGT AGT C**TG CAC CGT GAC TCC TAG GTA	1463-1485	Anti-sense	
[b]EN2F	GCT AGC A*GA GCC T*AT GCG TTG CAT AGG AAT ATC AA	301-320	Sense	1532
[b]ENR2	*GGG CCC C***CT TGT CAT CGT CGT CCT TGT AGT C**TG CAC CAT AAC TCC CAA ATA	1460-1482	Anti-sense	
[c,d]Phage.F	CAACGTGAAAAAATTATTATTCGC	2218	Sense	
[c]Phage.R	GTAAATGAATTTTCTGTATGAGG	2518	Anti-sense	

[a]Primers used to amplify full-length E gene
[b]Primers used to amplify E gene cassettes, the engineered restriction site are italic While the FLAG tag sequence are shown in bold
[c]Primers on the phagemid vector
[d]Primers used to synthesize cDNA and sequence E gene cassettes

2.2.3 CONSTRUCTION OF GENE CASSETTE

A purified gene cassettes amplicon from 2.2.2 was digested with *AlwN1* and *Bsp120I* restriction enzymes according to the manufacturer`s protocol. The digested fragments were inserted into phagemid, pCANTAB5E (Pharmacia, Sweden) corresponding to the restriction sites (*SfI* and *NotI*) (NEB, UK), to obtain a recombinant phagemid and then transformed into XL10-Gold® Ultracompetent *E. coli* cells (Figure 2.2).

The gene cassette was designed, constructed and displayed on M13 bacteria phage. The E was fused on M13 phages g3p minor coat protein to be displayed as attachment protein. E cassettes of DENV-1 (1232bp) and DENV-2 (1532 bp) strains were inserted into phagemid vector, pCANTAB5E. The phagemid "pCANTAB5E" vector has an origin of replication being the f1 origin for double and single-stranded DNA synthesis (plasmid and filamentous phage origins). It also has an ampicillin resistant gene for selection of transformed bacteria. The vector also contains a pIII fusion gene for expression of g3p-fused proteins. This is under the control of a *lacz* promoter. A signal sequence is fused to the g3p-fused protein to direct secretion of the fused protein. Each protein was then expressed with a FLAG-tag peptide sequence at the C-terminal end that allowed easy purification using affinity chromatography. The phagemid vector allows conversion to infective phage by super infection using phagemid-bearing cells containing helper phages.

2.2.3.1 LIGATION OF PCANTAB5E AND E GENE CASSETTE TEMPLATES

The following reaction mixture was prepared to ligate the phagemid vector to the E gene DNA template.

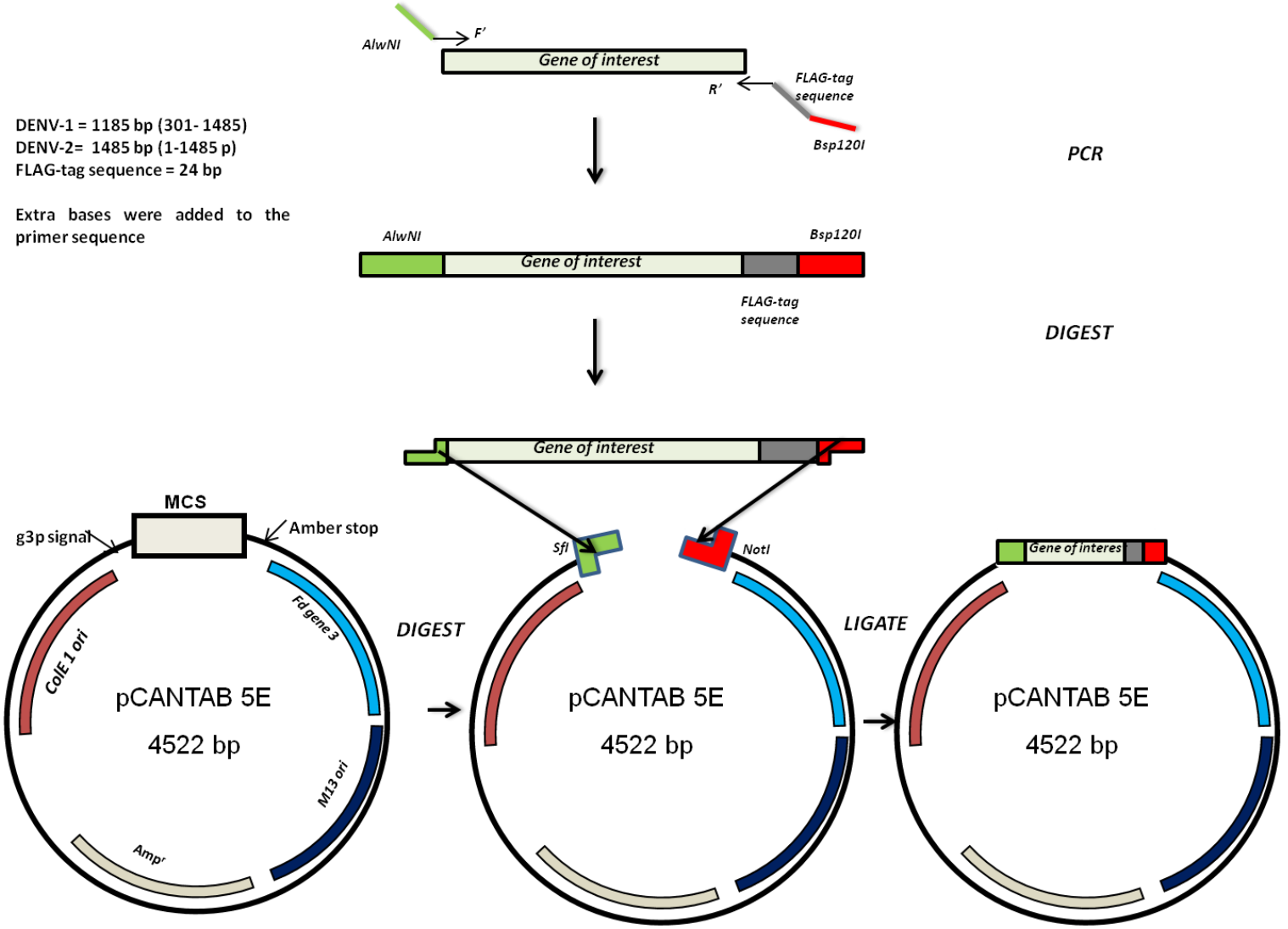
DNA Template	5.0 µl
pCANTAB5E	1.0 µl
T_4 ligase	1.0 µl
dNTPs mix	1.0 µl
H2O	4.0 µl
Total	12.0 µl

The reaction tube was incubated overnight at 16°C. The ligated products were transformed into XL10-Gold® Ultracompetent *E. coli* cells (Stratagene, USA) according to the manufacturers' protocol.

Figure 2.3. An overview of the E gene cassettes constructs. DENV-1 and DENV-2 E fragments were cloned into pCANTAB5E to obtain the recombinant phagemid.

DENV-1 = 1185 bp (301- 1485)
DENV-2= 1485 bp (1-1485 p)
FLAG-tag sequence = 24 bp

Extra bases were added to the primer sequence



2.2.4 TRANSFORMATION OF RECOMBINANT PHAGEMID INTO XL10-GOLD®

Chemical method for transformation of the bacteria was used. The technique is based on subjecting the plasmid DNA and chemically-treated ultracompetent bacteria into a mild heat shock that creates pores in the cell membrane promoting entry of the plasmid DNA into the bacteria. Ten microliter of ligation mixture is mixed with 100 µl of XL10-Gold® Ultracompetent cells treated with 1.0 µl of β-mercaptoethanol (Stratagene, USA) to inactivate surface exposed nucleases and proteins that may adversely affect transformation efficiency. The plasmid DNA and the bacteria were heat shocked in a heating block at 42°C for 2 minutes and briefly placed on ice. The mixture was then transferred into 1 ml Luria Beltane (LB) media (1.0 g of bacto-tryptone, 0.5 g of bactoyeast extract, and 0.5 g of NaCl) and incubated for 1 hour at 37°C with gentle shaking. Subsequently, 100 µl of the culture were poured on LB agar plates containing 100 µg per/ml ampicillin, and incubated overnight at 37°C.Positive clones were determined by colony PCR and sequencing using BigDye Terminator kit v.3.0 (Applied Biosystems, Bedford, MA). Phagemid containing recombinant gene cassettes of DENV-1 and DENV-2 were extracted and purified using the alkaline lyses of bacterial cells (Vogelstein and Gillespie 1979).

2.2.5 COLONY SCREENING

The proportion of colonies having an insert of the correct size was evaluated by amplification of a single colonies using forward primer from phagemid and reverse primers from gene insert (Table 2.2). Single colonies from agar plates were selected and boiled in 10 µl of distilled water for 5 minutes. The pellets were briefly centrifuged and 2 µl of the supernatant was transferred directly into an amplification reaction mixture. An initial denaturation step of 2 minutes at 94°C was followed by PCR amplification cycle.

2.2.5.1 COLONY AMPLIFICATION REACTION MIXTURE:

5 x PCR buffer	2.5 µl
10mM dNTPs mix	0.5 µl
Forward primer 10pmol/µl	0.25 µl
Reverse primer 10pmol/µl	0.25 µl
Taq DNA polymerase (5U/µl)	0.25 µl
Bacterial colony suspension	2.0 µl
Distilled H_2O	14.25 µl
Total	20.0 µl

2.2.5.2 AMPLICON AMPLIFICATION PROGRAM:

Denaturation	30 seconds	for	94°C	
Annealing	45 seconds	for	60°C	× 25 cycles
Extension	1 minute per kb	for	68°C	
Final extension	15 minutes	for	68°C	

2.2.6 SITE-DIRECTED MUTAGENESIS

Quick-change Light site-directed mutagenesis Kit (Stratagene, USA) was utilized to create single mutation on DENV-1 and DENV-2 E cassettes using primer presented in table 2.3 and table 2.4 respectively. The purified phagemid containing gene inserts were methylated circular dsDNA plasmids (pCANTAB5E – E) purified from (dam+) *E.coli* strains from 2.2.4. First, mutant strand is synthesized with mutagenic primers by primer directed replication of both plasmid strands using *Pfu* high fidelity DNA polymerase with highest fidelity. The double-stranded DNA containing gene insert were extended by the mutagenic oligos during temperature cycling to create a mutated phagemid containing staggered nicks. The product is treated with *Dpn* I endonuclease to digest the methylated and hemimethylated (5`-$Gm^6ATC$-3`) parental DNA template and to select the mutated synthesized DNA. The products that contain the mutant and nicked phagemid are transformed into competent cells.

2.2.7.1 SITE-DIRECTED MUTAGENESIS REACTION MIXTURE:

10 × reaction buffer	5.0 µl
10mM dNTPs	1.25 µl
Primer 1 (125ng)	1.0 µl
Primer 2 (125ng)	1.0 µl
Template dsDNA (50 ng)	1.0 µl
Quick solution reagent	1.5 µl
pfu HF DNA polymerase	1.0 µl
Distilled H_2O	38.25ul
Total	50.0 lµ

2.2.7.2 SITE-DIRECTED MUTAGENESIS CYCLE CONDITION:

Initial denaturation step	2 minutes	for 95ºC
Denature DNA strands cycle	30 seconds	for 95ºC × 18 cycles
Anneal	45 seconds	for 60ºC
Extension	30 seconds per kb	for 68ºC
Final extension	5 minutes	for 68ºC

Finally, DpnI was added to the amplified amplicon and incubated at 37ºC for 5 minutes to digest parental DNA template

Table 2.3: Site-directed mutagenesis primers used to create mutations on DENV-1 gene cassette

Name	Sequence 5` - 3`	Polarity
a481b	5'-gcacagaacatgggacabctgcaactataacaccc-3'	sense
a481b	5'-gggtgttatagttgcagvtgtcccatgttctgtgc-3'	antisense
t511b	5'-ccccaagctcctacgbcggaaatacagctga-3'	sense
t511b	5'-tcagctgtatttccgvcgtaggagcttgggg-3'	antisense
a814b	5'-ggagcgacagaaatccaabcgtctggaacgaca-3'	sense
a814b	5'-tgtcgttccagacgvttggatttctgtcgctcc-3'	antisense
a889b	5'-gacaaactgactttgaaagggbtgtcatatgttatgtgcacag-3'	sense
a889b	5'-ctgtgcacataacatatgacavccctttcaaagtcagtttgtc-3'	antisense
g1093h	5'-atagttactgacaaagaaaaaccahtcaacattgaggcagaacc-3'	sense
g1093h	5'-ggttctgcctcaatgttgadtggtttttctttgtcagtaactat-3'	antisense
a1168b	5'-ggtgaaaaagctttgaaactabgttggttcaagaaaggaagca-3'	sense
a1168b	5'-tgcttcctttcttgaaccaacvtagtttcaaagctttttcacc-3'	antisense

- Primers were designed using the quick-change primer design program on Stratagene official website https://www.genomics.agilent.com/CollectionSubpage.aspx
- First alphabet in the name box denotes mutated nucleotide underlined in the primer sequence, number denote mutated position and last alphabet denotes nucleotide introduced

Table 2.4: Site-directed mutagenesis primers used to create mutations on DENV-2 gene cassette

Name	Name Sequence 5` - 3`	Polarity
c154r	5'-tgatagaaacagaagccaaaraacctgccactctaagg-3'	sense
c154r	5'-ccttagagtggcaggttyttttggcttctgtttctatca-3'	antisense
g211h	5'-tgaccaacacaacaacahattctcgctgcccaaca-3'	sense
g211h	5'-tgttgggcagcgagaatdtgttgttgtgttggtca-3'	antisense
a490b	5'-acaggaaaacatggcaaggaaatcaaabtaacaccacagagtt-3'	sense
a490b	5'-aactctgtggtgttavtttgatttccttgccatgttttcctgt-3'	antisense
a526b	5'-cacagaagcagagttgbcaggctatggcactgt-3'	sense
a526b	5'-acagtgccatagcctgvcaactctgcttctgtg-3'	antisense
a1003b	5'-gggacggttctccatgtaagbtccctttgagataatg-3'	sense
a1003b	5'-cattatctcaaaagggavcttacatggagaaccgtccc-3'	antisense
a1178b	5'-aattgaagctcaactggtttabgaaaggaagttctatcggc-3'	sense
a1178b	5'-gccgatagaacttcctttcvtaaaccagttgagcttcaatt-3'	antisense

- Primers were designed using the quick-change primer design program on Stratagene official website https://www.genomics.agilent.com/CollectionSubpage.aspx
- First alphabet in the name box denotes mutated nucleotides underlined in the primer sequence, number denote mutated position and last alphabet denotes nucleotide introduced

2.2.8 TRANSFORMATION OF PHAGEMID ON TG1 *E. COLI*

To obtain mutant of recombinant phagemid, competent TG1 *E. coli* strain were transformed with the mutated synthesized DNA from 2.2.7. Briefly, 10 ml of overnight culture of TG1 was transferred into 90 ml of 2×YT (17 g trypticase peptone, 10 g yeast extract, 5 g NaCl)/L broth incubated at 37°C until A_{600} = 0.5. The cells were sedimented at 2500 x g for 20 minutes at 4°C. The pellets were gently resuspended in 10 ml of cold TSS buffer (LB media containing 10.0 g of polyethylene glycol, 5 ml of Dimethyl sulfoxide (DMSO), and 5 ml of 1M $MgCl_2$ in 100 ml) and placed on ice. The mutated recombinant phagemid (2 µl) were mixed with 1 ml of competent TG1 cells and kept on ice for 1 hour. The mixture was heat-pulsed for 2 minutes at 42°C, and then placed on ice for another 2 minutes. One hundred microliters was transferred into 10 ml of LBG medium (LB media containing 20 mM glucose) and incubated for 1 hour at 37°C shaking at 200 x g. One microliter of the transformed bacteria were plated out into SOBAG plates ((20 g trypticase peptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 1M $MgCl_2$, 55.6 ml 2M glucose, 20 mg/ml ampicillin)/900 ml). Phagemid containing recombinant gene cassettes of DENV-1 and DENV-2 were extracted and purified using the alkaline lyses of bacterial cells and sequenced using BigDye Terminator kit v.3.0. The remaining bacteria were used to rescue the recombinant phages.

2.3 RESCUE OF THE RECOMBINANT PHAGES DISPLAYING GENE CASSETTES

2.3.1 RESCUE OF THE RECOMBINANT PHAGES

The transformed *E. coli* TG1 containing the mutant recombinant phagemid from 2.2.8 were grown in 2×YT-A media (17 g trypticase peptone, 10 g yeast extract, 5 g NaCl)/L, 100 µg/ml Ampicillin) overnight at 37ºC. The recombinant bacteria in 2×YT-A containing 2% glucose (1:10) were cultured at 37ºC until they reached mid-log phase. Helper phage, 3×10^{10} pfu/ml of M13KO7 (Invitrogen, USA) was added to the culture.

The mixture was incubated for another one-hour at 37ºC, and the phage-infected *E. coli* were sedimented at 1000 x g for 10 minutes and resuspended in 2×YT-AK media (2×YT-A, 50 µg/ml Kanamycin). The package phage-infected *E. coli* TG1 was then kept at –70ºC in 20% glycerol until needed.

2.3.2 PURIFICATION OF RECOMBINANT PHAGE PARTICLES

To prepare purified recombinant phage particles, the infected *E.coli* from 2.3.1 sedimented at 1000 x g for 10 minutes and resuspended in 2×YT-AK media and were incubated at 37°C overnight. The culture was then re-sedimented at 10000 x g for 10 minutes. One in a ratio of four (1:4) volume of PEG/NaCl ((200 g Polyethylene glycol 6000, 146.1 g NaCl)/L) was added to the supernatant. The mixture was kept on ice for 60 minutes. Precipitated phages were sedimented at 20000 x g for 20 minutes at 4ºC, and the supernatant was discarded. The precipitation was repeated three times. The phages were subsequently resuspended in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The resulting suspension were filtered through 0.45 µm filters (Sartorius, Germany) to remove bacterial cells and stored at 4ºC until needed.

2.3.3 DETERMINATION OF RECOMBINANT PHAGE TITRE

Purified recombinant phage particles from 2.3.2 were diluted in a series of ten-fold dilutions with sterile TE buffer. A small volume from each dilution (10 µl) was mixed together with mid-log phase *E. coli* TG1 (90 µl; A_{600} = 0.5) and the mixture was incubated for 30 minutes at 37 ºC. The mixture of *E. coli* and serially diluted phage (100 µl) was then plated out onto 2×YT-AG plates and incubated overnight at 37ºC. The number of colonies formed was counted the next day for the determination of the recombinant phage titre.

2.3.4 SUPER INFECTION OF HB2151 WITH RECOMBINANT PHAGE PARTICLES

Because of the amber stop codon between the E-tag sequence and fdg3p protein on the phagemid vector, a non-suppressor bacterial strain was used for expression of the recombinant E-g3p proteins. Briefly, 100 µl of log phase HB2151 bacteria were infected with 10 µl of purified phage precipitated particles and incubated with intermittent shaking for 30 minutes at 37°C. To ensure that resulting colonies were true nal^r (Nalidixic acid resistant) transductions and not carry over from infected TG1 cells, *E. coli* strain HB2151 were culture on SOBAG-N agar plates (SOBAG plates containing 100 µg/ml nalidixic acid). Using sterile inoculating loop, the infected bacteria were spread onto the SOBAG-N and incubated at 30°C overnight. Positive clones from the plate were determined by colony screening and sequencing. Positive colonies were sub-cultured into SOBAG-N plates and stored at 4°C for one month until used.

2.3.5 EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEIN

To express soluble recombinant proteins, HB2151 positive clones on SOBAG-N plates from 2.3.4 were used. Briefly, 1 ml of overnight culture of a single positive colony from the SOBAG-N plate was incubated in 9 ml of 2×YT-AG broth for 1 hour at 30°C. The culture was later centrifuged at 1500 x g for 15 minutes. The pellet was resuspended in 100 ml of 2×YT-A medium supplemented with 0.1mM IPTG and incubated at 30°C for 5 hours with gentle shaking at 200 rpm. Twenty-five grams of lyses powder (CelLytic Express), (Sigma-Aldrich, USA) were added onto the culture directly. The bacterial cells were incubated at 37°C for 1 hour with intermittent shaking.

Prior to that, SigmaPrep Spin Columns (Sigma-Aldrich, USA) were equilibrated at room temperature with 50 mM TBS (50 mM Tris-HCl-pH 7.4 and 150 mM NaCl, pH 8.0). The columns were embedded with 400 µl affinity gel (anti-FLAG™ M2) (Sigma-

Aldrich, USA). Following incubation, 800 µl of lysed cell solution were added into SigmaPrep Spin Columns (Sigma-Aldrich, USA). The columns were incubated for 1 hour at room temperature. Following that, the columns were washed three times with 50 mM of 1 × TBS. Protein samples were eluted into a new collection tube containing 10 µl of 0.5 M Tris-HCl pH 7.4, using 100 µl of 0.1 M glycine, pH 3.0. All centrifugation were performed at 8200 x g for one minute at room temperature using microcentrifuge. Eluted samples were analysed using Bradford BCA reagent (Pierce, USA) and stored at -20°C until needed. The purified protein was incubated with enterokinase (0.02 units per 1mg of purified protein) at 22°C for 16hours to produce the digested fusion protein.

2.3.6 SDS-PAGE AND IMMUNOBLOTTING

Twenty-five microgram per millilitre (25 µg/ml) of purified proteins were mixed with 3 × SDS sample buffer, boiled at 95°C for 5 minutes and resolved in 10% polyacrylamide gels. The proteins were transferred onto nitrocellulose membrane (Millipore, USA) using a wet transfer system (Bio-Rad, USA) at 100 Volts for 2 hours. Membranes were blocked with 3% bovine serum albumin (BSA) for 2 hours and then probed with mouse anti-FLAG monoclonal antibody for 2 hours followed by washing in 1 × TBS buffer for 10 minutes. The membrane was further incubated with goat anti-mouse IgG conjugated with HRP (Sigma, USA). The membrane was washed 2 times with 1 × TBS containing 0.05% Tween (PBS-T) and 1 × TBS buffer for 10 minutes each time. The membrane was developed using NBT-BCIP (KPL, USA) for five minutes and rinsed with distilled $_{H2O}$ for 2 minutes.

2.4 BINDING ASSAY

2.4.1 PREPARATION OF DENGUE VIRUS ANTIGEN IN VERO CELLS

Vero cells in 75 cm^3 tissue culture flask at 90% confluence was infected with respective DENV shown in Table 2.1 at a MOI = 0.1 for six days at 37°C. The supernatant of the infected cells harvested seven days later. DENV particles contained in the supernatants were clarified by centrifugation at 10000 x g for 15 minutes at 4°C. Subsequently, one volume of sterile PEG/NaCl was added to four volumes of the supernatant and kept on ice for one hour. The precipitated virus was then centrifuged at 10000 x g for 30 minutes at 4°C. The pellets consisting of the PEG-virus complexes were resuspended in sterile TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and centrifuged for 20000 x g for 15 minutes at 4°C. Supernatant were filtered through 0.22 µm to ensure only virus particles were collected. The virus suspension was aliquoted and stored at -70°C in 20 % glycerol. The virions were quantified using the Bradford BCA reagent according to the manufacturer's instructions.

2.4.2 OPTIMUM BINDING OF PHAGE PARTICLES ON C6/36 AND VERO CELLS

C6/36 and Vero cells were harvested at 90% confluence and washed three times in 1 × PBS with repeated centrifugation at 800 x g. C6/36 cells and Vero cells at1×10^5 cells/ml were seeded to occupy all possible non-specific binding sites into 96-well plates and fixed with 4% paraformaldehyde for one hour at room temperature. Wild type purified recombinant phage particles (2.5×10^5, 2.5×10^6 and 2.5×10^7 cells/ml) were added. After an hour of incubation at 37°C, the unbound phages were vigorously washed 10 times with 1 × PBS-T and five times with 1 × PBS. Mid-log phase *E. coli* TG1 at A_{600} = 0.5 was added and the plate was further incubated at 37ºC for 30 minutes. The infected *E. coli* (100 µl) was then plated onto 2×YT-AG plates and incubated

overnight at 37ºC. The number of colonies, which represents the number of recombinant phages particles bound to the cells proteins, was counted 24 hours later.

2.4.3 BINDING OF VARIANT RECOMBINANT PHAGE PARTICLES

The binding of recombinant phage particles to DENV susceptible cell lines was performed as previously described (Abd-Jamil et al. 2008). Briefly, recombinant phage particles (2.5×10^6 cfu/ml) were added to C6/36 or Vero cells fixed in 96-well plates using 4% paraformaldehyde. The plates were incubated at 37°C for two hours with gentle agitation at every 15 minutes. The unbound phages were removed by extensive washing with 1x PBS-T and PBS. The cells were then incubated with TG1 E. coli at mid-log phase (A600 = 0.5) at 37ºC for 30 minutes. The *E. coli* (100 µl) was then plated onto 2×YT-AG plates and incubated overnight at 37ºC. The number of colonies, which represents the number of recombinant phage bound to the cell surface proteins, was counted the day after.

In similar binding study, 2.5×10^6 cfu/ml of the recombinant phage particles were precipitated with the pAb described in 2.4.3 using two different dilutions (1:80 and 1:640) for one hour at 37 °C. Subsequently, the mixture was centrifuged at 40000 x g for 15 minutes to pellet the antibody-phage complexes. The supernatant (100 µl) was transferred into wells containing the fixed cells. The binding study was performed as above (2.4.3).

2.4.4 BINDING OF POLYCLONAL ANTIBODY TO DENGUE VIRION

The binding of polyclonal antibodies to DENV was determined as previously described by (Wong et al. 2007). Briefly, twenty five µg/ml of purified viruses prepared above were coated into 96 well plate using 0.5M Na2CO3, pH 9.6, and overnight at 4ºC.

Following the removal of excess buffer, the coated proteins were blocked with 100 µl of 3% BSA in TBS-T for 2 hours at room temperature and incubated with the heat inactivated pAb diluted in 1% BSA for one hour at 37°C. After extensive washing with TBS-T, 100 µl of alkaline phosphatases-labelled goat anti-mouse IgG (1:2000 in 1% BSA) was added. The reaction was developed by addition of p-nitrophenyl phosphate (KPL, USA). The OD absorbance was read at 410 nm. The cut-off value of absorbance was calculated as average mean OD of negative samples ± 2 SD. The DENV-1 isolates showing 2-fold greater ELISA OD with pAb was identified as positive and strong binding.

2.4.5 COMPETITION ELISA

Equal amount and concentration of DENV-1 and DENV-2 virions (1:1) were mixed and pre-coated into the 96 well plates overnight as in 2.4.4. Excess buffer was removed and blocking of non-specific binding sites were done by adding 3% BSA, heat inactivated pooled pAb from DENV-1 and DENV-2 diluted with 1% BSA were added and incubated for 2 hours at 37°C. The plate was washed 3 times with 1 × TBS-T and 3 times with 1 × TBS. Subsequently, 100 µl of alkaline phosphatases-labelled goat anti-mouse IgG diluted 1:2000 in 1% BSA solution was added. Then, p-nitrophenyl phosphate substrate (100 µl) was added to develop the reaction. The absorbance value was determined at 410 nm with an ELISA micro plate reader. The cut-off value of absorbance was calculated as average mean OD of negative samples ± 2 SD.

2.5 IMMUNE RESPONSE

2.5.1 MICE IMMUNIZATION WITH RECOMBINANT PROTEINS

A group of four BALB/c female adult mice (4 weeks old) (MP/23/02/2011/MBD®) was immunized intraperitoneally with a 50% emulsion of complete Freud's adjuvant mixed

with 100 µg of purified recombinant proteins. The animals were housed in a temperature-controlled environment at 22°C – 24°C with 12 hours day-night cycles. The immunization was boosted twice: first with the same dose of recombinant proteins in 50% incomplete Freud's adjuvant and the final without using adjuvant. The interval between the first, second and final immunization was ten days. Blood samples were collected through tail bleeding seven days after the last immunization. ELISA method was used to determine immune response. Final blood samples were obtained ten days after last immunization through heart puncture. pAb was harvested by centrifugation of clotted blood at 1800 x g for 5 minute and stored at -20°C until use. ELISA method was used to determine polyclonal antibody titres. The absorbance was read at 490 nm with an ELISA micro plate reader (Tecan Sunrise™, USA). The cut-off value for positive binding was calculated as average mean OD of negative samples ± 2 SD.

2.5.2 IMMUNE RESPONSE AGAINST RECOMBINANT PROTEIN ON MICE

Briefly, purified wild type mutant recombinant E (25 µg/ml) and their respective phage particles (2.5×10^6 cfu/ml) were pre-coated onto 96-well plates (Costar, USA) in a carbonated buffer at 4°C overnight. The plates were blocked with 3% BSA for 2 hours at room temperature. Heat inactivated pAb two-fold dilution in 1% BSA was added after 5 times washing with TBS-T. The alkaline phosphatase-labelled goat anti-mouse IgG (100 µl) diluted 1:2000 solution was added. Then, 100 µl of p-nitrophenyl phosphate substrate was used to develop the plate. The absorbance was read at 410 nm with an ELISA micro plate reader. The cut-off absorbance value was calculated as average mean OD of standard negative samples ± 2SD. The ± 2SD was determined from microneutralization assay using mouse sera. The test serum showing a 2-fold rise in ELISA OD than that of the negative control was positive and significant, while serum

samples which shows two folds lower ELISA OD than that of the negative is negative and not significant for IgG.

2.6 MICRONEUTRALIZATION ASSAY

DENV isolates (5×10^2 ffu/ml) were mixed in equal volumes of heat-inactivated serially diluted pAb (1:20 – 1:1280) and incubated at 37°C for one hour. Neutralization assay was performed as previously described(Twiddy et al. 2003). The cut-off value was determined by subtracting ± 2 SD from the average OD of standard negative control. The neutralizing titre is the maximum dilution below the cut-off value. To test the ability of pAb from immunized mice to neutralize or block dominant DENV-1 and DENV-2 circulating in Malaysia, microneutralization assays were performed. The pAbs were heat-inactivated for 30 minutes at 56°C using heating block.

2.6.1 MICRONEUTRALIZATION OF DENGUE WITH POLYCLONAL SERUM

Fifty microliters of each DENV isolates (5×10^2 ffu/ml) was incubated with equal volume of serial dilution (1:20 – 1:1280) of the heat inactivated polyclonal serum samples and incubated at 37°C for one hour. Mixture of the virus and serum (100 µl) was transferred to overnight monolayers of C6/36 and Vero cells in a 96 well plates (3×10^4cells/ml). The plates were incubated at 28°C and 37°C in 3% and 5% CO_2 for 4 days respectively. Then, the cells were fixed with 100 µl of cold ethanol/methanol (1:1) for 30 minutes at 4°C. After removing the fixative and vigorously washed 10 times with 1 × PBS, the plates were incubated with rabbit raised hyperimmune serum for another 1 hour at 37°C. After washing 10 times with 1xPBS, anti-rabbit IgG conjugated with alkaline phosphatase was added and incubated at room temperature for 2 hours. Subsequently, 100 µl of AP-substrate, p-nitrophenyl phosphate was added. The OD was read at 410 nm / 465nm wavelength. The cut-off value was determined by subtracting ±

2 SD from the average OD of standard negative control. The neutralizing titre is the highest dilution below the cut-off value.

2.6.2 FOCI REDUCTION NEUTRALIZATION TEST ($FRNT_{50}$)

The $FRNT_{50}$ assay was performed according to (Zandi et al. 2012) with minor modification. Briefly, heat-inactivated serum samples were diluted four-fold (1:20 to 1:1,280) in tubes, with maintenance medium containing 2% FBS used as diluent. The serially diluted pAbs were incubated with an equal volume of virus suspension 3000 ffu/ml for an hour at 37°C. Following that, the virus and serum mixture (200 µl) were added to the monolayers of C6/36 and Vero cells. The plates were incubated for 2 hours, at room temperature. Overlay medium (1ml), containing 2% FBS and 1.5% carboxymethylcellulose (Sigma-Aldrich, USA) was added to the wells. The plates were incubated at 28°C and 37°C for 4 days, respectively. The foci were visualized as described in 2.1.4. The antibody titre was expressed as the reciprocal of highest serum dilution yielding50% reduction in foci formed ($FRNT_{50}$).

2.6.3 NEUTRALIZATION ESCAPE

DENV virions were continuously propagated in growth medium supplemented with pAb at normalized dilution (1:80) that yields 50% reduction in foci formed. Briefly, 3000 ffu/ml of DENV virions were mixed with 50 µl of diluted pAb for one hour at 37°C and then briefly centrifuged before transferred onto C6/36 and Vero cells for one hour at room temperature with gentle rocking. After removing excess media, the plate was incubated at 28°C or 37°C in 3% and 5% CO_2 for 4 days corresponding to the insects or mammalian cell line. The supernatant was harvested and 100 µl were mixed with 50 µl of the diluted pAb followed by passaging for the second time. This propagation was repeated thrice. After the each round of propagation, focus formation

assay was performed as previously described (Russell and Nisalak 1967; Wong, et al. 2007) to determine the foci reduction. The viral RNA from passage four was obtained and E was sequenced and compared.

2.7 SEQUENCING ANALYSIS

2.7.1 SEQUENCING AND ANALYSIS OF E

DENV-1 and DENV-2 strains shown in Table 2.1 were obtained from UMMC virology repository. Sequencing was performed in-house using BigDye Terminator kit v.3.0 (Applied Biosystems, Bedford, MA) according to the manufacturer`s. Analysis of nucleotide and amino acid sequences were performed using Clustal × V3.1 (Larkin, Blackshields et al. 2007), GeneDoc, MEGA 5.0 (Tamura, et al., 2011) programme respectively.

2.7.2 SEQUENCING ANALYSIS OF SITE DIRECTED MUTAGENESIS CLONES

Similarly, purified phagemid displaying DENV-1 and DENV-2 recombinant gene cassette and site directed mutagenesis clones were sequenced as in 2.7.1. The purified phagemid were sequence to determine whether variant mutants were generated and whether there was shifting in the reading frame.

2.8 MOLECULAR MODELLING

The nucleotide sequence of the DENV E and the molecular location of amino acid substitutions were modelled on the 3D structure of the DENV-2 E (SWIS-prot PDB code: 1ok8) using the program FirstGlance in Jmol version 1.44 (http://bioinformatics.org/firstglance/fgij/). The program was used to determine the locations of the major amino acid residues substituted with the minor residues. The

relative distance between the amino acid residues, at the same monomer on envelope homodimers were equally read.

2.9 STATISTICAL ANALYSIS

The ELISA and FRNT results were analysed using Graph Pad PRISM 5.0 (Graph Pad Software Inc., San Diego, CA, 2005). Mean differences were calculated using ANOVA followed by Tukey's post-hoc test where appropriate. The levels of significance are denoted as the following: *P<0.05.

2.10 CHEMICALS AND REAGENTS

All chemicals except when stated were purchased from Sigma, USA and all organic solvents or solutions were purchased from BDH, UK. All culture media and tissue culture reagents were obtained from Flowlab, Australia. Monoclonal antibodies were purchased from BDH Pharmgen, USA; Cell Signaling Technology, USA and Sigma, USA.

3.0 RESULTS

The overall objective of the proposed study is to investigate the antigenic evolution of DENV in Malaysia. To achieve this, site directed mutagenesis approach were used to create mutations on E gene cassettes displayed on phage g3p minor coat protein. The mutated phage particles and their corresponding E were used in competitive binding assays to determine the effects of mutations engineered on E on the mosquito (C6/36) and mammalian cells (Vero). The polyclonal antibodies were generated against gene cassettes displaying recombinant E of the Malaysian DENV-1 and DENV-2 in BALB/c mice. The corresponding polyclonal antibodies were used for binding and neutralization escape studies using dominant DENV-1 and DENV-2. This is important for determining positions on E likely to influence future antigenic evolution of DENV-1 and DENV-2.Functional assays were employed to test the immunological properties of the polyclonal antibodies and to identify the antigenic site on E of the dominant DENV-1 and DENV-2 circulating in Malaysia that could play a significant role in neutralization escape and selection of dominant strains likely to evolve in the future.

In the present study, DENV-1 and DENV-2 antigenic evolution were investigated *in vitro*. The results and data analysis between DENV-1 and DENV-2 used in the study were reported simultaneously. However, the results and data presented are independent of each genotype, because the two genotypes have different evolutionary patterns. The results obtained from the study were inferred between the two genotypes in the course of this study.

3.1 DENV-1 AND DENV-2 E GENE CASSETTES

The following series of experiments were performed to construct gene cassettes displaying the envelope-coding regions of DENV-1 and DENV-2. Dominant strains from DENV-1 and DENV-2 were explored from the Malaysian genome-sequencing

project. The genetic variability was determined. A variable sites coding for predicted surface exposed amino acids known to influence antigenic evolution were selected. Successful mutations on these positions were created by site-directed mutagenesis and determined using Sanger sequencing. The gene cassettes were displayed as recombinant phage particles. Phages were rescued using helper phage and rescue phage was titrated. The titres were quantified by colony forming unit/viable plate count. The antigenic proteins displayed by the recombinant phage particles were expressed following infection of *E.coli* HB2151. The expressed proteins were visualized using immune blot assay. The proteins were purified using affinity gel matrix.

3.1.1 VARIABLE ANTIGENIC SITES ON E CODING REGION OF DENV-1 AND DENV-2

The dominant DENV strains were defined as the major endemic genotypes circulating in natural population. The sequences of the dominant strains presenting with one or more variable site on the predicted surface exposed amino acid were considered as an antigenic informative site. From the analysis of both DENV-1 and DENV-2 dominant strains, six antigenic informative sites each were determined across the three major antigenic domains (Table 3.1 and 3.2). The six positions were within or adjacent to DENV-1 and DENV-2 known antigenic determinants spread across the E. The amino acid sequences of the dominant dengue virus strains were shown in appendix B (DENV-1) and appendix C (DENV-2). Mutations introduced into the gene cassettes were based on these selected positions and they mimic published data and rare amino acids, amino acid infrequent in the strains that co-circulate along with the dominant strains (Table 3.1 and 3.2).

Table 3.1: Surface exposed amino acid on the E of dominant DENV-1 strains

Antigenic informative site and their amino acid residues						
Antigenic domain	EDI			EDII	EDIII	
AAs position	E161	E171	E297	E272	E305	E390
Major AAs residue	T	S	M	T	S	S
D1.36000/05/GIa	T	S	M	T	P	N
D1.11177/97/GIb	T	S	M	T	S	S
D1.59280/05/GIc	T	T	M	T	S	S
D1.10245/97/GII	I	S	I	T	S	S
D1.59266/97/GIII	I	S	V	M	L	S
Minor AAs residue	S	A	L	S	P	R

- ✓ Major AAs are amino acids found on the dominant isolates frequent during an outbreak
- ✓ Minor AAs are amino acids found on the less frequent isolates circulating with the dominant isolates during an outbreak

Table 3.2: Surface exposed amino acid on the E of dominant DENV-2

Antigenic informative site and their amino acid residues						
Antigenic domain	EDI		EDII		EDIII	
AAs position	E164	E176	E52	E71	E335	E393
Major AAs residue	I	T	H	A	I	K
D2.14281/97/Cosmopolitan	I	T	H	A	I	K
D2.14245/97/Cosmopolitan	V	T	H	A	I	K
D2.16041/98/Asian I	I	T	Q	E	V	K
D2.1704378/07/Cosmopolitan	I	T	H	A	I	K
D2.1877347/07/Cosmopolitan	I	T	H	A	I	K
Minor AAs residue	L	S	K	N	L	M

- ✓ Major AAs are amino acids found on the dominant isolates frequent during an outbreak
- ✓ Minor AAs are amino acids found on the less frequent isolates circulating with the dominant isolates during an outbreak

3.1.2 AMPLIFICATION OF E

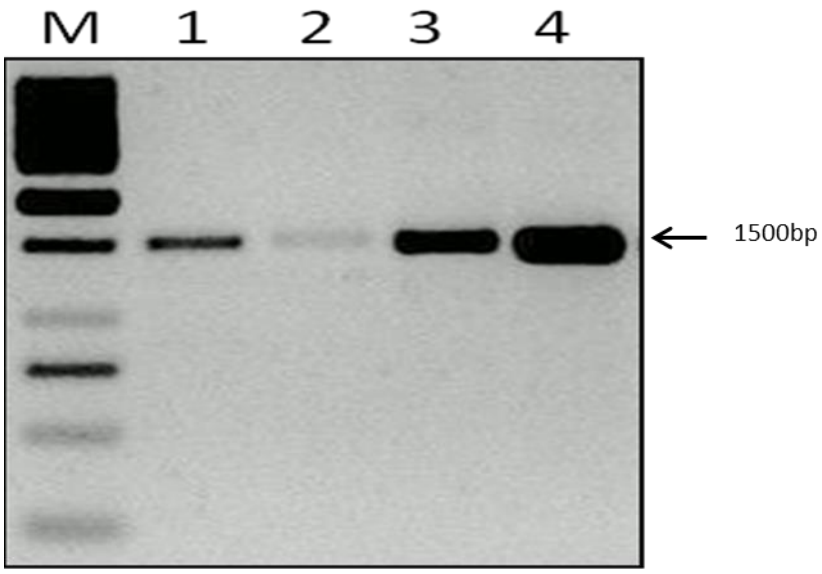
The envelope-coding regions of the virus were amplified from total RNA extracted from one of the circulating prototype of the respective dominant strains. The amplification uses combinations of degenerate primers. As shown in Figure 3.1A, all primer amplified DENV-1 and DENV-2 E (1485 bp).

3.1.3 AMPLIFICATION OF GENE CASSETTES

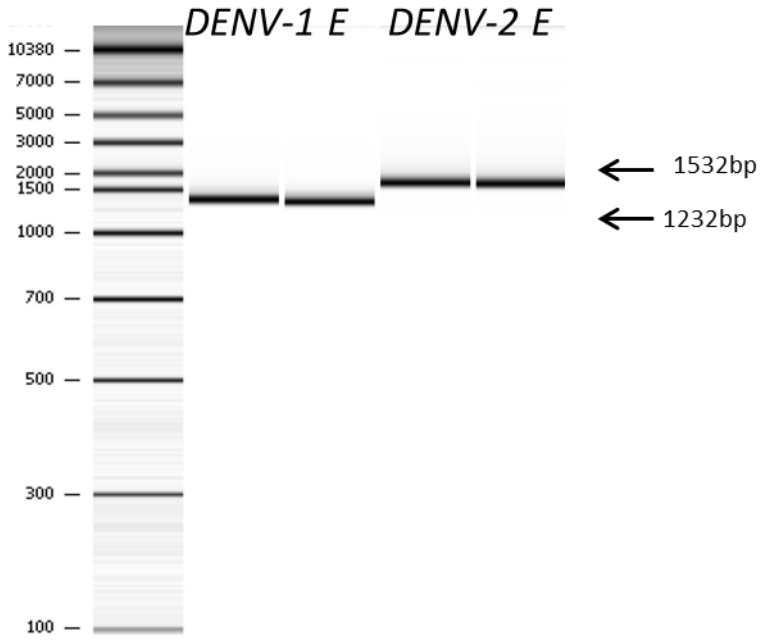
Amplification of gene construct was performed as in 3.1.2 using specific primers. The combinations amplified a 1232 bp and 1532 bp fragments corresponding to DENV-1 and DENV-2 respectively (Figure 3.1B &3.1B). The total amino acid residues for the respective gene cassettes were 403 and 505 (excluding restriction sites) with estimated molecular mass of 44 kDa and 56 kDa for DENV-1 and DENV-2, respectively. The amplified gene cassettes shown in Figure 3.1B and 3.1C contained the engineered *AlwNI* and *Bsp1208* restriction sites at N-terminal and C-terminal ends corresponding to the *sfI* and *NotI* restriction sites on pCANTAB5E phagemid vector accordingly. This enables fusion of the gene cassettes to the M13 minor g3p coat protein and displayed as antigenic proteins. There was also 24bp FLAG M2® tag fusion peptide at the 3` ends of the gene cassettes. The FLAG M2® tag was engineered to enable purification and detection of purified proteins with anti- FLAG M2® monoclonal antibody. The results showed the primers amplified the required E and the gene cassettes at expected band sizes.

Figure 3.1**.** DENV-1 and DENV-2 amplified E coding regions. The Figures show amplified genes coding for DENV-1 and DENV-2 E. (A) Amplification of genes coding for DENV-1 and DENV-2 envelope regions: M= marker, lane 1 and 2= DENV-1, lane 3 and 4= DENV-2. (B) Gel electrophoresis of amplified DENV-1 truncated E coding region from DV1.rEwt.pCANwith an approximate DNA band size of 1232 bp and DENV-2 complete E coding regions DV2.rEwt.pCANwith an approximate band size of 1532 bp in duplicate obtained using Bioanalyzer (Implen, Germany). The band varies due to RNA concentration used from 1ul to 2ul respectively.

(A)


M
1
2
3
4
1500bp

(B)


DENV-1 E
DENV-2 E
10380
7000
5000
3000
2000
1500
1000
700
500
300
100
1532bp
1232bp

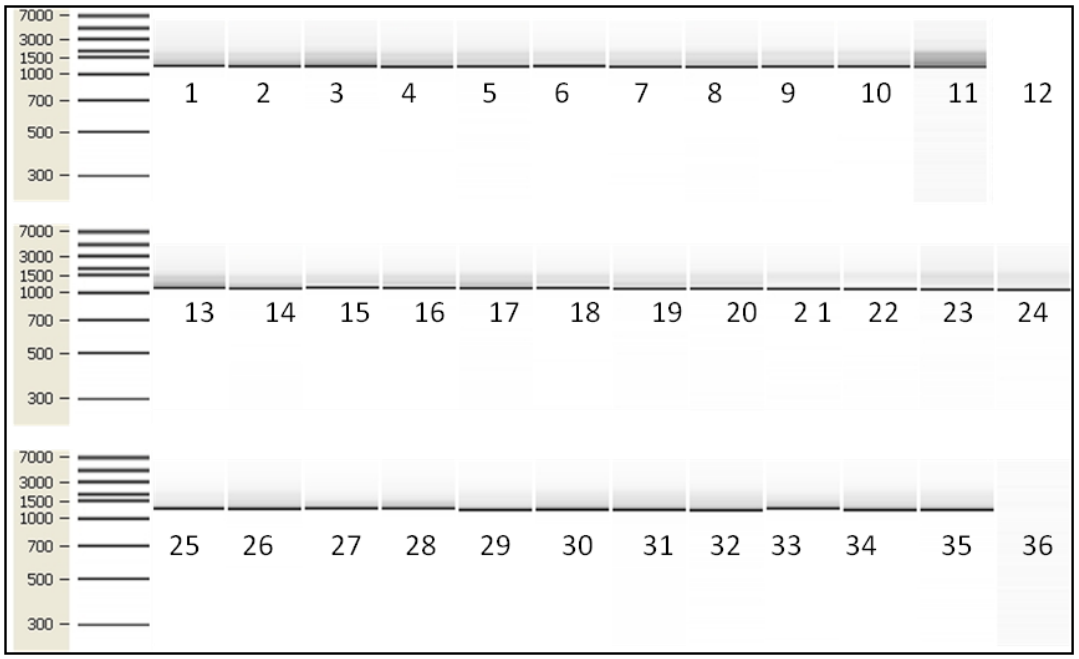
3.1.4 RECOMBINANT PHAGE MUTANTS

To generate the recombinant phage mutant, the wild type recombinant phagemid (rEwt.pCAN) were mutated by site directed mutagenesis. The recombinant phagemid displaying mutated gene was transformed into TG1 bacteria cells. To determine whether the transformed TG1 bacteria contained the recombinant phagemid with desired mutations on the gene cassette, direct colony amplification was performed on randomly selected single colonies. Gene specific primers were designed to amplify only a fragment of the DENV-1 and DENV-2 gene inserts (~ 1203 and 1503 bp). The results showed that 90 percent of the bacterial colonies screened contained a recombinant phagemid with the desired gene cassette construct at the expected sizes. The results suggest the recombinant phagemid had been successfully mutated (Figure. 3.2 A/B). Analyses of the amplified amplicons showed with the exception of clones rE171.pCAN (5) and rE390.pCAN (5) from the DENV-1, rE176.pCAN (5) andrE393.pCAN (4) from the DENV-2, all six clones tested were positive and had the correct gene insert. The confirmed positive colonies were sequenced and generated mutations were confirmed.

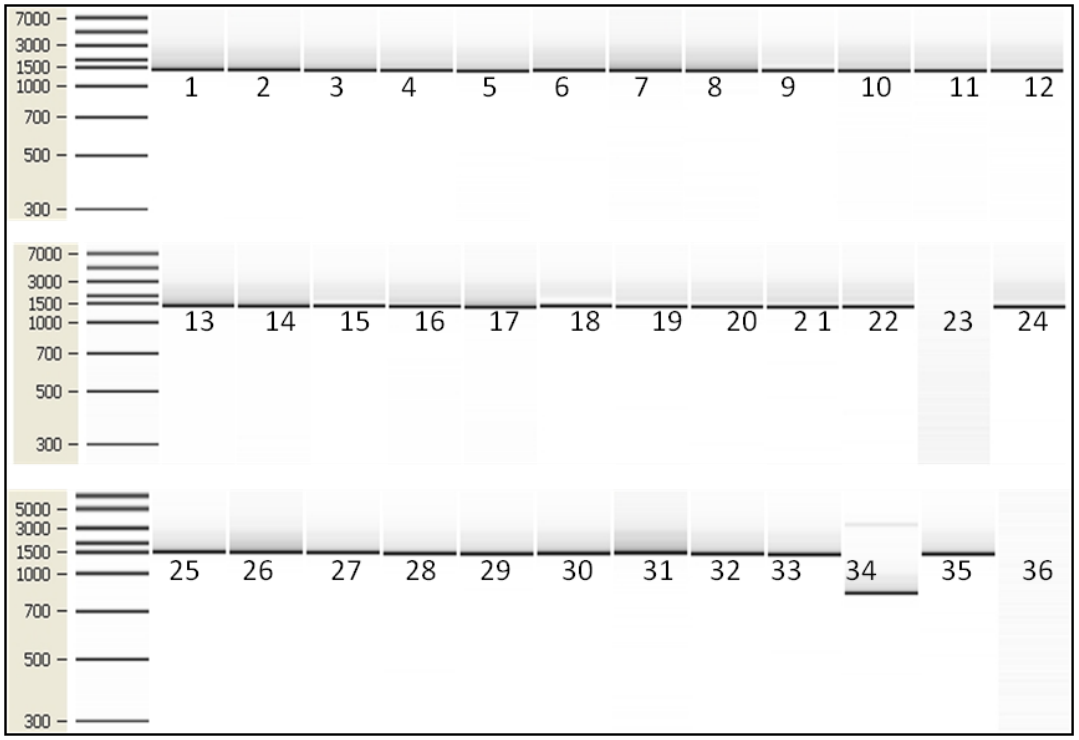
Figure 3.2. Screening of amplified amplicons after site-directed mutagenesis. (A) DENV-1 and (B) DENV-2 amplified amplicons from the recombinant phagemid after site-directed mutagenesis: The DNA size markers and expected band sizes determined using Bioanalyzer electrophoresis gel are shown on the gel picture. The test samples were labelled as follows;

Lanes	(A) DENV-1	(B) DENV-2
1-6	rE161.pCAN	rE52.pCAN
7-12	rE171.pCAN	rE71.pCAN
13-18	rE272.pCAN	rE164.pCAN
19-24	rE297.pCAN	rE176.pCAN
25-30	rE305.pCAN	rE335.pCAN
31-36	rE390.pCAN	rE393.pCAN

(A)


7000
3000
1500
1000
700
500
300
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 2 1 22 23 24
25 26 27 28 29 30 31 32 33 34 35 36

(B)


7000
3000
1500
1000
700
500
300
5000
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 2 1 22 23 24
25 26 27 28 29 30 31 32 33 34 35 36

3.1.5 SEQUENCING AND CONFIRMATION OF MUTATION

DENV sequencing and analysis was performed to identify the nucleotide sequences of each mutated position after site directed mutagenesis. As mentioned above, six surface exposed rare amino acids on the gene cassettes expressing DENV-1 and DENV-2 E were mutated. Purified phagemid from the positive colonies in 3.1.4were sequenced. They were evaluated to confirm amplification and mutation at the desired antigenic position positions. From result analysis of the sequencing results it was confirmed that the nucleotides at the selected positions and their corresponding amino acids were successfully mutated compared with the wild type recombinant phagemid with the original prototype gene. The sequence traces for the wild type and that of the mutated recombinant gene cassettes showed conserved sequences. Mutations were observed at the expected mutated amino acid positions (see appendix D (DENV-1) and appendix E (DENV-2) for chromatograms). These results confirmed a successful mutation and introduction of the single amino acid at the targeted positions on the gene cassettes of both DENV-1 and DENV-2.

3.1.6 PHAGE TITRE OF RECOMBINANT PHAGE PARTICLES DISPLAYING ANTIGENIC PROTEINS

In order to determine the wild type and mutated phage titre, the TG 1 bacteria transformed with phagemid were rescued using the M13K07 helper phage. The wild type recombinant phage particles were designated as DV1.rEwt.phand DV2.rEwt.phand their titre was determined. After phage rescue by serial dilutions and viable plate count, the titres were found to be approximately2.3 x 10^{10} and 5.4 x 10^{9} cfu/ml for DENV-1 and DENV-2 respectively.

The phage titre of the mutated phage particles from DENV-1 and DENV-2 after rescue with helper phage were determined and quantified. From the results, phage titres of the mutants were slightly lower in comparison with the wild type phages. The titre ranges from 1 x 10^{9} to 1 x 10^{10} colony forming unit per millilitre (Table 3.3). Results obtained showed that from a known amount of helper phage of 3.0 x 10^{10} cfu/ml added as the starting material to infect the transformed bacteria, 1 x 10^{9} – 1 x 10^{10}cfu/ml (30% - 80%) of the helper phage infect the transformed bacterial cells and were packaged into mature phage particles. The mean difference in the phage titre between the wild type and the mutants was 10% compared with the starting phage titre of approximately 55%. These results confirmed the successful infection of the transformed bacteria with the helper phages. An average recovery rate of 1 x $10^{9.5}$cfu/ml of phage particles in both DENV-1 and DENV-2 were packaged accordingly. The purified recombinant phage particles were used for binding tests as well as expression of the respective recombinant antigenic proteins.

Table 3.3: Quantitative phage titre of wild type and mutant phage particles after rescue with helper phages

DENV-1	DV1.rEwt.ph	rE161.ph	rE171.ph	rE272.ph	rE297.ph.ph	rE305.ph	rE390.ph	+ve ctr
Phage titre (cfu/ml)	2.3×10^{10}	5.3×10^{9}	8.2×10^{9}	4.2×10^{9}	3.2×10^{9}	7.7×10^{9}	9.2×10^{9}	2.2×10^{10}
DENV-2	DV2.rEwt.ph	rE52.ph	rE71.ph	rE164.ph	rE176.ph	rE335.ph	rE393.ph	+ve ctr
Phage titre (cfu/ml)	5.4×10^{9}	5.2×10^{8}	6.6×10^{9}	6.0×10^{9}	2.1×10^{10}	9.2×10^{9}	7.0×10^{9}	2.2×10^{10}

3.1.7 RECOMBINANT PHAGE PARTICLES EXPRESSING WILD TYPE ANTIGENIC PROTEINS

For the expression of the recombinant proteins cloned in the mid phage particles, the recombinant phage were precipitated and purified. The expression host HB2151 *E. coli*, was infected with the purified phage particles. Single colony from the infected bacteria was grown and induced by IPTG; expressed proteins were purified from the lysate prepared using a bacterial non-denaturing extraction formulation. The purity of the protein from both columns was comparable and was read to be in the range of 210µg – 275µg per litre of an induced bacterial culture (Table 3.4).

Twenty-five micrograms of the purified proteins were electrophoresed on 10% SDS-PAGE. The wild type antigenic proteins were designated rEnvWT. The expected bands were confirmed by immuno blotting using anti-FLAG M2® monoclonal antibody. As shown in Figure 3.3 A/B, the FLAG-tagged antigenic proteins were separated at the correct molecular mass of 44-kDa for DENV-1 and 56-kDa for DENV-2. The results showed the wild type DENV-1 and DENV-2 E expressed as soluble proteins, detected, and recognized by the anti-FLAG M2® monoclonal antibody (Figure. 3.3 A/B). The recombinant proteins were purified using the affinity column which bound to the FLAG M2® tag peptide engineered on the proteins.

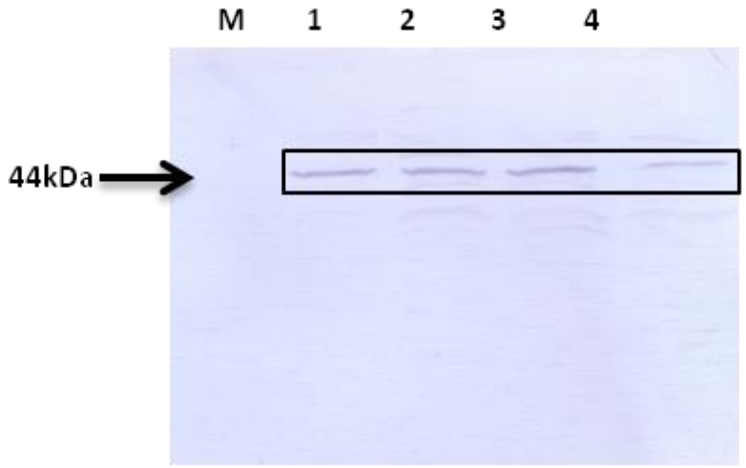
Table 3.4: The expressed recombinant protein concentrations

DENV-1	DV1.rEnvWT	rEnv161*	rEnv171	rEnv272	rEnv297*	rEnv305	rEnv390
Protein Conc.	210µg/L	-	78µg/L	196µg/L	-	254µg/L	207µg/L
DENV-2	DV2.rEnvWT	rEnv52	rEnv71	rEnv164	rEnv176	rEnv335	rEnv393
Protein Conc.	270µg/L	211µg/L	201µg/L	116µg/L	218µg/L	202µg/L	271µg/L

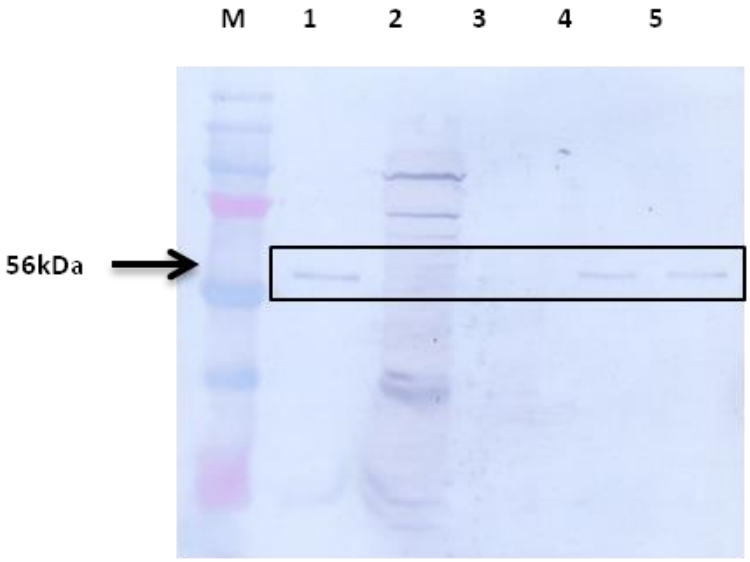
- Not successfully expressed

Figure 3.3. Expression and purification of phage displayed recombinant DENV (A) Immuno blot of DV1.rEwt expressed and displayed on phage M13 g3p attachment protein. Peroxidase conjugated anti-FLAG M2® monoclonal antibody was used as the secondary antibody. The monoclonal antibody bound specifically to the 44 kDa recombinant E antigenic proteins indicated by the arrows. (1: purified protein, 2: unpurified lysate (3 hours induction), 3: unpurified (6 hours induction), 4: uninduced lysate).). (B) Immuno blot of DV2.rEwt expressed and displayed on phage M13 g3p attachment protein. The monoclonal antibody bound specifically to the 56 kDa of the recombinant E antigenic proteins indicated by the arrow. Detection was performed as described in (a) above (1: unpurified, 2: whole cell lysate (3 hours induction), 3: uninduced lysate, 4-5 purified lysate (6 hour induction).

(A)



(B)



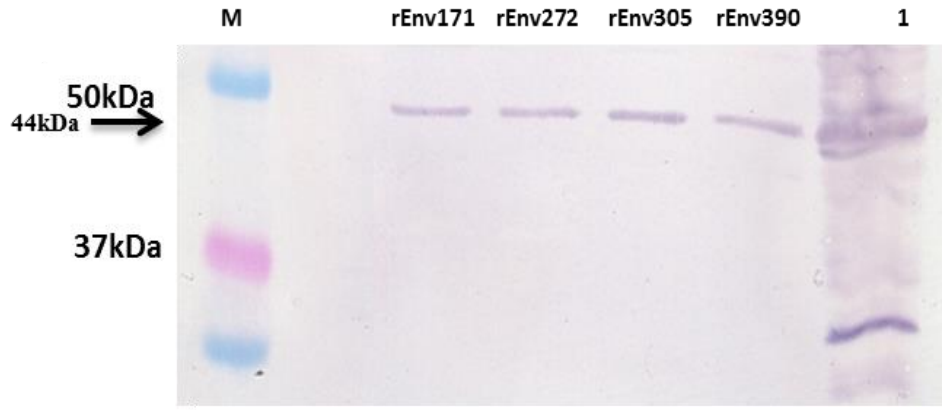
3.1.8 RECOMBINANT PHAGE PARTICLES EXPRESSING MUTANT ANTIGENIC PROTEINS

The expression of soluble proteins displayed by the mutated gene cassettes was also determined. The positive recombinant phagemid verified after site-directed mutagenesis were transformed into HB2151 *E. coli*, rescued and purified as in 3.1.7. Twenty-five micrograms of the expressed proteins were electrophoresed on 10 % SDS-PAGE. The recombinant protein was designated according to their respective amino acid positions (Table 3.4). The expected polypeptides were confirmed by immuno blotting using anti-FLAG M2® monoclonal antibody. In figure 3.4 A/B, eluted fraction contained 44kDa and 56 kDa polypeptides, which corresponded to pure recombinant protein as observed in the wild type proteins. Four recombinant Es from DENV-1 (rE171, rE272, rE305 and rE390) and all six recombinant Es from DENV-2 (rE52, rE71, rE164, rE176, rE335 and rE393) displayed, expressed and purified as a soluble protein. They were detected and recognized by the anti-FLAG M2® monoclonal antibody. The purified proteins were quantified using the micro BCA kit. The quantities of the purified proteins ranged between 78 µg to 270 µg of proteins per litre of bacterial culture. The expression efficiency varies between each clone. The proteins were used for both binding assay and generating pAb in mice. The rE161 and rE297 were not successfully expressed. Taken together, the results confirmed the expression of soluble antigenic proteins by HB2151 *E. coli* and displayed on g3p minor proteins coat of the M13K07 infected bacteriophages.

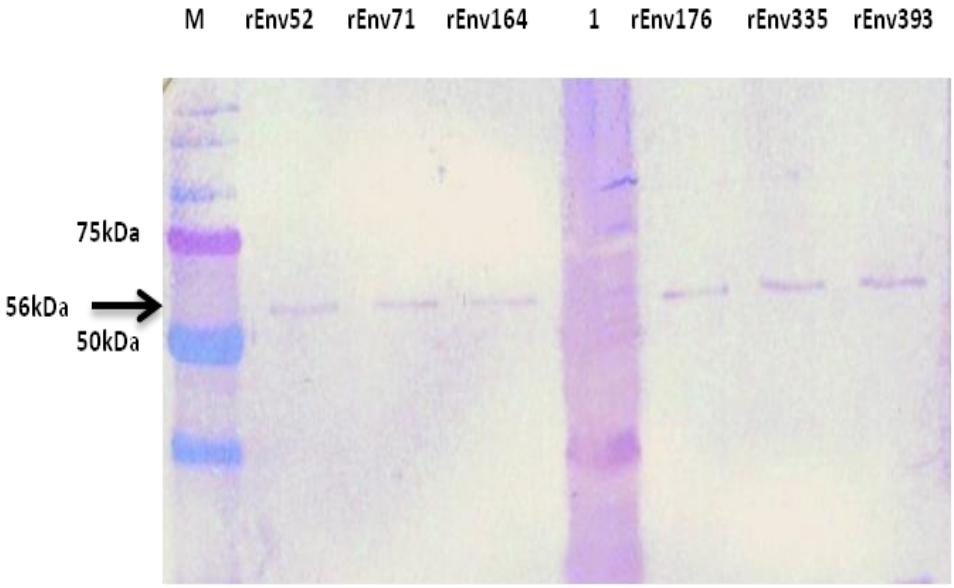
Figure 3.4. Expression and purification of recombinant E from mutated DENV-1 and DENV-2

(A) Immuno blot of mutant DENV-1 recombinant proteins expressed and displayed on phage M13 g3p attachment protein. Mutant recombinant proteins were detected by anti-FLAG M2$^{(R)}$ antibody. Arrows indicate the presence of the 44kDa recombinant protein. The monoclonal antibody binds specifically to the 44 kDa of the recombinant E antigenic indicated by the arrow (2= pooled rEnv161 and rEnv297). (B) Immuno blot of mutant DENV-2 recombinant E antigenic proteins expressed and displayed on phage M13 g3p attachment protein. The monoclonal antibody binds specifically to the 56 kDa of the recombinant E antigenic proteins indicated by the arrow (1= pooled follow through from the purified proteins). All purified proteins were indicated by their designated names.

(A)


M
rEnv171
rEnv272
rEnv305
rEnv390
1
50kDa
44kDa
37kDa

(B)


M
rEnv52
rEnv71
rEnv164
1
rEnv176
rEnv335
rEnv393
75kDa
56kDa
50kDa

3.2. CHARACTERIZATION OF RECOMBINANT PHAGE DISPLAYING MUTATED CLONES

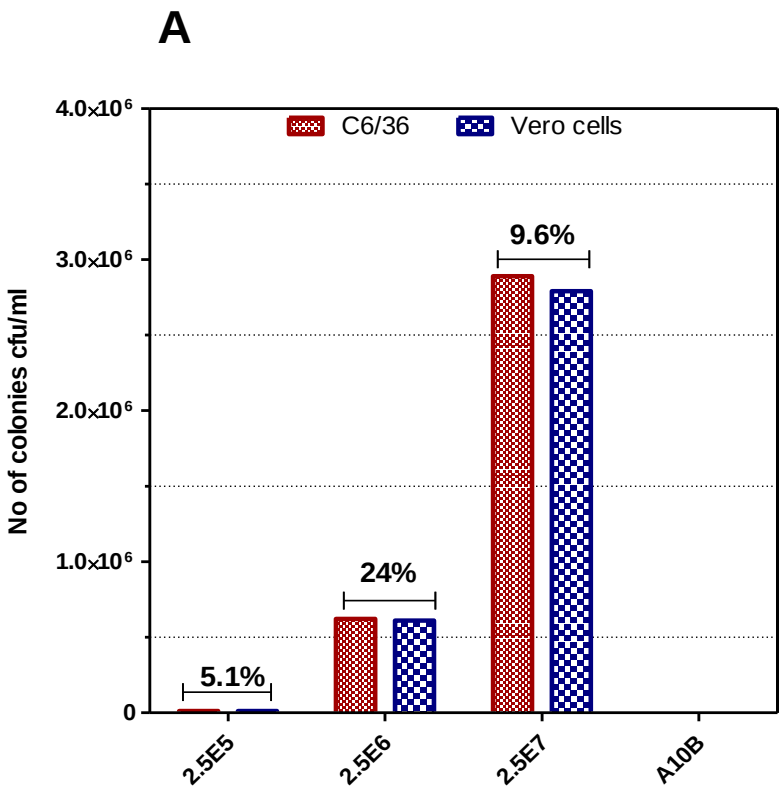
Having successfully generated the starting materials for the proposed study, the following series of binding studies were performed. Firstly, to determine the optimum binding of the wild type and mutated phage particles by bacteria colony forming count/viable plate count. Secondly, to determine the effects of altered amino acids on binding affinity and specificity of the phage particles to mosquito (C6/36) and mammalian cells. Finally to evaluate the binding affinity of recombinant proteins and dengue virions against polyclonal antiserum generated in mice immunized with the respective recombinant proteins. The binding was assessed using ELISA method. The objectives of this experiment are to establish the role of the altered amino acid positions on the recombinant mutants on receptor binding to C6/36 and Vero cells.

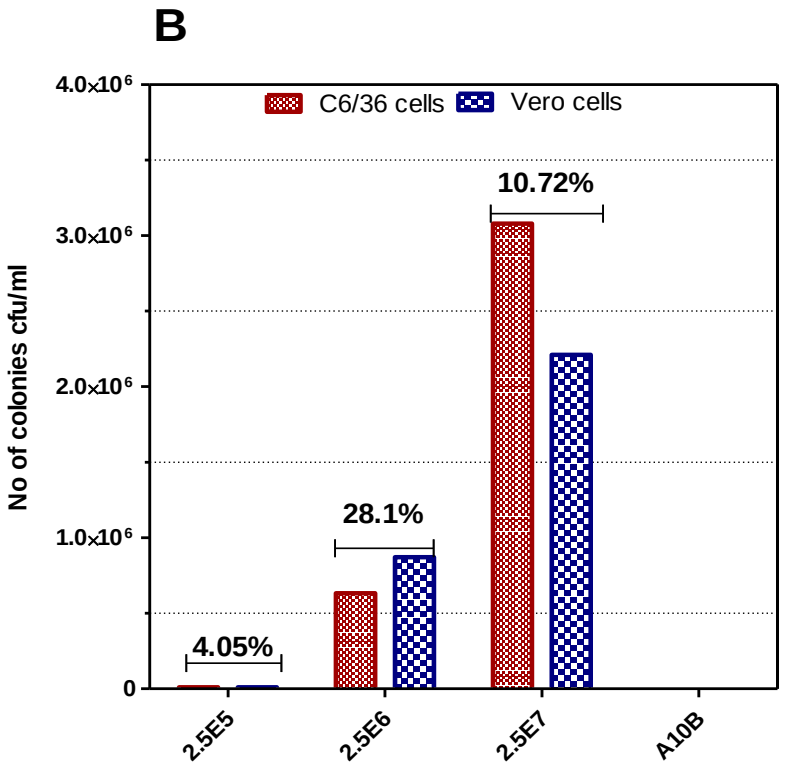
3.2.1 OPTIMUM BINDING CONCENTRATION OF RECOMBINANT PHAGE PARTICLES

Binding studies were first performed to determine the optimum titre of the recombinant phage particles that would bind to C6/36 and Vero cell lines. Following incubation of the wild type recombinant phage particles with C6/36 and Vero cell lines using three different phage titres; 2.5 x 10^5 cfu/ml, 2.5 x 10^6 cfu/ml and 2.5 x 10^7 cfu/ml; the recombinant phage particles recovery varies as the titre of the phage particles differed across the two cell lines (Figure 3.5A/B). An estimated average percentage of phage titre recovery, which bound to the targeted cell receptors on C6/36 and Vero cell lines in increasing order, was 5.1%, 24% and 9.6% for phage displaying DENV-1 and 4.05%, 28.1% and 10.72% for DENV-2 accordingly. The binding was relatively similar in both C6/36 and Vero cells with no significant differences. The only notable difference between the cell lines were observed at the maximum phage titre of 2.5 10^7 cfu/ml in DENV-2. From the results, the optimum phage titre on the C6/36 and Vero cells was

2.5×10^6 cfu/ml. The 2.5×10^6 cfu/ml was used throughout the subsequent binding assay.

Figure 3.5**.** Binding of (A) DV1.rEwt.ph and (B) DV2.rEwt.ph.C6/36 cell lysate coated onto 96-well plates were used. The cells were fixed using paraformaldehyde. Initially cells were incubated with 2.5 x 10^5, 2.5 x 10^6 and 2.5 x 10^7 cfu/ml, for 2 hours at 37°C. TG1 bacteria (mid-log) were added and further incubated for 30 minutes at 30°C after vigorous washing with 1x PBS buffer to remove unbound phage. The binding was quantified using colony forming count/viable plate count and expressed as colony count per unit (cfu). The control used 2.5 x 10^6 cfu/ml of A10B. Binding of truncated DENV-2 E fragments displayed on M13 phages.

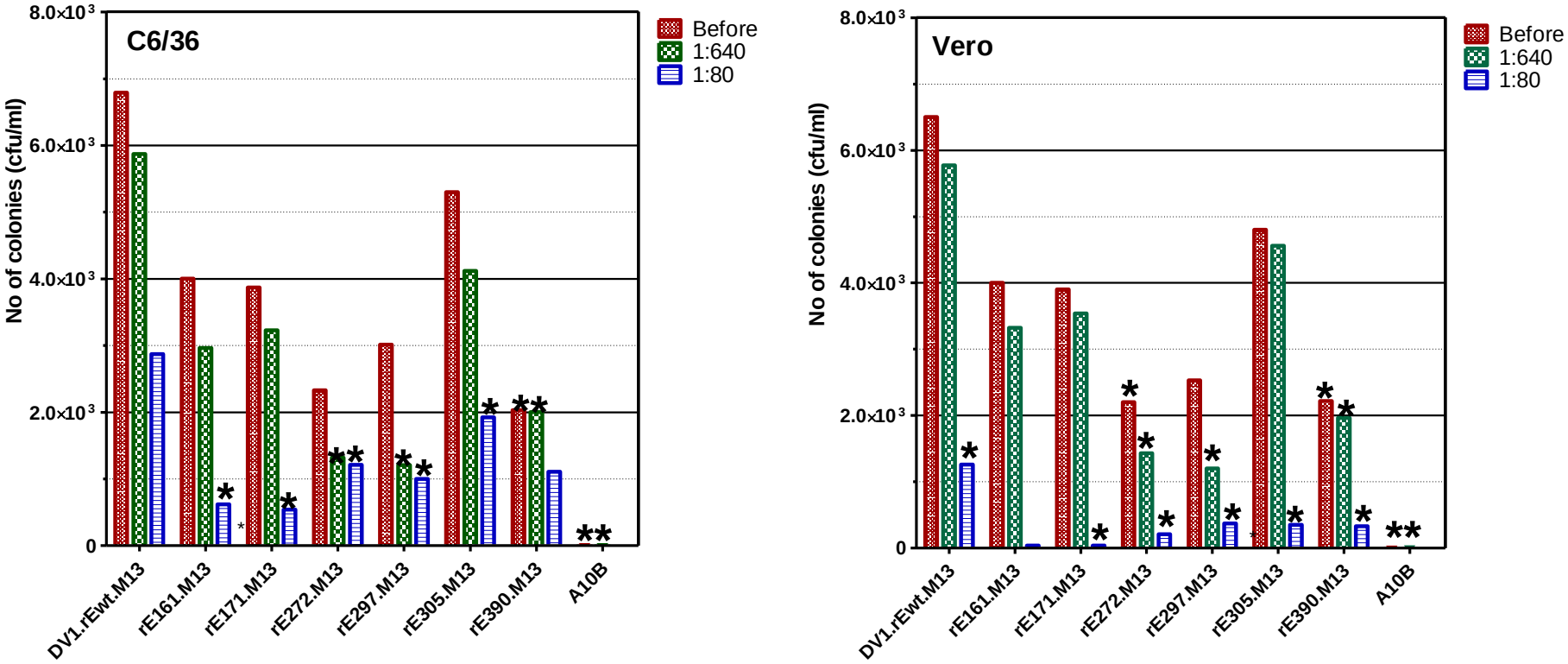

A
4.0x10⁶
3.0x10⁶
2.0x10⁶
1.0x10⁶
0
No of colonies cfu/ml
C6/36
Vero cells
5.1%
24%
9.6%
2.5E5
2.5E6
2.5E7
A10B


B
4.0x10⁶
3.0x10⁶
2.0x10⁶
1.0x10⁶
0
No of colonies cfu/ml
C6/36 cells
Vero cells
4.05%
28.1%
10.72%
2.5E5
2.5E6
2.5E7
A10B

3.2.2 EVALUATION OF BINDING ASSAY OF RECOMBINANT PHAGE PARTICLES

DENV-1: The mutations on E are specific on each individual clone. Their effects were investigated. To this aim, the binding of DENV-1 recombinant phage on C6/36 and Vero cell lines were evaluated. 2.5×10^6 pfu/ml of purified recombinant phage particles were added to fixed cells in 96 well plates and unbound phage were vigorously washed and mid-log bacteria were added and spread onto 2×YT-AG plates overnight at 30°C. The phage binding to two DENV-1 susceptible cells lines, C6/36 and Vero did not differ significantly (Figure 3.6). All E mutant phage displayed reduced binding compared to the DV1.rEwt.ph in both cell lines. The binding efficacies of rE272.ph and rE390.ph to both C6/36 and Vero cells were reduced significantly compared to DV1.rEwt.ph. Although the binding efficacies of rE161.ph, rE297.ph and rE305.ph were also reduced compared to DV1.rEwt.ph, the differences were not statistically significant, suggesting that mutation at these positions did not affect binding. No measurable binding with negative control (A10B) was observed. These results demonstrated that the binding affinity and interaction of rE272.ph and rE390.ph to C6/36 and Vero cell receptors were affected by the alteration of single amino acid at these positions on the DENV-1 recombinant E gene.

Figure 3.6. Binding affinity of DENV-1 recombinant phage particles to C6/36 and Vero cells: Binding of the phage particles to the cells before precipitation and after phage precipitation with pooled polyclonal antibodies from the wild type and recombinant E at 1:80 and 1:640 dilutions. Cell viable count was used and average mean differences were calculated using ANOVA followed by Tukey's multiple comparisons for each sample with DV1.rEwt.ph before precipitation. (*) Denotes level of significance at $p<0.05$.



* Significant at p <0.05

Since the primary function of the neutralizing antibodies is to bind to antigenic determinant and block proteins binding to cell receptors, we next assessed how the pAb generated against the recombinant phage displaying the E mutant will affects binding to insects and mammalian cells. The pAb generated from the wild type and mutant E were each pooled and used to precipitate the recombinant phage at 1:80 and 1:640 dilutions. The specificities of these interactions were assessed, for the recombinant phages, by comparing against the binding efficacies of DV1.rEwt.ph prior to precipitation. After precipitation with the pooled polyclonal antibodies at 1:640 dilutions, all recombinant phage displayed reduced binding to C6/36 cells (Figure3.6). However, only rE272.ph and rE390.ph showed significant reduction. Similarly, rE272.ph and rE390.ph exhibited significantly reduced binding compared to rEwt.ph binding to Vero cells. At 1:80 dilutions, all recombinant phages showed up to four-fold reduction in the binding to both cells, except for rEwt.ph binding to Vero cells. These results suggest that the pooled pAb could block and reduce the binding of the DV1.rEwt.ph and the mutated E, specifically rE272.ph and rE390.ph from binding to C6/36 and Vero cells.

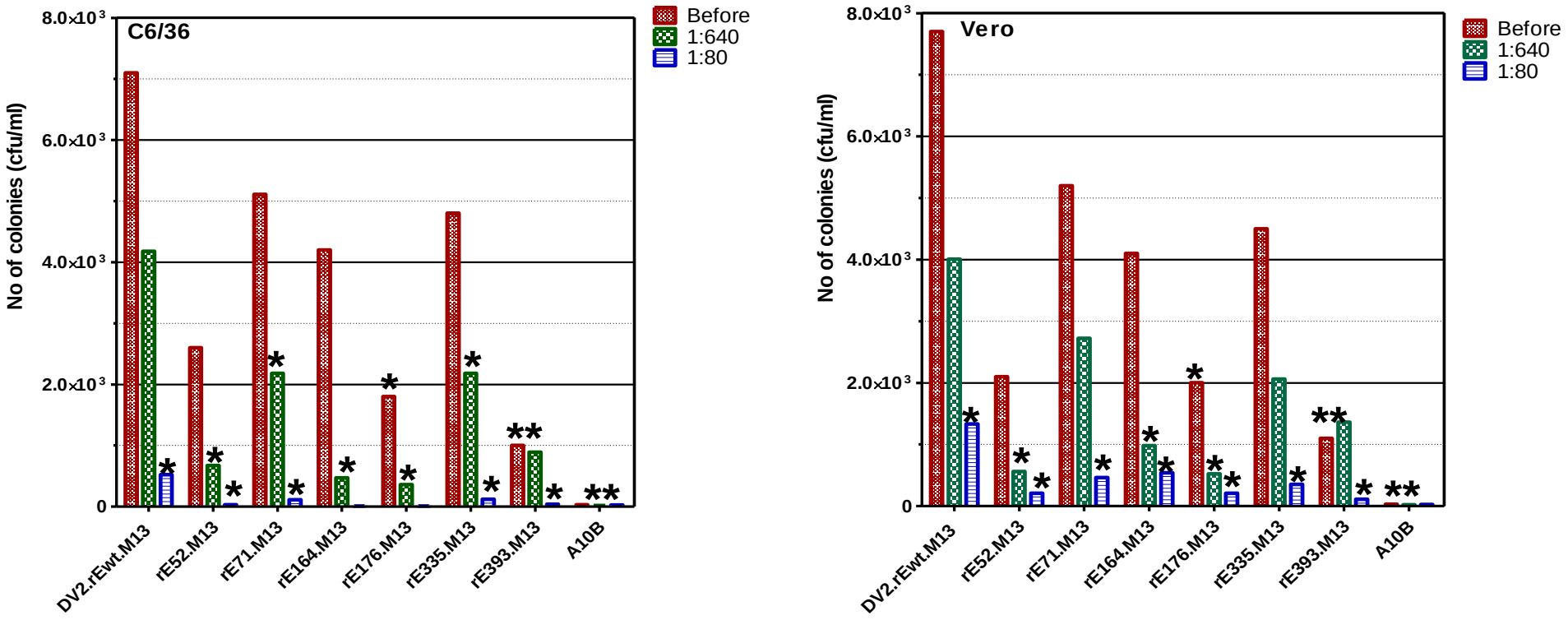
DENV-2: In a similar setting, binding using DENV-2 recombinant phage to fixed C6/36 and Vero cells was evaluated. The phage binding to two DENV-2 susceptible cells lines, C6/36 and Vero did not differ significantly (Figure 3.7). From the results, rE71.ph, rE164.ph and rE335.ph mutants displayed highest binding compared to the DV2.rEwt.ph recombinant phage binding to both cell lines. The binding of rE176.ph and rE393.ph to both C6/36 and Vero cells were significantly reduced compared to DV2.rEwt.ph. No measurable binding with negative control (A10B) was observed. These results demonstrated that the binding affinity and interaction of

rE52.ph, rE176.ph and rE392.phto C6/36 and Vero cells were affected by the alteration of single amino acid at the respective positions on the DENV-2E cassettes.

The DENV-2 recombinant phage particles were precipitated with the pooled pAb generated against the displayed proteins. The specificities of these interactions were assessed by comparison with the DV2.rEwt.ph prior to precipitation. However, in DENV-2, after precipitation with 1:640 dilutions of the pooled polyclonal antibodies, two-fold reduction in the number of colonies formed at ≤1:640 were observed (Figure 3.7). All recombinant phage displayed showed four-fold reduction at 1:80 dilutions for both cell lines. The rE71.ph, rE164.ph and rE335.ph did not exhibited significantly reduced binding compared to DV2.rEwt.ph before precipitation, but significantly reduced after precipitation with pAb (Fig. 3.7). These results suggest that the pooled pAb could block and reduce the binding of the recombinant phage displaying the wild type and E mutants, especially rE71.ph, rE164.ph and rE335.ph from binding to C6/36 and Vero cells.

Taken as a whole, these results showed binding of the phage particles expressing DENV-1 and DENV-2 E was affected by the mutations introduced at certain positions of the DENV recombinant E.

Figure 3.7. Binding of DENV-2 recombinant phage particles to C6/36 and Vero cells: Binding of the phage particles to the cells before precipitation and after phage precipitation with pooled polyclonal antibodies from the wild type and recombinant E at 1:80 and 1:640 dilutions. Bacterial cell viable count was used and average mean differences were calculated using ANOVA followed by Tukey's multiple comparisons for each sample with DV2.rEwt.ph before precipitation. (*) Denotes level of significance at $p<0.05$.



* Significant at p <0.05

3.2.3 REACTIVITY OF DENV ANTIGEN WITH POLYCLONAL ANTIBODY

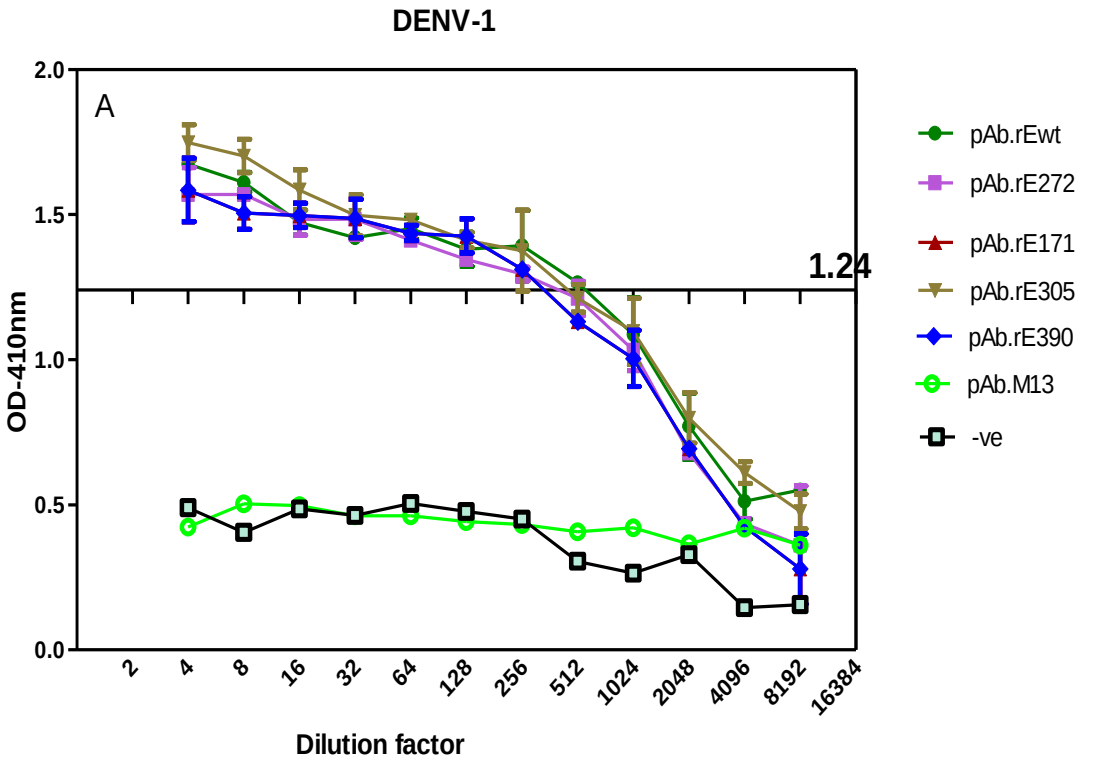
The recombinant phage particles were able bind to mosquito and mammalian cell lines. The binding was affected when amino acids were altered. Precipitating the phages with antibodies showed statistically reduced binding of the phages. Next, BALB/c mice were immunized with the purified recombinant proteins to assess the specific antibody response against the E mutants. This is to test whether the mutations affects recombinant proteins binding to pAb. The results are shown in figure 3.8 A/B. Overall, the recombinant E from DENV-1 induced high immune responses in BALB/c mice, producing pAb as detected by ELISA (Figure 3.8A). The DV1.rEnvWT produced antibodies at titre up to 1:1024 dilution above the ELISA cut-off value of 1.24±2SD. Amongst the recombinant E mutants, the pAb from the mice immunized with rEnv305 showed a slightly higher titre value at 1:256 serum dilutions compared to rEnv272, rEnv171 and rEnv390 (all at >1:256 respectively). Hence, the read antibody responses induced from DV1.rEnvWT and mutants were at comparable levels. The negative control containing recombinant phage particles expressing the gene cassette alone (pAb.M13) did not induce detectable immune response in the BALB/c mice.

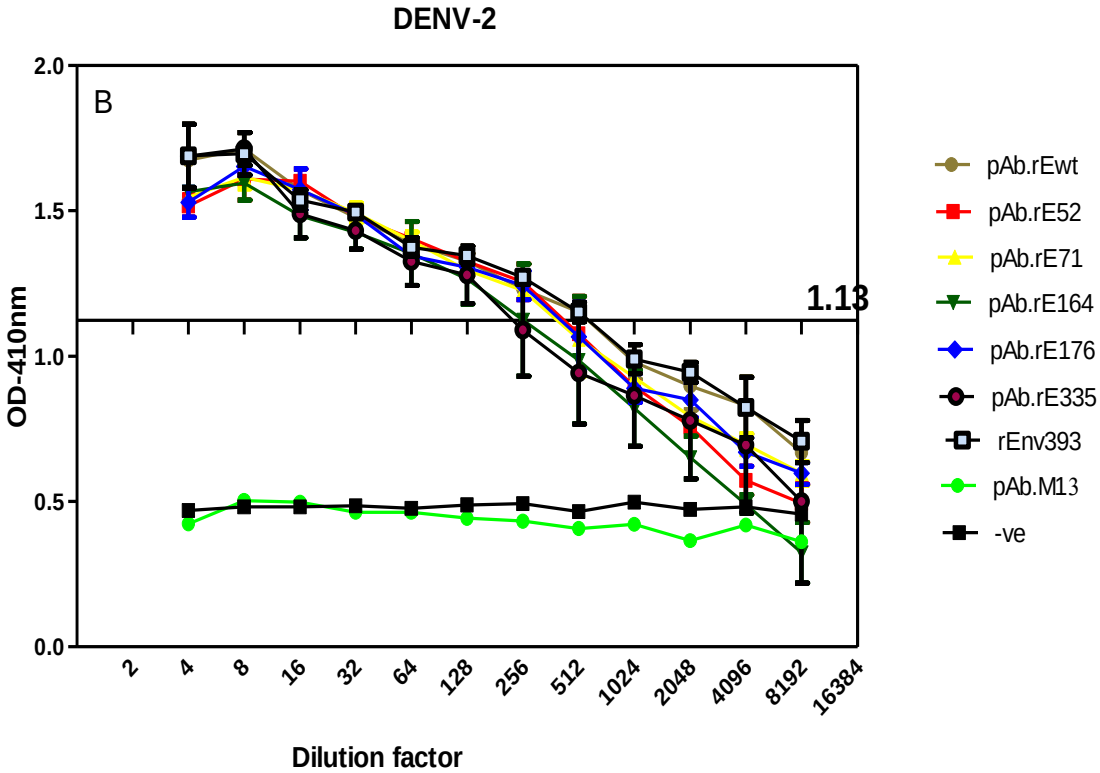
Similarly, immune responses against the recombinant E from the DENV-2 induced high immune responses in BALB/c mice as detected above the ELISA cut-off value of 1.13±2 SD. The pAbs titre were in the range of >1:256, >1:512, >1:512, 1:128, >1:256, 1:512 and 1:256 for pAb.rEnv52, pAb.rEnv71, pAb.rEnv164, pAb.rEnv176, pAb.rEnv335 and pAb.rEnv393respectively (Figure 3.8B).

The result suggests that the DENV-1 and DENV-2 recombinant antigenic proteins displayed by the phage particles induced immune responses in BALB/c mice. It is

apparent also, that the recombinant phage particles expressing the gene cassettes does not induced detectable immune response in BALB/c mice. The immune response was significant at $p<0.05$ and below the ELISA cut-off value of 1.24±2 SD and 1.13±2SD for DENV-1 and DENV-2 respectively.

Figure 3.8. Dose response curve of pAb in BALB/c mice immunized with the (A) DENV-1 and (B) DENV-2 wild type or mutant recombinant E. Sera from 4 mice per group were collected 1 week after last immunization and tested by ELISA. Plates were coated with wild type E and immobilized with pAb against wild type and mutant E. Antibody titres were expressed as the average mean OD of negative samples ± 2 SD. The ELISA cut-off value was 1.24±2SD and1.13±2SD for (A) DENV-1 and DENV-2 respectively. The values shown are means of duplicate assays. pAb.M13 is pooled recombinant phage particles and (-ve) represents serum samples from non-immunized mice.


DENV-1
A
OD-410nm
2.0
1.5
1.0
0.5
0.0
1.24
2
4
8
16
32
64
128
256
512
1024
2048
4096
8192
16384
Dilution factor
pAb.rEwt
pAb.rE272
pAb.rE171
pAb.rE305
pAb.rE390
pAb.M13
-ve


DENV-2
B
OD-410nm
2.0
1.5
1.0
0.5
0.0
1.13
2
4
8
16
32
64
128
256
512
1024
2048
4096
8192
16384
Dilution factor
pAb.rEwt
pAb.rE52
pAb.rE71
pAb.rE164
pAb.rE176
pAb.rE335
rEnv393
pAb.M13
-ve

3.2.4 BINDING AFFINITY OF ANTISERA AGAINST DENV

The pAb generated by immunizing mice with recombinant protein may not bind to the dominant DENV-1 and DENV-2 strains since they were generated using recombinant E with mutations at the antigenic regions. To identify the antigenic determinants the pAb binding sites were mapped by determining the binding to DENV-1 and DENV-2 strains. The aim is to examine the mutated residues leading to reduced or loss of binding to DENV-1 and DENV-2.

In DENV-1, the neutralization efficiency of the pAb varied across the different DENV-1 strains tested (Figure 3.9A). The pAb from DV1.rEwt displayed reduced neutralization to two DENV-1 strains: the D1.59266 and D1.59280. The pAb.rE272 and pAb.rE390 also exhibited significantly decreased neutralization to D1.59266 and D1.59280. The pAb recognized their corresponding antigenic proteins with high affinity. The results indicate that mutations at E272 and E390 reduced the binding of the pAb generated to the antigenic determinant in DENV-1 (D1.59266 and D1.59280 genotypes). The pAb.rE171 and pAb.rE305 displayed efficient binding to all of the DENV-1 isolates tested, suggesting that mutation at these residues were antigenically less crucial. There was significantly reduced binding when the polyclonal antibody were mapped against the D2.14281 genotype.

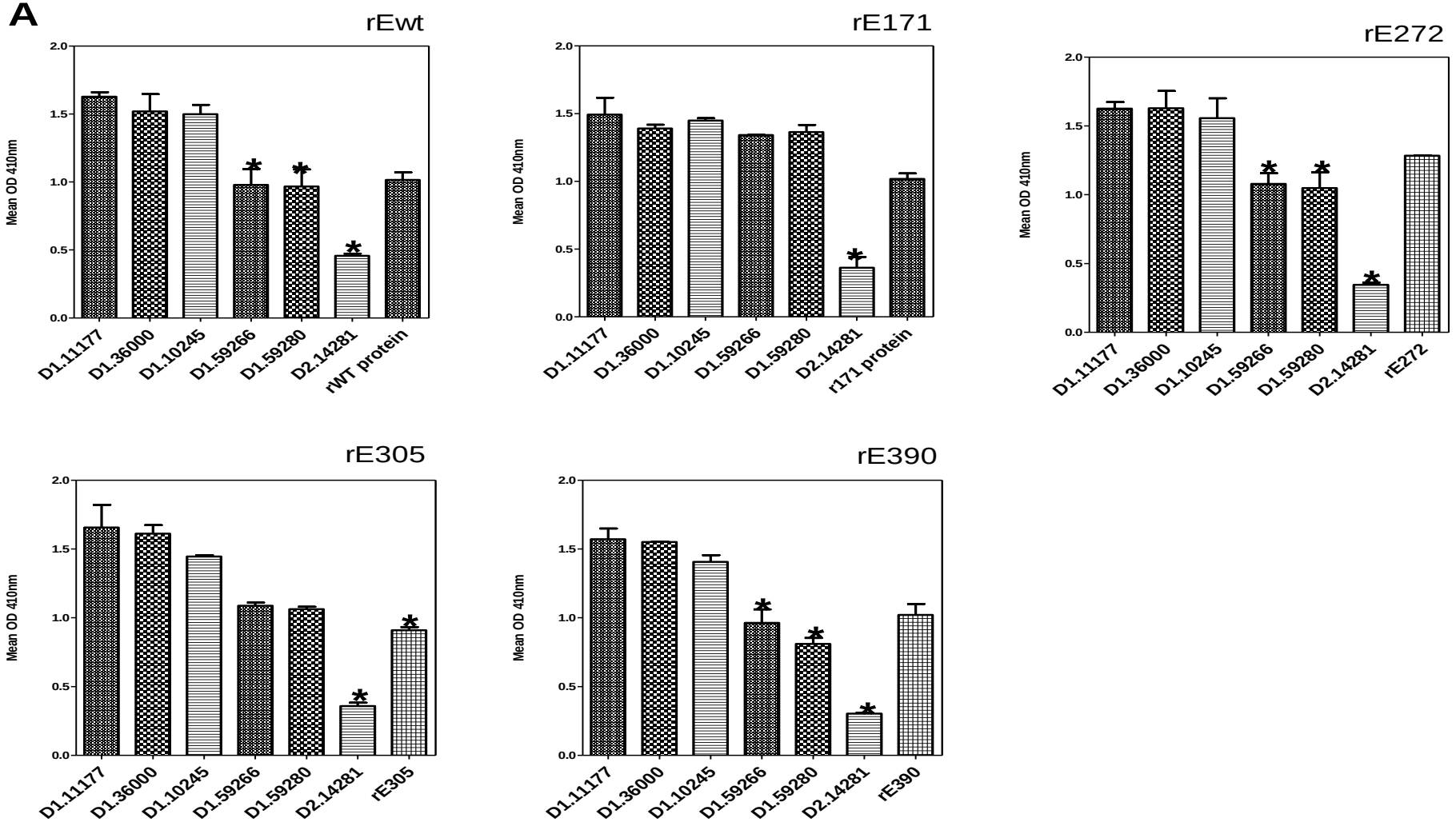
Using similar approach, the binding efficiency of the pAb varied across the different DENV-2 strains tested (Figure 3.9B). The pAb.DV2.rEwt displayed significant binding to all DENV-2 strains. The pAb.rE52, pAb.rE164 and pAb.rE176 displayed reduced binding when mapped against DENV-2 (D2.16041) Asian 1 strain (Figure 3.9B). The pAb.rE164 and pAb.rE335 also exhibited significantly decreased binding to (D2.1877347) Cosmopolitant strain. Consequently, the pAb recognized their corresponding antigenic proteins with high affinity. But, pooled pAb raised from

recombinant DENV-1 E did not bind to any of the DENV-2 isolates, suggests that the pAb rose using the DENV-2 E here are not cross-reactive with DENV-1. Therefore, the results here suggest that mutations on rE52, rE164 and rE176 affected the binding of the pAb to the antigenic determinant on D2.16041 genotype. Subsequently, mutations generated on rE164 and rE335 affected binding to D2.1877347 strain. The pAb.rE71 and pAb.rE393 displayed efficient binding to all of the DENV-2 isolates tested, suggesting that mutation at these residues were antigenically less crucial.

In a competitive binding ELISA of equal ratio of DENV-1 and DENV-2 virion binding to polyclonal antibody DENV-2 virion does not alter the affinity of DENV-1 pAb binding towards antigenic determinant on DENV-1. The result was same as the result on 3.2.4 as shown in figure 3.9A/B. The same was observed in DENV-2 binding in the presence of DENV-1.

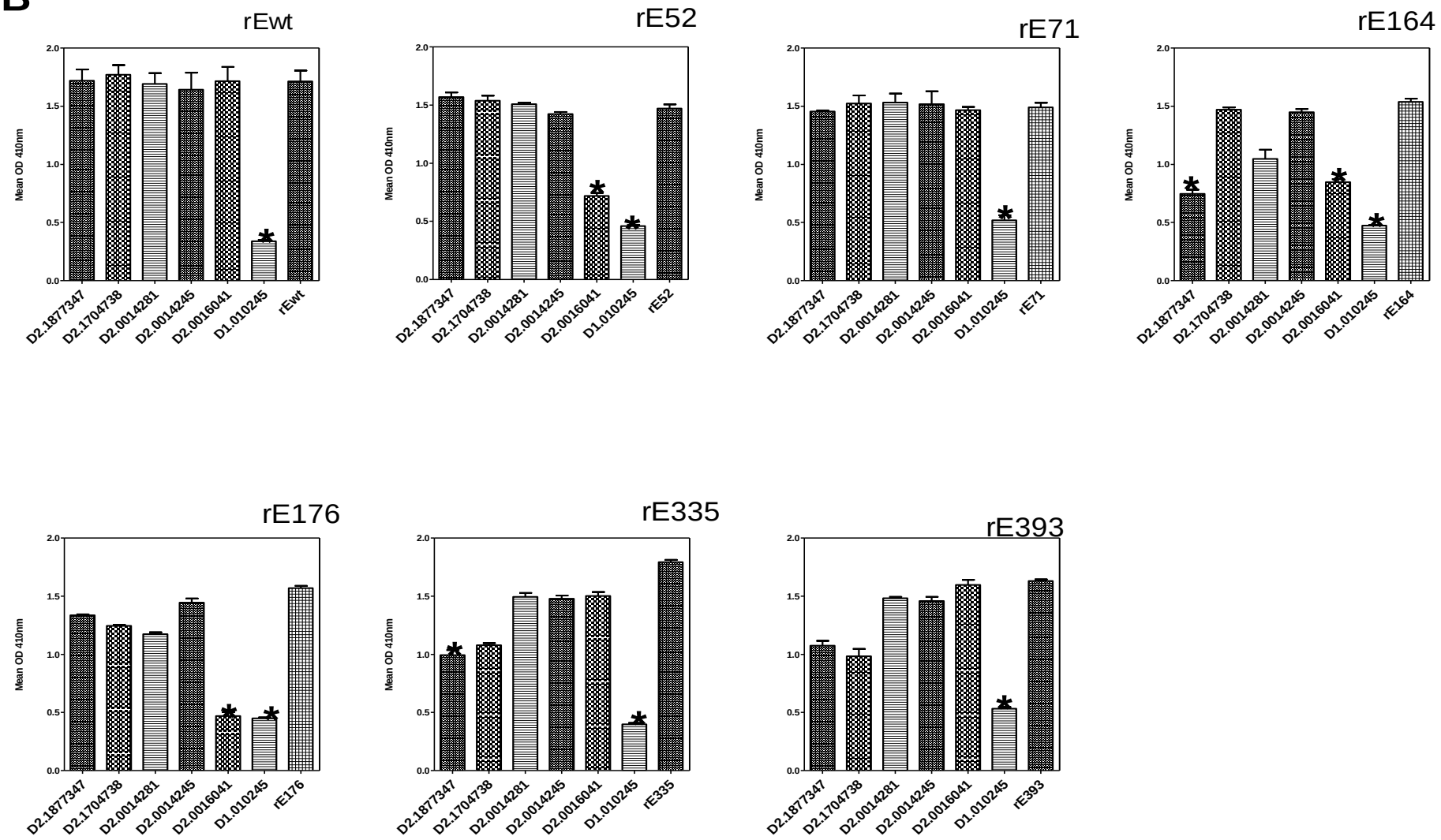
Figure 3.9A/B**.** Binding of pAb to DENV: The binding was determined using ELISA. Briefly, (A) DENV-1 and (B) DENV-2 virions were coated onto 96-well plate followed by immobilization with the indicated pAb. The corresponding antigenic proteins were used as positive control. Data represent mean of SEM of the triplicates. The data were analysed using Tukey's multiple comparisons test. (*) Denotes significant difference ($p<0.05$).

A



* significant at p<0.05

B



* significant at p<0.05

3.3 NEUTRALIZATION ASSAY

The results presented so far demonstrated that mutations at positions E272 and E390 of the DENV-1 E affected binding of the respective recombinant phages to C6/36 and the Vero cells. The same mutation generated pAb that had reduced binding to two of the dominant DENV-1 strains. In DENV-2, mutations at E52, E164 and E 393 affected phage binding to C6/36 and Vero cells. On the contrary, binding of pAb generated using recombinant E52, E164 and E176 affects binding on Asian I strain and E164 and E335 on one of the Cosmopolitan strain of the dominant DENV-2. Thus, these mutations on E affected the respective antibody binding to some, but not all the dominant dengue strain of the same serotypes. In the following investigation microneutralization assay was performed to determine the neutralizing effects of the pAbs against DENV-1 and DENV-2 *in vitro* using C6/36 cells. The microneutralization results were validated using Focus Reduction Neutralization Test ($FRNT_{50}$) on Vero cells.

3.3.1 MICRONEUTRALIZATION CAPACITY OF POLYCLONAL ANTIBODIES AGAINST DOMINANT DENV STRAINS

The investigation aimed to determine possible DENV strains that could evade antibody neutralization in the future. The results from Microneutralization/$FRNT_{50}$ assays are shown in Table 3.5. Microneutralization result performed on C6/36 was presented as the highest pAb titre whereas the $FRNT_{50}$ results using Vero cells were presented as the percentage of the total number of foci formed corresponding to the highest titre on microneutralization. From the result, the pAbD1wt and the mutant recombinant E from DENV-1 neutralized and blocked infection of D1.11177, D1.10245, and D1.36000 to the C6/36 cell line (Table 3.5). The dilution varied across pAbs as well as between the strains; the range was from 1:80 to 1:640 antibody titres.

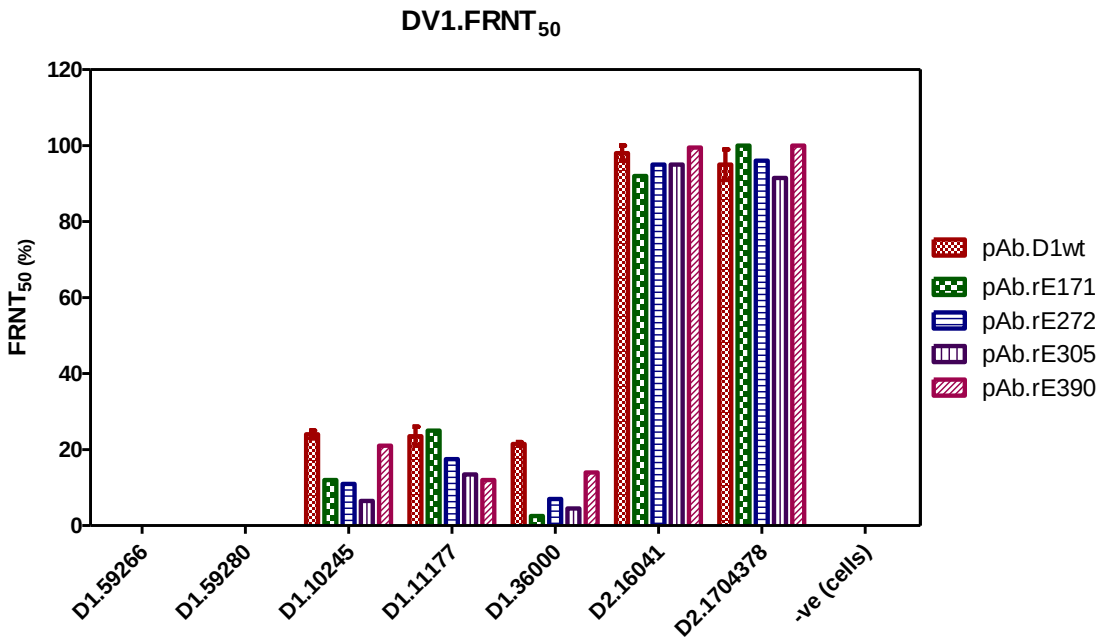
In the Focus Reduction Neutralization Test on Vero cells similar effects were observed when compared with the corresponding microneutralization assay results (Table 3.5, Figure 3.10). The reduction in the number of foci is equivalent to two-fold reduction (74% – 95% foci reduction) of the virus titre of D1.11177, D1.10245, and D1.36000 in the presence of all the pAbs ($FRNT_{50}$). However, D1.59266 and D1.59280 were completely neutralized by all the pAbs tested at pAb titre >1:320 – 1:640. An unrelated polyclonal serum antibody was included as negative control that did not neutralize any of the DENV strains. The pAbs raised against DENV-1 antigenic proteins did not block DENV-2 infections even at increased serum concentration (1:40) (data not shown). These results suggest that the pAbs generated using the recombinant DENV-1 E had neutralizing effects by inhibiting D1.59266 and D1.59280 completely and the D1.11177, D1.10245 and D1.36000 at varied serum antibody ≥1:180.

Table 3.5: Microneutralization and Foci Reduction Neutralization Test$_{50}$ of polyclonal serum antibodies generated using the DENV-1 recombinant E

Strain / **E domain**		**EDI**	**EDII**	**EDIII**		
Polyclonal serum	pAb.D1wt	pAb.rE171	pAb.rE272	pAb.rE305	pAb.rE390	Genotypes
D1.59266/87	1:320/1	1:640/0	1:320/6	1:640/0	1:640/6	GIII
D1.59280/87	1:640/7	1:320/1	1:320/5	1:640/0	1:320/1	GIa
D1.10245/97	1:80/23	1:80/10	>1:80/10	>1:80/5	>1:80/19	GII
D1.11177/97	1:160/26	1:160/23	1:320/16	1:320/14	1:320/12	GIb
D1.36000/05	1:160/22	1:320/4	1:640/7	1:640/6	1:640/12	GIc
D2.16041	>1:20/100	>1:20/97	>1:20/95	>1:20/100	>1:20/100	Asian I
D2.1704378	>1:20/98	>1:20/97	>1:20/89	>1:20/97	>1:20/99	Cosmopolitan
-ve control	<1:20/00	<1:20/00	<1:20/00	<1:20/00	<1:20/00	

- The microneutralization and FRNT results are presented as (microneutralization/FRNT$_{50}$)
- Microneutralization results are presented as highest titre of serum dilution
- FRNT$_{50}$ presented as percentage of foci formed corresponding to the highest titre of pAb observed in microneutralization
- Polyclonal serum antibody was generated in mice following inoculation with respective recombinant DENV E

Figure 3.10. $FRNT_{50}$ of mouse pAb against dominant DENV-1: The graph explains the Foci Reduction Neutralization Test as presented in Table 3.5. The results correspond to the pAb titre observed in microneutralization assay. The bars represent the percentage in total number of foci and expressed as reduction in foci count.


DV1.FRNT50
FRNT50 (%)
120
100
80
60
40
20
0
D1.59266
D1.59280
D1.10245
D1.11177
D1.36000
D2.16041
D2.1704378
-ve (cells)
pAb.D1wt
pAb.rE171
pAb.rE272
pAb.rE305
pAb.rE390

In the following study the binding inhibitory studies of pAb to block dominant DENV-2 strains in Malaysia was investigated. DENV-1 was used to serve as control and look for any cross-reactivity against DENV-2 pAbs. Results shown in Table 3.6 suggests that the pAb.D2wt, pAb.rE52 and pAb.rE71 from EDII could not block or neutralize the Asian I strains. The pAb.rE164 and pAb.E176 all on EDI failed to neutralize virus at any given concentration. The pAbs generated against the EDII and EDIII domain of the mutated E of DENV-2 neutralized and blocked infection of all dominant Cosmopolitan strains (Table 3.6). The titre varies across pAbs as well as between the strains ranging from 1:80 to 1:320.

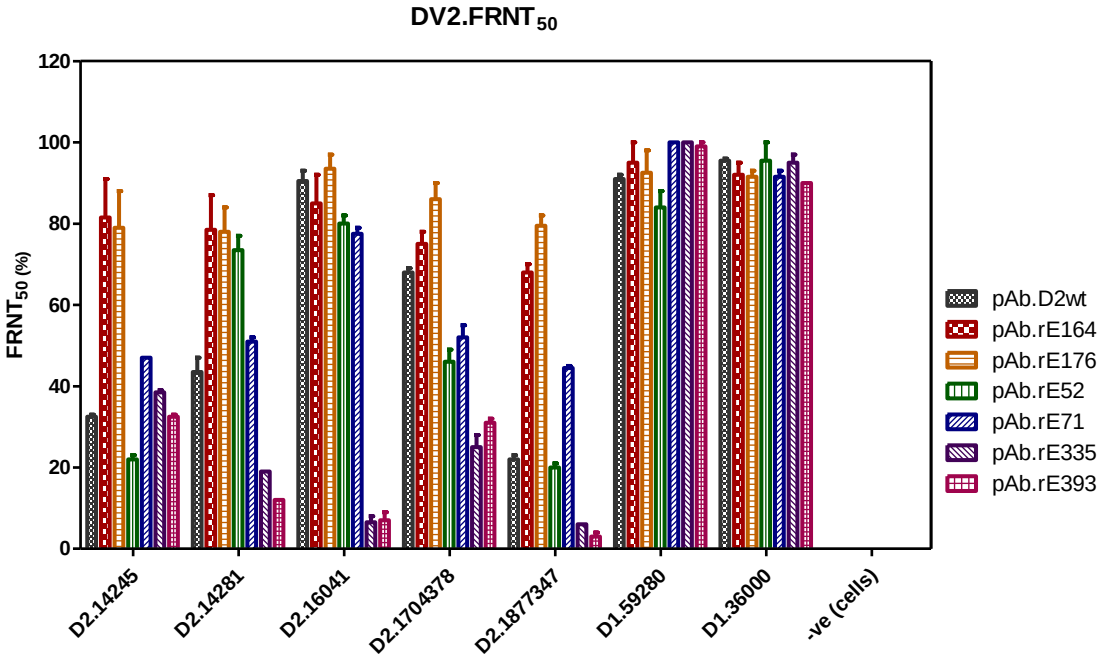
In the Focus Reduction Neutralization Test performed using Vero cell lines, the following results when compared with the microneutralization were observed as shown in Table 3.6 and Figure 3.11. The reduction in the number of foci is equivalent to two-fold reduction of the virus titre in the presence of all the pAbs ($FRNT_{50}$) corresponding to the highest titre of pAb observed in microneutralization. The average neutralizing dilution of all the Cosmopolitan DENV-2 strains was in the range of 1:80 – 1:320 accordingly. The percentage of the total foci formed at the minimum dilution of 1:320 obtained from microneutralization varies slightly across antigenic domains (Table 3.6) (Figure 3.11). An unrelated polyclonal serum antibody included as negative control did not neutralize any of the DENV strains. The polyclonal antibody generated against DENV-1 antigenic proteins failed to block DENV-2 infections even at increased serum concentration. These results suggest that the pAbs recognizes sites on DENV-2 EDII and EDIII domains of E consisting of antibody neutralizing sites of the Cosmopolitan strains and EDIII for the Asian I strain. No cross-reactivity of the antibody against DENV-1 was observed. The results suggest the neutralizing influence of the pAb from both DENV-1 (Table 3.5) and DENV-2 (Table 3.6) are type-specific.

Table 3.6: Microneutralization and Foci Reduction Neutralization Test$_{50}$ of polyclonal serum antibodies generated using the DENV-2 recombinant E

Strain	**E domain**	**EDI**		**EDII**		**EDIII**		
Polyclonal serum	pAb.D1wt	pAb.rE164	pAb.rE176	pAb.rE52	pAb.rE71	pAb.rE335	pAb.rE393	Genotypes
D2.14245/97	>1:80/33	<1:20/84	<1:20/79	>1:320/23	>1:320/48	>1:320/39	>1:320/32	Cosmopolitan
D2.14281/97	1:160/47	<1:20/79	<1:20/78	1:160/77	1:320/52	1:320/19	1:320/12	Cosmopolitan
D2.16041/98	>1:20//88	<1:20/85	<1:20/94	<1:20/78	<1:20/79	1:80/8	1:80/5	Asian 1
D2.1704378/07	>1:80/69	<1:20/75	<1:20/86	>1:320/43	>1:320/49	>1:320/28	>1:320/32	Cosmopolitan
D2.1877347/07	>1:80/23	<1:20/68	<1:20/79	>1:320/21	>1:320/45	>1:320/6	>1:320/4	Cosmopolitan
D1.59280/87	<1:20/92	<1:20/95	<1:20/93	<1:20/88	<1:20/100	<1:20/100	<1:20/100	GIc
D1.36000/05	<1:20/95	<1:20/92	<1:20/92	<1:20/89	<1:20/100	<1:20/93	<1:20/97	GIa
-ve control	<1:20/00	<1:20/00	<1:20/00	<1:20/00	<1:20/00	<1:20/00	<1:20/00	

- The microneutralization and FRNT results are presented as (microneutralization/FRNT$_{50}$)
- Microneutralization results are presented as highest titre of serum dilution
- FRNT$_{50}$ presented as percentage of foci formed corresponding to the highest titre of pAb observed in microneutralization
- Polyclonal serum was generated in mice following inoculation with the respective recombinant DENV E

Figure 3.11. $FRNT_{50}$ of mouse pAb against dominant DENV-2: The graph explains the foci reduction neutralization test as presented in table 3.6. The results correspond to the pAb titre observed in microneutralization assay. The bars represent the percentage in total number of foci and expressed as reduction in foci count.


DV2.FRNT50
FRNT50 (%)
120
100
80
60
40
20
0
D2.14245
D2.14281
D2.16041
D2.1704378
D2.1877347
D1.59280
D1.36000
-ve (cells)
pAb.D2wt
pAb.rE164
pAb.rE176
pAb.rE52
pAb.rE71
pAb.rE335
pAb.rE393

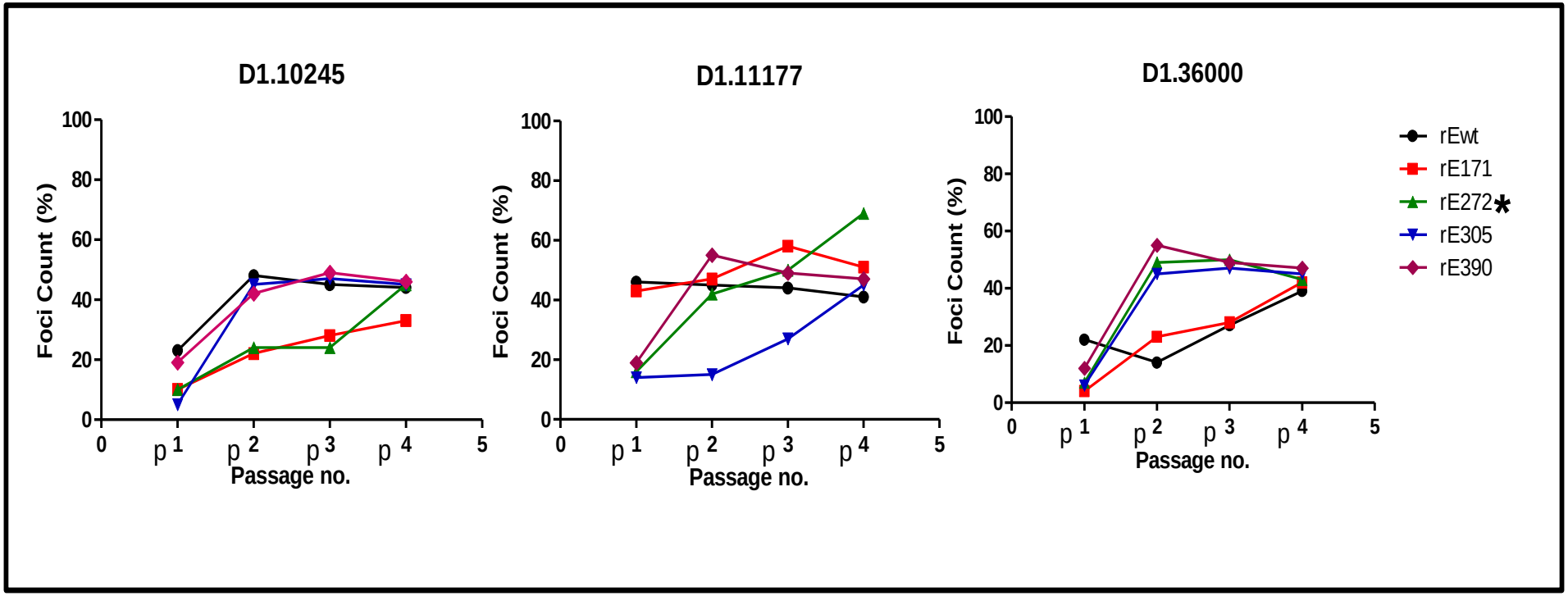
3.3.2 NEUTRALIZATION ESCAPE MUTANT

Almost all the pAbs generated against the mutated DENV-1 E neutralized and blocked D1.11177, D1.10245, and D1.36000. In addition, the pAb that recognized sites on EDII and EDIII of DENV-2 neutralized all the Cosmopolitan DENV-2 strains. However, pAb.rE335 and pAb.rE393 have potential neutralizing activity against the dominant DENV-2 of Asian I origin. The potential mutation sites on E that are likely to promote antibody neutralization were investigated. DENV-1 and DENV-2 virions were propagated three times in the presence of the pAb diluted at 1:80 dilutions (highest achievable dilution observed in microneutralization test) in C6/36 and Vero cells. Focus formation assay was performed at every round of passages, and the foci reduction was quantified following the fourth round of passage. The nucleotide sequences of the virus tested were also determined after the fourth passage. Out of the three DENV-1 strains tested, the neutralization of D1.10245 was consistent in the presence of all pAb tested except for pAb.rE272, in which the foci number increased steadily after the fourth passage (Figure 3.12A). D1.11177 also exhibited large increase in foci number above the $FRNT_{50}$ in the presence of the pAb.rE272 (Figure 3.12A). It also showed slight increase in foci number in the presence of the pAb.rE171 and pAb.rE305 and remain steady at passage four. However, in the presence of pAb.D1wt and pAb.rE390 the foci number remained consistently below the $FRNT_{50}$. D1.36000 foci formation remained stable after fourth passage below the $FRNT_{50}$ (Figure 3.12A). A steady increase was observed in the presence of pAb.rE305 but still below $FRNT_{50}$. These results suggest that the viruses D1.10245 and D1.11177 have potential to escape neutralization in the future in the event of mutation at rE272, with D1.11177 more likely than the remaining two dominant strains. No statistically significant differences were observed between infection of C6/36 and Vero cell lines (Figure 3.12A/C).

DENV-2 was serially propagated in sub neutralizing antibody concentration (1:80). After fourth round of propagation, reduction in foci formed was observed for virus propagation in the pAb.D2wt across the Cosmopolitan and Asian I strains. A steady increase in focus formation numbers in the two cosmopolitan strains D2.14245 and D2.1877347 was maintained in the presence of the entire polyclonal antibody tested except pAb.rE52 and pAb.rE71. There was gradual increase in focus formation with every increase of serial passage of the viruses (Figure 3.12B). At passage 1 through 4, two-fold linear increase was observed in the presence of pAb.rE335 and pAb.rE393. These results suggest the mutation in E52 and E71 of the EDII would likely enable the Cosmopolitan strain to escape neutralization in future than mutation in EDIII. No statistically significant differences were observed between infection of C6/36 and Vero cells (Figure 3.12B/D). Collectively, the antibody neutralization result from DENV-1 and DENV-2 from above suggest that mutation at EDII of the respective DENV could confer more fitness advantage for the circulating dominant DENV to emerge in the future. The exception is the Asian I strains which is likely to emerge because of mutation in both EDII and EDIII with EDII showing more likely possibility.

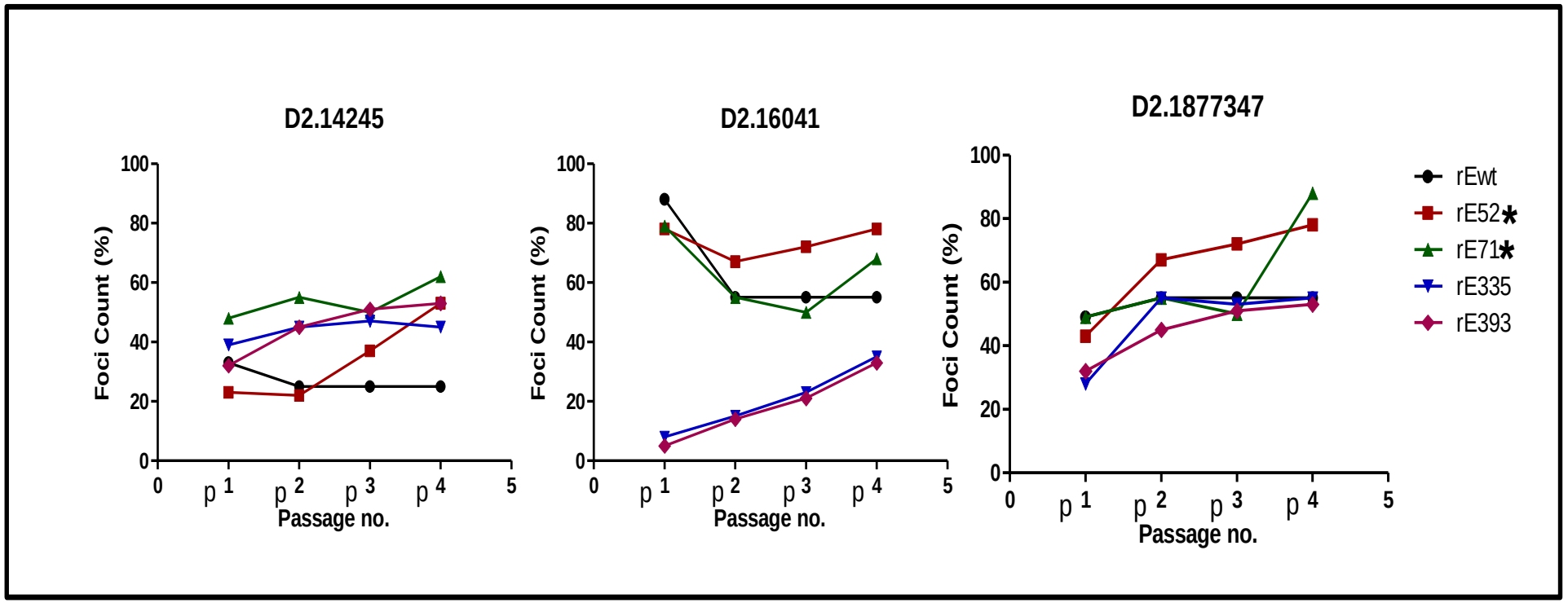
Figure 3.12**.** Selections of DENV-1 and DENV-2 neutralization escape after serial propagations in polyclonal sera. The neutralizing potential immune escape strains were propagated in C6/36 (A/C) and Vero cell (B/D) lines in the presence of pAb at neutralizing titre of 1:80. The * denotes antibody escape after fourth round. The values shown are means of duplicate assays. Foci counts were expressed as percentage.

A



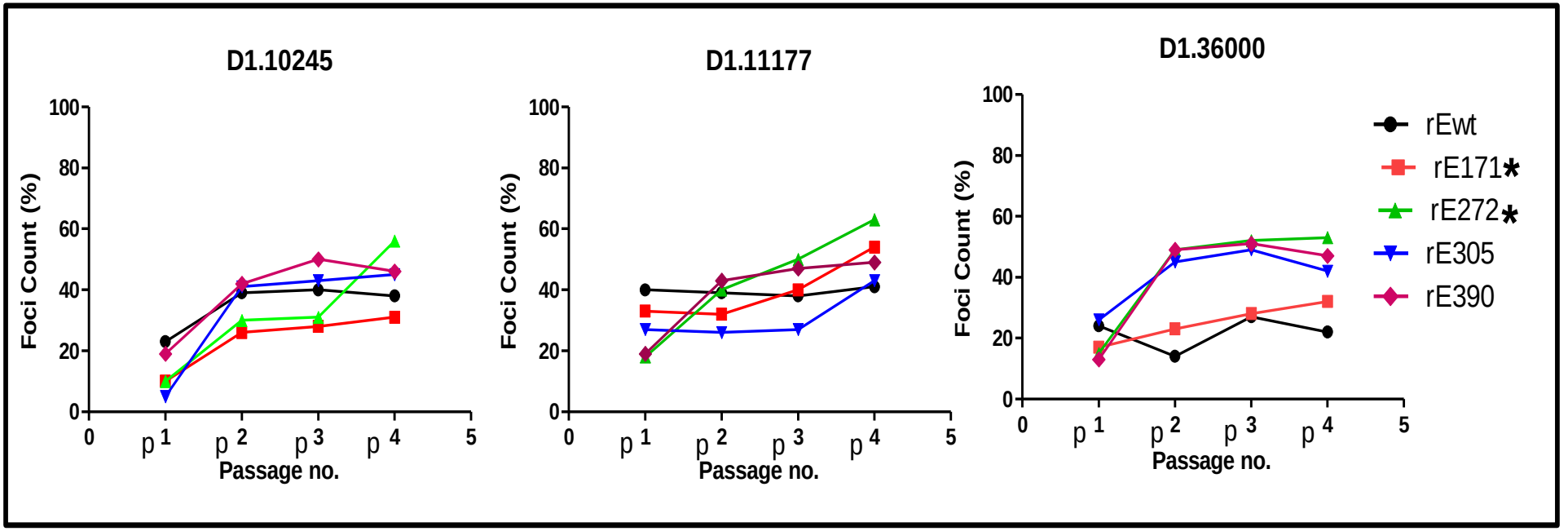
* Neutralizing antibody escape after fourth passage

B



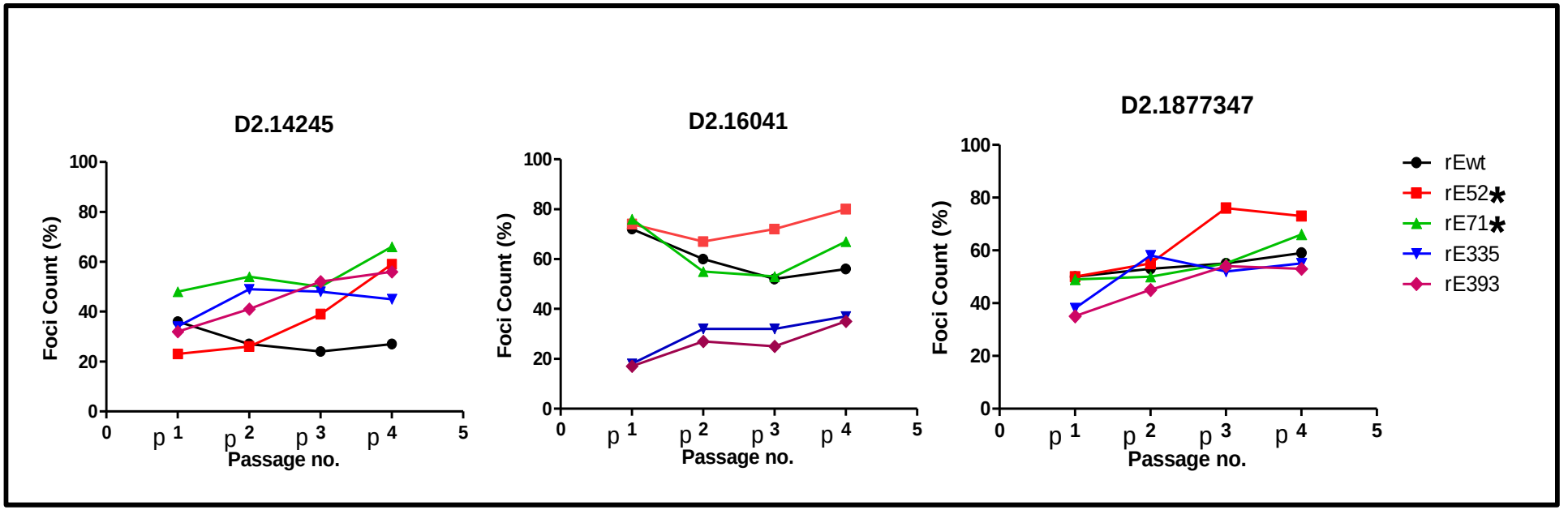
* Neutralizing antibody escape after fourth passage

C



* Neutralizing antibody escape after fourth passage

D



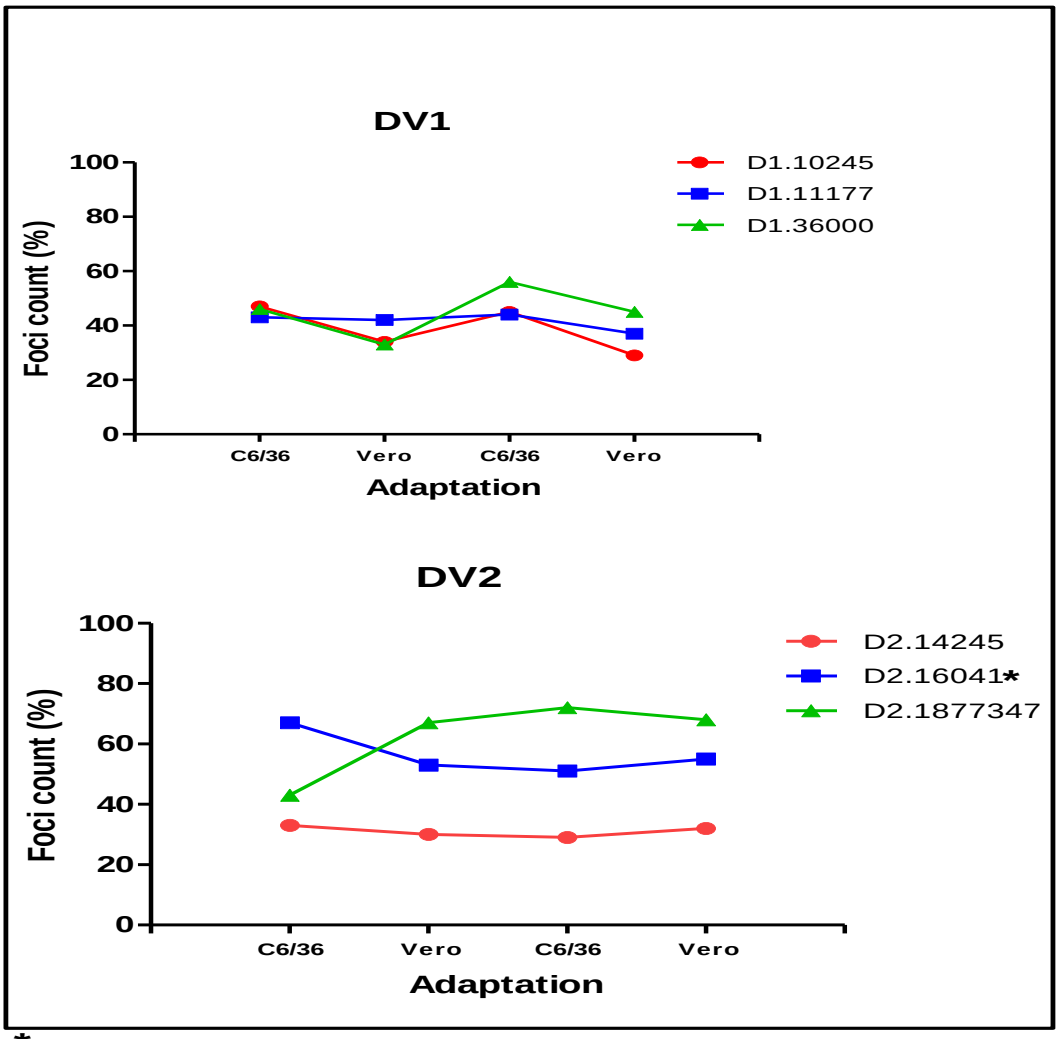
* Neutralizing antibody escape after fourth passage

3.3.3 ADAPTATION OF THE NEUTRALIZATION ESCAPE MUTANT IN C6/36 AND VERO CELLS

The DENV-1 and DENV-2 strains that showed an increase in the number of foci after serial propagation in the pAb were cultured in alternating C6/36 and Vero cell lines to mimic mosquito and human cycle in the presence of pooled pAb from EDII (pAb.rE272) and EDIII (pAb.rE305 and pAb.rE390) of DENV-1 and from EDII (pAb.rE52, pAb.rE71) and EDIII (pAb.rE335 and pAb.rE393) of DENV-2. All the DENV-1 strains tested displayed a fluctuating pattern of higher foci number in C6/C36 cells and lower foci number in Vero cells (Figure 3.13). The ability of the virus strains to grow differently in the two alternating host may be an attribute for adapting to different receptors on the cell surfaces.

However, in DENV-2 the titre of the dominant strains maintained in the two cells remained steady after the fourth passage except D2.1877347 which shows a slight fluctuation in the foci formation at passage three and four. The results therefore suggested that DENV-1 alternating between two different hosts adapt differently, and the replication efficiency varies according to the host irrespective of the strain. Analysis of nucleotide and deduced amino acid of the E sequence from the viruses revealed sequence conservation of all the DENV-1 and DENV-2 propagated in the presence of pAbs. Silent nucleotide substitution at position 690 (A – T) of the Asian I strain of DENV-2 was observed at passage 4. This finding suggests that DENV E alone may not play a major role in the dominant endemic virus ability to adapt to different hosts.

Figure 3.13. The mosquito and human cycle adaption of DENV-1 and DENV-2 strains**.** Foci formation assay of the DENV-1 and DENV-2 dominant viruses after alternating passage between C6/36 and Vero cell lines with pooled ED II and ED III antibody titre (1:80) of the respective strains.



* Mutation observed after passage 4

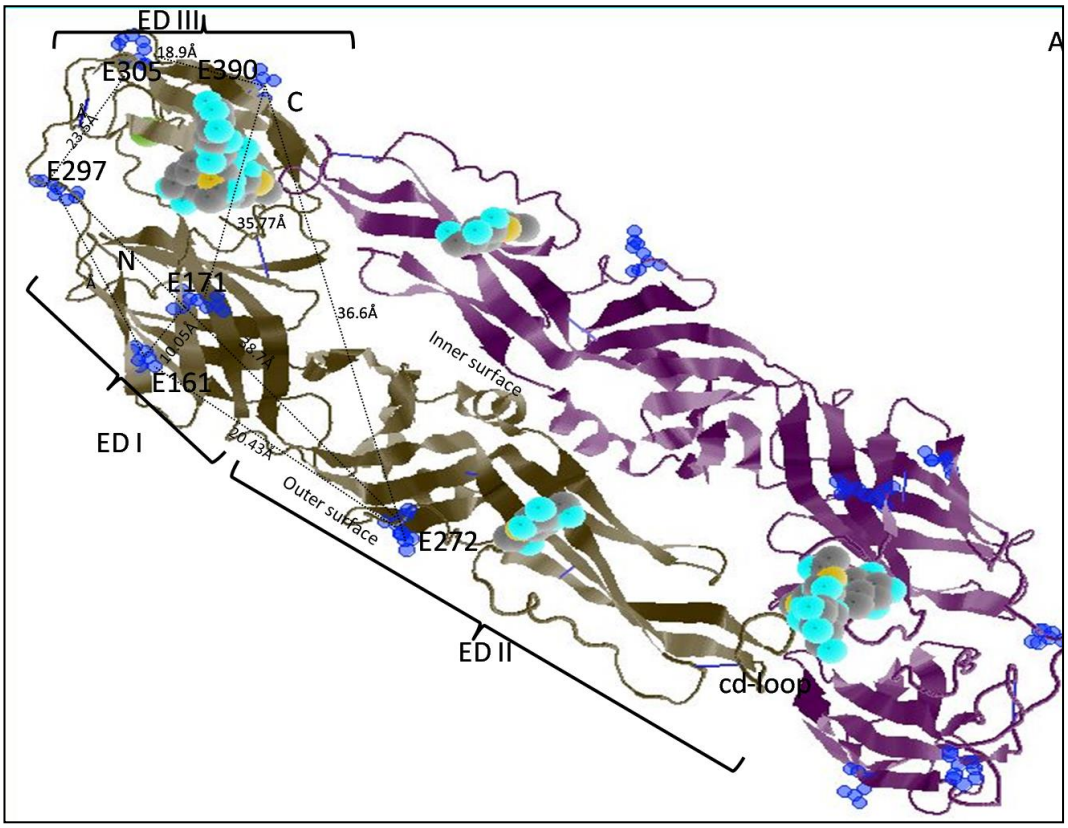
```
D2.14281/97    : 670                                  700 :  30
D2.14245/97    :                                          :  30
D2.16041/98    : ..G..T........G....T.....                :  30
D2.1704378/07  :                                          :  30
D2.1877347/07  :                                          :  30
                 GCAGACACACAAGGATCAAATTGGATACAG
```

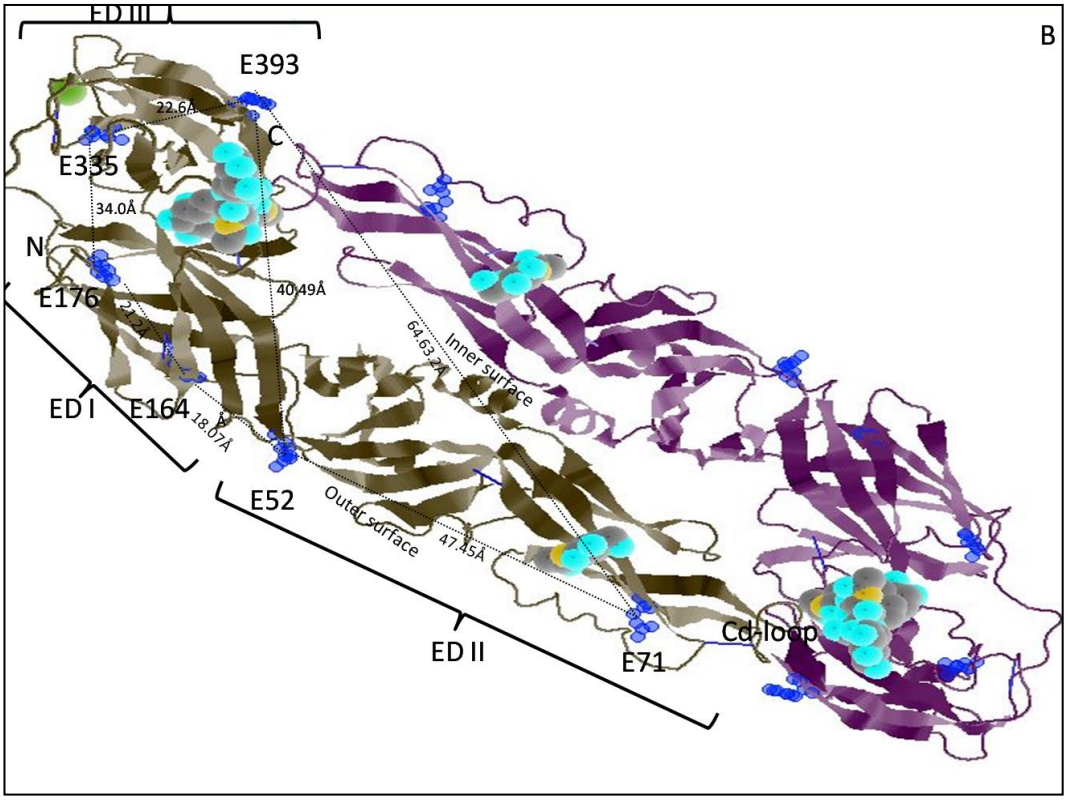
Nucleotide substitution observed at position 690 of the DENV-2 Asian I strain in Malaysia after serial propagation

3.3.4 LOCATION OF MAJOR AMINO ACID USED IN THIS STUDY

Using the crystal structure of DENV-2 all the six substitutions identified to be associated with the emerging antigenic variable strains of the dominant DENV-1 and DENV-2 in Malaysia were located. Because the relative distance of antigenic determinants to one another correlates with ability of neutralizing antibody to have access, their relative positional influences on the monomeric and homodimeric protein were highlighted. As shown in Figure 3.14A/B, amino acid residue E161, E171 and E297 are located on EDI, E272 on EDII and E305 and E390 on EDIII of DENV-1. The substituted amino acids, E164 and E176 are located in EDI, E52 and E71 in EDII, E335 and E393 on EDIII of DENV-2 E accordingly. The molecular relative distance observed on the amino acid residues on EDI of DENV-1 was 10.05-25.8Å.The two amino acids on EDIII were separated apart by 18.07Å. The closest of the EDI and EDIII to amino acid residue E272 on EDII was 20.43Å and 36.6Å respectively. In DENV-2, the relative distances between the two amino acids residues on the same domain was 21.24Å on EDI, 47.45Å on EDII and 22.63Å on EDIII. The relative distances among the residues of different antigenic domains on DENV-2 were EDI - EDII 18-35.11Å, EDII – EDIII 40.49-64.3Å and EDI – EDIII 34.04-51Å accordingly. These results showed the position and the proximity of the altered amino acid residues on the exposed surface to the monomeric E varies.

Figure 3.14**.** Major amino acid residues substituted on E highlighted in the dimeric structure. (A) DENV-1, (B) DENV-2. The structures were modified based on PDB file 1OANon FirstGlance program in Jmol version 1.44 (http://bioinformatics.org/firstglance/fgij/). The dotted lines represent the relative spatial distance in Armstrong (Å).

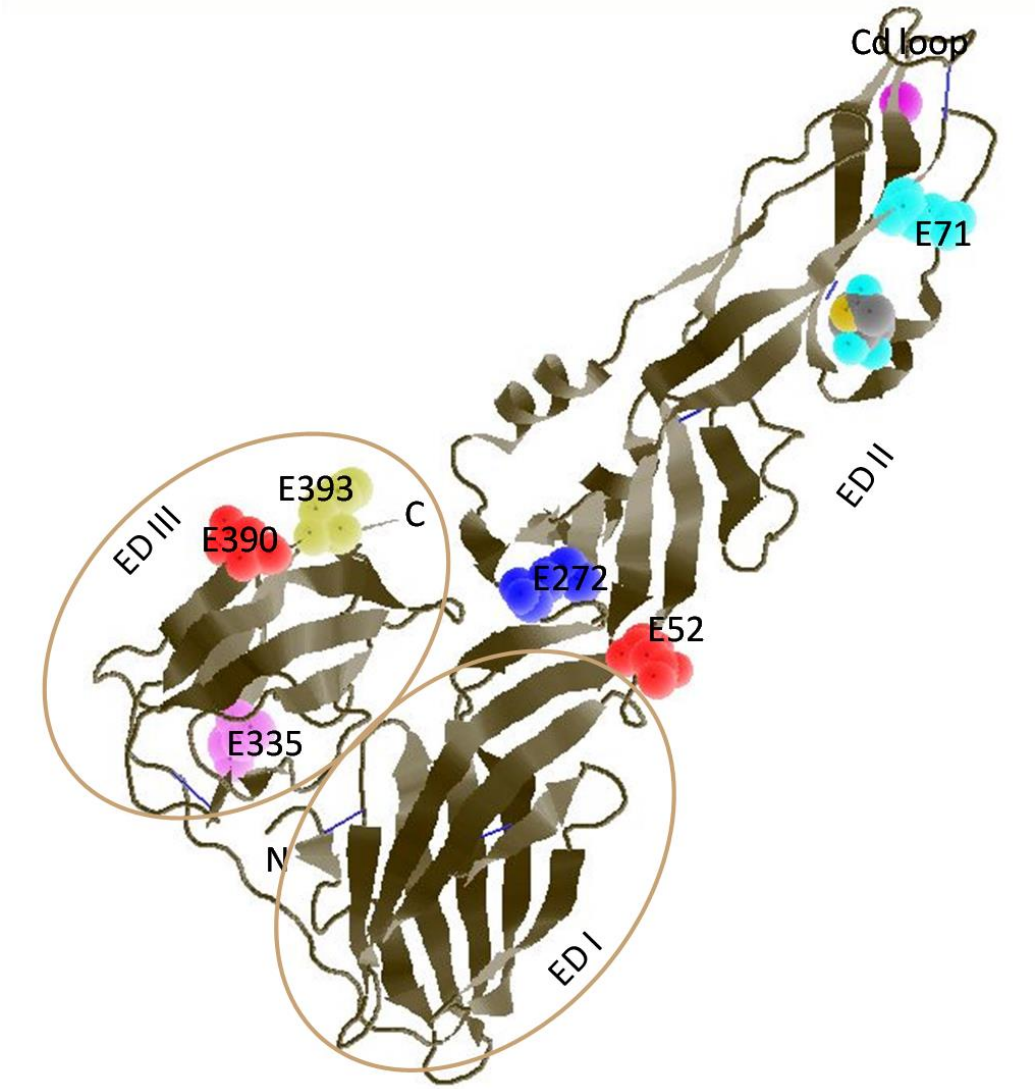

ED III
A
18.9Å
E305
E390
C
23.5Å
E297
35.77Å
N
E171
36.6Å
10.05Å
48.7Å
Inner surface
E161
ED I
20.43Å
Outer surface
E272
ED II
cd-loop


ED III
B
E393
22.6Å
C
E335
34.0Å
N
40.49Å
E176
27.27Å
64.63 Å
Inner surface
ED I
E164
18.07Å
E52
Outer surface
47.45Å
ED II
E71
Cd-loop

3.3.5 THE RELATIVE POSITION AND SIGNIFICANCE OF THE SUBSTITUTED AMINO ACID RESIDUES

The relative positions of amino acid residues on EDI and EDIII, EDI in association with EDII of DENV-1 that select for neutralization escape could participate in forming an antigenic determinant. The relative distance is within the surface area accessible by the neutralizing antibody molecule, 15-20Å × 20-30Å (Figure 3.14A). Similarly, EDI and EDIII together with amino acid residues on EDI (E164) in association with EDII (E52) could equally participate in forming an antigenic determinant on both the monomeric and homodimeric E of DENV-2 (Figure 3.14B). The remaining amino acid residues are unlikely to form antigenic determinants in association with other amino acid residue (relative distance is greater than 30Å).They lie within or adjacent to the antigenic determinant accessible by neutralizing antibodies on their own. These results suggest that strains with multiple substitutions would likely escape neutralization faster than the strains with single mutations. Overall, the amino acids associated with significant binding to both mammalian and mosquito cell lines as well as neutralization and selection of neutralization escape from dominant DENV-1 and DENV-2 strains are highlighted in Figure 3.15. Based on the data reported in this study and their locations on the E monomeric protein, the residues are distributed on EDII and EDIII. The T272M (DENV-1) and H52K and A71N (DENV-2) residues illustrated in blue, red and cyan on EDII influenced neutralization escape. The S390R (DENV-1) and I335L (DENV-2), and K393M (DENV-2) illustrated by red, pink and yellow colour balls on EDIII fall within putative binding sites and the attachments to cells flaviviruses. Mutations created at these positions could influence neutralizing antibody binding.

Figure 3.15. Location of significant amino acid residues on monomeric E: Positions of E amino acid residues substituted on monomeric E structure. The structure was modified using SWIS-prot PDB code: 1ok8on First Glance program in Jmol version 1.44 (http://bioinformatics.org/firstglance/fgij/).These positions and antigenic domains are likely to affect future antigenic evolution of DENV-1 and DENV-2 in Malaysia.


Cd loop
E71
ED II
E393
C
ED III
E390
E272
E52
E335
N
ED I

4.0 DISCUSSION

The present study hypothesized that the dominant endemic DENV strains may acquire the characteristics of minor strains with the ability to escape immunity and perhaps be the progenitors of future outbreak. This hypothesis was investigated here and the specific amino acid residues that are conceivably involved in the observations are further discussed. Prior to this study, little is known as to how emerging variants of DENV-1 and DENV-2 escape neutralization and cause major outbreaks in Malaysia. This study identified and described the effect of the variation in single amino acids on the antigenic determinants of DENV-1 and DENV-2 in Malaysia. Results presented here highlight and detail the effects of these mutations on the antigenic evolutions of DENV-1 and DENV-2 in Malaysian natural population.

The continuous transmission of different DENV serotypes within the Malaysian population has led to several major outbreaks (Abubakar and Shafee 2002; AbuBakar et al. 2002b; Teoh et al. 2010) and this established DENV with dynamic antigenic variation (Abubakar and Shafee 2002; AbuBakar et al. 2002b; Teoh et al. 2010). During a 30 year period, a reasonable number of different DENV strains were isolated from patients who visited the University of Malaya Medical Centre (UMMC) and were IgM positive for DENV infection (Teoh et al. 2010). University of Malaya Medical Centre is the largest teaching hospital in the country and a major referral health facility for dengue cases. High numbers of dengue cases exceeding 4000 are reported at the hospital yearly, especially during dengue outbreaks. Using the DENV isolates retrieved from 30 years of dengue activity in the country, the sequencing project in our laboratory has generated a nationwide dengue genome database that is easy to access and hence, providing an ideal opportunity for us to study the antigenic evolution of DENV.

The antigenic recombinant DENV E used in this study was obtained using a non-denaturing and highly efficient protein extraction formulation for in-culture bacterial cell lyses with reduced proteolytic degradation. Hence, the functional and biological activity of the conformationally sensitive antigenic determinants was preserved (Roehrig et al. 2004; Tripathi et al. 2008). These recombinant proteins were used to ensure that the antibody generated from immunized mice is based on the E in its native conformation as opposed to the denatured conformation (Serafin and Aaskov 2001; Wahala et al. 2012). Furthermore, the binding affinity and specificity of the antibody generated against the target protein or cell surface receptors are largely dependent on the conformation of the antigen in which the antibody was raised (Roehrig et al. 2004; Wahala et al. 2009). Thus, this approach was useful for the specific functional assays employed in this study to establish the effects of the altered amino acid residues on the antigenic capability of the E, which largely depends on this conformation. Phage display system was used here, allowing for the quantitative estimation of direct binding of the recombinant E displayed on the g3p minor coat attachment protein to its cellular targets which allowed us to determine the effects of the engineered E mutations (Abd-Jamil et al. 2008; Hashemi et al. 2010). The intraperitoneal immunization strategy in mice was reported to induce strong neutralizing antibodies following inoculation with recombinant E (Liu et al. 2010; Liu et al. 2006; Wang et al. 2006). Both mosquito and mammalian cell types have different cellular receptors that recognize DENV (Mercado-Curiel et al. 2006). The ability of DENV E to interact with different cell types during attachment is essential for DENV survival (Smith 2012).

Several studies have used binding assay to demonstrate DENV binding to the surfaces of mammalian (Vero) (Chen et al. 2003; Hanley et al. 2003; Thaisomboonsuk et al. 2005) and mosquito (C6/36) cells(Mercado-Curiel et al. 2006; Munoz et al. 1998; Salas-

Benito et al. 2007). In the present study, we detected differences in the ability of specific mutations to affect virus binding to mammalian and mosquito cells. Similarly, when the recombinant phage particles were precipitated with pAb generated in this study, there was significant reduction in the binding of the recombinant phage particles to mammalian and mosquito cell lines depending on the altered residue on the DENVE. The effects of the pAb were noticeable in the binding interaction against the dominant dengue virions tested. These results are in line with previous reports, suggesting that the interaction of the DENV E with the cell surface receptor plays a significant role in determining tissue tropism (Rico-Hesse 2003; Thaisomboonsuk et al. 2005). The inability of certain amino acid positions on E to affect virus binding to both the mosquito and mammalian cells demonstrated the influence of the altered positions on E in viral mediated cell membrane fusion (Martina et al. 2009; Rodrigo et al. 2009; Thaisomboonsuk et al. 2005).

On the other hand, successful transmission of DENV involves virus replication alternating between mosquito vector and human hosts (Twiddy et al. 2002a; Twiddy et al. 2002b). The virus must be able to recognize cell surface receptors in both cell types. Therefore, it is not surprising that the DENV-1 used in this study adapted to the mosquito cells faster than mammalian cells when allowed to infect two cell lines in alternative succession to mimic the mosquito and human cycle in the presence of sub-neutralizing antibodies. This result confirmed the previous reports that suggest the cell surface receptors used during human infection are different from those used in mosquitoes and are independent of the infecting serotype (Smith 2012; Thaisomboonsuk et al. 2005). The mammalian cell surface receptor contains sialic acid and heparan sulfate, in which the absence of these two molecules in C6/36 cells may facilitate receptor binding in mosquito cell (Smith 2012). The repeated passage in mammalian cells may select for net positive charge on the E, which favours interaction

with the heparan sulfate on mammalian cell surface. The adaption of the virus in sub-neutralizing concentrations of antibody mimics antigen-antibody interaction, which takes place during the viral entry. Antigenic variation of DENV E therefore, is likely to occur in mammalian cells due to the variation in antigenic determinants on E that favour net positive charge to negative charge residues as influenced by heparan sulfate in the cell surface receptor (Smith 2012). Besides, the pre-existing antibody and cross-neutralizing antibodies against DENV and other flaviviruses in humans, which limits DENV replication, are absent in the mosquito cycle. Hence, in the process of adaptation to the human host, the virus changes its E antigenic determinant to adjust to the new environment while the mosquito transmits it to the next susceptible host. The difference between the DENV genotypes, which carries the different residues in the E, in adapting to the alternate cell lines was not evident here. Therefore, there may be other genes that are not studied here affecting the adaption of DENV in mammalian or insects host(Rico-Hesse 2003).

The dominant strains that had caused major outbreaks were used to help select for neutralization escape against the mouse pAbs generated from the altered E. In the present studies, amino acid residues on ED II (E272) of DENV-1 and EDII (E52, E71) on DENV-2 were surface exposed variable amino acid (Lin et al. 2012; Wahala et al. 2010). The residues on the E associated with DENV-1 genotype GIb and GII and DENV-2 Cosmopolitan and Asian I strain neutralization escape. The EDI/EDII junction was reported to influence mouse monoclonal antibody binding. The EDII lateral ridge was reported to contain antigenic determinant that recognize type specific neutralizing antibodies (Gromowski and Barrett 2007). Polyclonal antibody against altered E272 resulted in loss of binding to two dominant DENV genotypes D1.59280 and D1.59266 (GIII). Similarly, the antisera inhibited and neutralized the two DENV-1 genotypes. When the sensitive genotypes were selected against the antisera, pAb against E272

exhibited reduced activity against D1.10245 (GII) and D1.11177 (GIb) genotypes. The remaining polyclonal antibodies generated against the altered positions E171, E305 and E390poorly inhibited these viruses. It its evident thatT272S substitution is involved in selection of neutralization escape of D1.10245 (GII) and D1.11177 (GIb). The remaining residues are essential parts of the antigenic determinants recognized by the pAb. Specifically, S305P substitution does not prevent binding but showed a decreased infectivity. The residue lies within the hydrophilic residues that are important for antibody-antigen interaction. The E272 is within the hydrophobic pocket (268-280 residues) known to affect membrane fusion (Butrapet et al. 2011). Mutation at this position induces conformational change and virus characteristics essential for DENV entry (Butrapet et al. 2011). The S305P residue lies within the negatively charged putative heparan sulfate binding site (E294-E310) on A-strand of the EDIII. This substitution is the major residue that differentiates D1.10245 (GII) and D1.11177 (GIb) with D1.36000 (GIa) on E. The hydrophobic nature of the antigenic determinant at this position in the presence of nonpolar residue plays a significant role in maintaining protein conformation during binding. The serine residue increases the hydrophilic pocket of the antigenic determinant around E305 residue. Because of steric hindrance of low pH-induced dimeric prefusion rearrangement, EDI and EDIII conformational rearrangement might blocks EDII. This prevents EDII raised mouse pAb accessibility to the antigenic determinant. This facilitates neutralization escape (Butrapet et al. 2011; Chu et al. 2005; Wahala et al. 2010; Wahala et al. 2012).

In the same way, both cosmopolitan and Asian I genotypes used in the study escaped neutralization because of mutation at E52 and E71 of EDII of DENV-2. The Cosmopolitan and Asian I differs at position E52 (His-Gln) and E71 (Ala-Glu) in the E. The two residues are conserved in the respective Cosmopolitan and Asian I dominant strains. These conserved amino acid residues lie within the EDI/EDII interface and

internal fusion peptide on EDII (E98 – E110) respectively (Lin et al. 2012). Recently, mutation at E52 was reported to alter antigenic structure, and decrease stability that affects membrane fusion in DENV-2 (Butrapet et al. 2011). Previously, mutated E52 was found to alter the structure of a neutralizing antibody epitope in JEV (Nga et al. 2004) and increase mouse virulence in YFV (Chambers and Nickells 2001). Equally, E71 was reported to be associated with neutralization escape mutant in combination with other mutations on E of YFV (Vlaycheva et al. 2005). Similar observations were made in this study. The studies showed the two residues could form different antigenic determinant that could play a role in neutralization antibody escape. Any mutation in this region is likely to alter fusion activity and membrane fusion. The mutations on E52 and E71 on EDII of the two dominant genotypes of DENV-2 in Malaysia are an important determinant for DENV-2 neutralization escape. The mutated E52 and E71 block virus binding to mammalian and mosquito cells when exposed to pAb. The ability of the pAb to select these viruses in primary cell lines, suggested that mutation are important component of future DENV-2 antigenic evolution. The antisera against altered EDIII (E335 and E393) showed moderate effects as observed in DENV-1. Even though there was strong binding and neutralizing antibody effects, the antisera failed to select neutralization escape mutant in both the Cosmopolitan and Asian I strain used in the study. The E335 residue lies adjacent to the neutralization antigenic determinant on A-strand (319-333) (Wahala et al. 2010). The E393 is within the Flavivirus virulence determinants on the EDIII lateral ridge (383-394) (Howard 2006; Hung et al. 2004). Mutation at these positions affects antibody binding, which allows virus attachment to cell receptors and possible infection. The ability of the antisera to neutralize both genotypes suggests the mutation on the altered protein does not have any effects on the two viruses.

Previously, it was reported that *in vitro* analysis is not an ideal model host for *in vivo* DENV infection studies. The uptake mechanism during antibody absorption had been proposed for JEV (Butrapet et al. 1998), WNV (Gollins and Porterfield 1986) and in DENV (Chen et al. 1996; Crill and Roehrig 2001; Huang et al. 2006). A correlation has been found to exist among the three antigenic domains (Alen et al. 2011; Chen et al. 1996; Mercado-Curiel et al. 2006). In mice, antibodies against EDI antigenic protein were reported to be non-neutralizing (Aaskov et al. 1989; Howard 2006; Serafin and Aaskov 2001). But antibodies against EDII determinant can either be neutralizing or non-neutralizing (Crill and Roehrig 2001). EDIII, on the other hand were reported to be associated with host cell binding (Roehrig et al. 1998), and acted as a major site for neutralizing antibody activity (Gromowski et al. 2008). In humans, previous studies have reported that, polyclonal human antibody may recognize and react to antigenic determinant on EDI and EDII better than to EDIII (Wahala et al. 2009).

Previously, immune responses of DENV E generated in BALB/c mice were reported (Howard 2006; Serafin and Aaskov 2001). In DENV-1 and DENV-3, less than 20% of anti-DENV-1 and DENV-3 were able to neutralize dengue infection. Approximately, 40% and 80% were reported to neutralize DENV-2 and DENV-4 respectively (Howard 2006; Serafin and Aaskov 2001; Simantini and Banerjee 1995). Recent reports showed that the most potent neutralizing antibodies are presented on the lateral ridge of EDIII (Wahala et al. 2010). In this study, not all the engineered residues influenced the pAb neutralizing activity against the dominant strains. The antisera generated from mutated E of EDI and EDIII failed to select neutralization escape variants in both DENV-1 and DENV-2. Altering the E sequences lead to changes in the conformation of antigenic domain of the E. All other residues on the mutant strains are of the wild type strain. The mutant may have an altered conformation that presents changes to existing or may have

hidden the antigenic determinant. This leads to a varied binding and antibody-antigen interaction when presented to the mosquito and mammalian cells (Lin et al. 2012).

This study showed that dominant DENV were sensitive to neutralization by pAb developed against E with the acquired characteristics of the minor strains, which co-circulate along with these dominant strains. Neutralization escape mutants were also selected using the pAb with altered EDII E. Previously, antigenic determinants in DENV-1 associated with neutralization escape were identified in EDI (E293) and EDII (E279) (Aaskov et al. 1989; Howard 2006; Serafin and Aaskov 2001). The same antigenic determinant were reported in EDII (69) and EDIII (307 and 311) of DENV-2 (Lin et al. 1994; Lok et al. 2001), EDIII (E386) of DENV-3, EDII (E95) (Aaskov et al. 1989) and ED III (E329) of DENV-4 (Howard 2006; Serafin and Aaskov 2001). The residues are within or adjacent to the antigenic determinant identified. The pAb were derived from non-compromised mice. Therefore, this may not necessarily reflect the neutralizing antibody response in humans. The antibody repertoire against different DENV genotypes in humans remains unknown. The mechanism by which these residues influence antibody and block virus infection is still disputable. However, the neutralization is either because of the binding of a single antibody to one antigenic determinant or because of binding pAb to multiple antigenic determinants (Burton et al. 2001). On the other hand, recent studies suggest the humoral response against flaviviruses in humans may be directed away from EDIII neutralizing epitopes. During DENV infections *in vitro,* the antigenic determinants are presented in the cell endosomes during fusion that allows for antibody binding and blockage of viral entry and infection (Allison et al. 2001; Butrapet et al. 2011; Modis et al. 2004; Schibli and Weissenhorn 2004).

Before this study, little was known as to how newly emerged DENV of DENV-1 and DENV-2 escape neutralization and cause major outbreak in Malaysia. Regardless of the mechanism, the results presented in this study identified and described the effect of antigenic determinant in EDII (E272) and EDIII (E390) of DENV-1. Equally, similar results were identified and described in EDII (E52) and EDIII (E335 and E393) of DENV-2. The study showed that DENV-1 and DENV-2 are expected to escape neutralization under sub-neutralizing antibody concentration. The result directly underlines the effects of these mutations on naturally emerging DENV-1 and DENV-2 in Malaysian population keeping in mind the current global DENV vaccine strategy. Although the proposed dengue vaccines are heterogeneous mixture of viral populations, it might likely not work against naturally occurring neutralization escape mutants.

CONCLUSION

In conclusion, this study presents the first attempt to identify and replace the major variable, surface-exposed amino acid residues in the E of the dominant DENV-1 and DENV-2 strains with that from the co-circulating minor strains in the Malaysian natural population. Polyclonal antibodies generated from these mutants E produced markedly segregated binding and neutralization specificity against the dominant DENV-1 and DENV-2 strains. Specifically, two dominant strains in both DENV-1 and DENV-2 that could likely escape neutralization were identified. Overall, the mutations engineered and the mouse pAbs generated against the mutated E were grouped into two groups: (i) consist of the mutations on E, which reduced antibody binding and neutralizing activity, and (ii) mutations on E, which selects for neutralization escape variants. The mutated EDII engineered E selects for neutralization escape, while the EDIII neutralized and blocks infection *in vitro*. Going forward, this strategy and with improved design can be adopted to predict dengue virus evolution from present to the future in hyperendemic countries. In this way, the predicted strain could be beneficial especially to the vaccine developers and manufacturers in the future.

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Appendix A: University of Malaya use of Animal Ethics Approval Letter

UNIVERSITY OF MALAYA
KUALA LUMPUR

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The Leader In Research and Innovation

PEJABAT KETUA

21 Mac 2011

Mohammed Bashar Danlami
Jabatan Mikrobiologi Perubatan
Fakulti Perubatan

Tuan,

IMMUNE RESPONSE AGAINST DENGUE VIRUS RECOMBINANT PROTEINS

Dengan sukacitanya Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan, Fakulti Perubatan, Universiti Malaya telah meluluskan permohonan untuk penyelidikan tersebut di atas.

No rujukan etika: **MP/23/02/2011/MBD (R)**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh **dua tahun sehingga 23 Februari 2013.**

Sekian, terima kasih.

Yang benar,

Dr. Haji Azizuddin Bin Haji Kamaruddin
Ketua
Pusat Haiwan Makmal
Fakulti Perubatan
Merangkap Setiausaha Jawatankuasa Etika Penjagaan dan Penggunaan Haiwan

SK : Puan Zura Syazleena Hamizan
Setiausaha MCRC
Pejabat Dekan
Fakulti Perubatan

MS ISO 9001:2000 REG. NO. AR 2760

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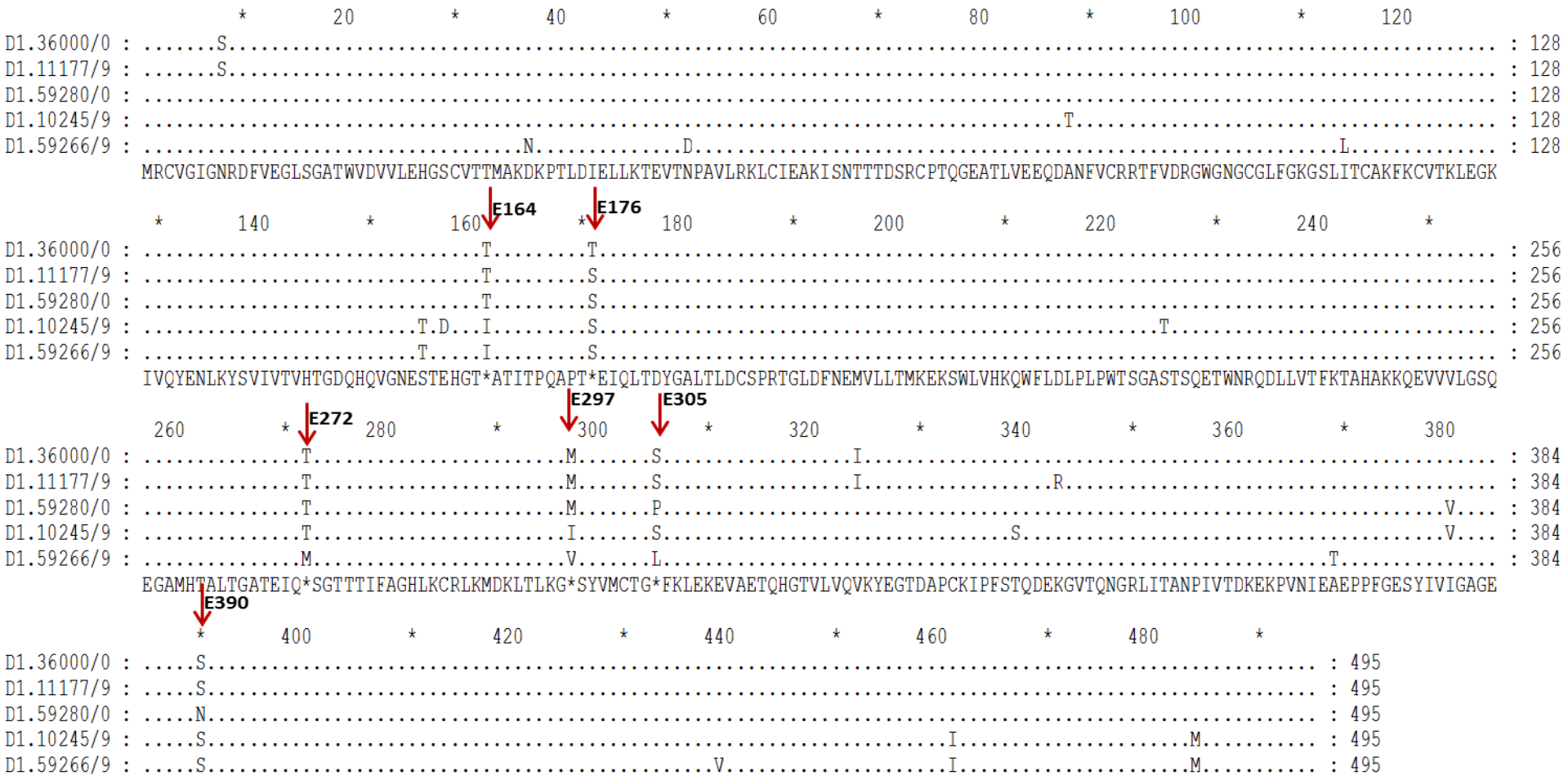
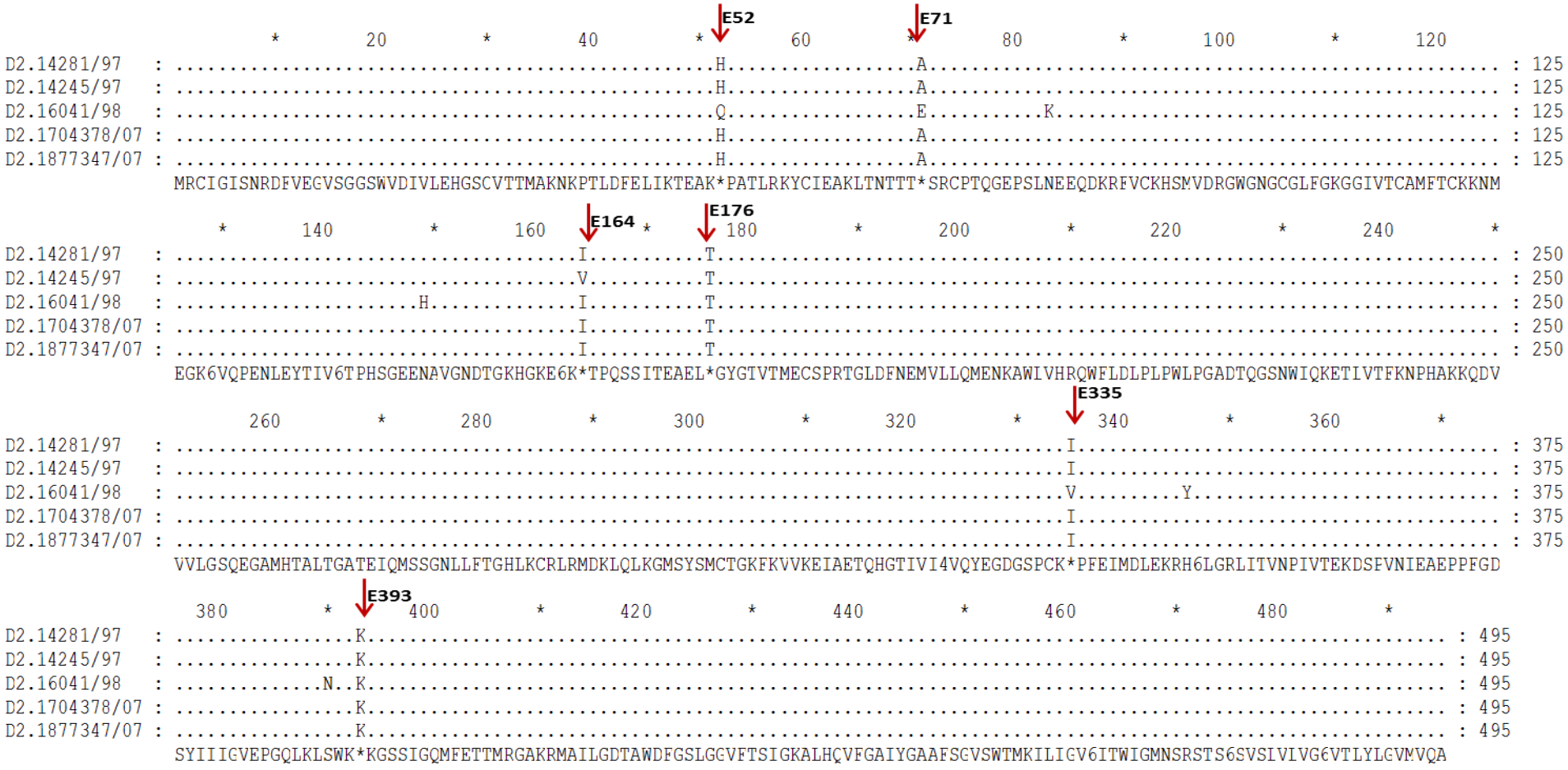
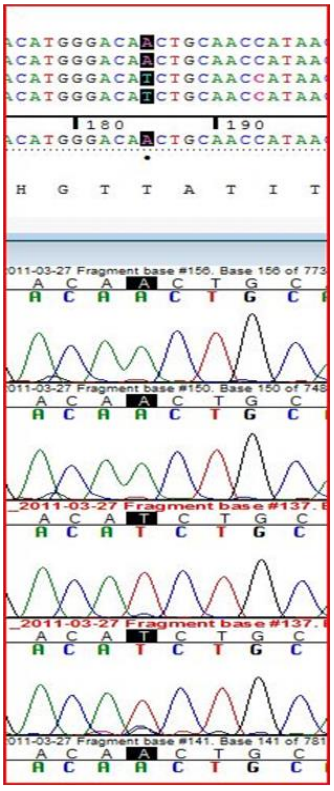
Appendix B: Amino acids sequence of the DENV-1 dominant strains used in the study



Appendix C: Amino acids sequence of the DENV-2 dominant strains used in the study



Appendix D: Chromatograms files of DENV-1 E after site-directed mutagenesis



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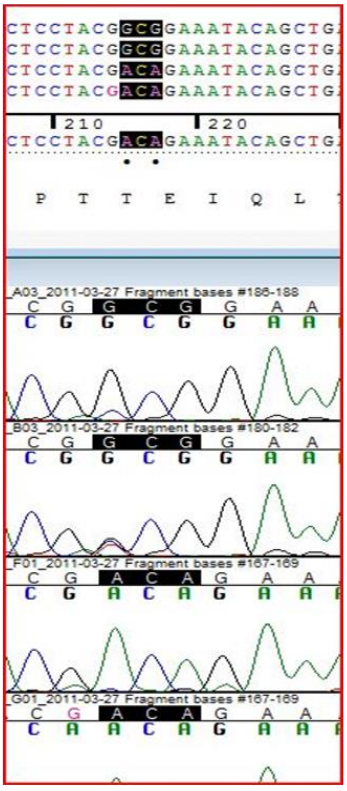

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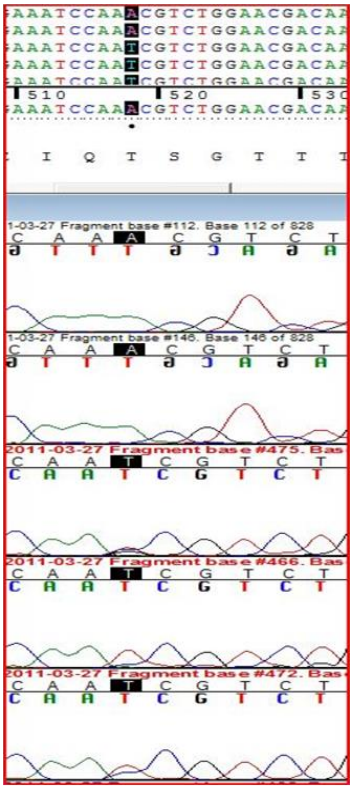

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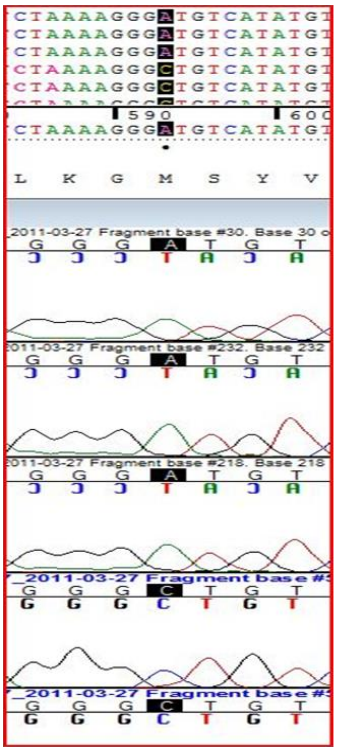

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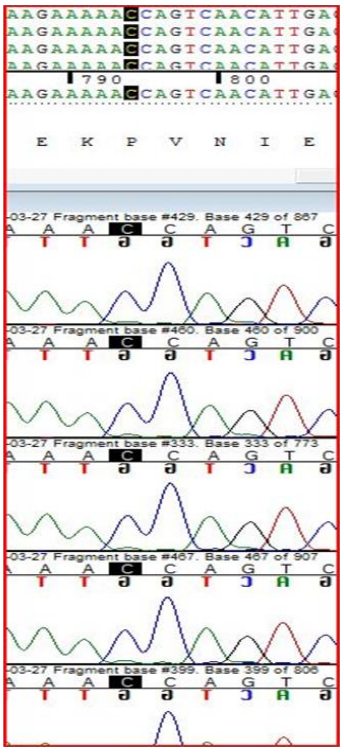

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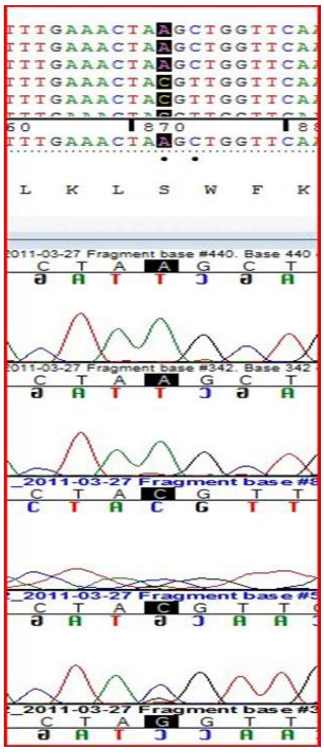
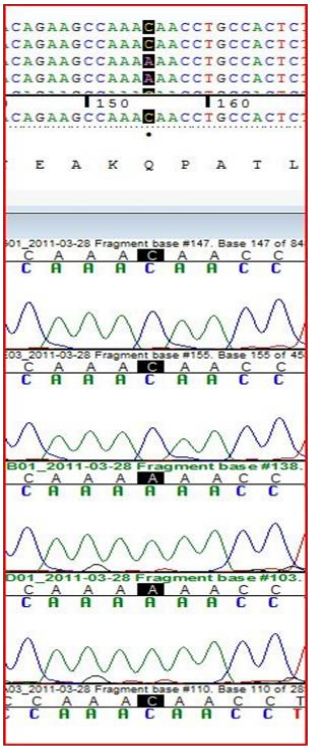


E390

Appendix E: Chromatograms files of DENV-1 E after site-directed mutagenesis



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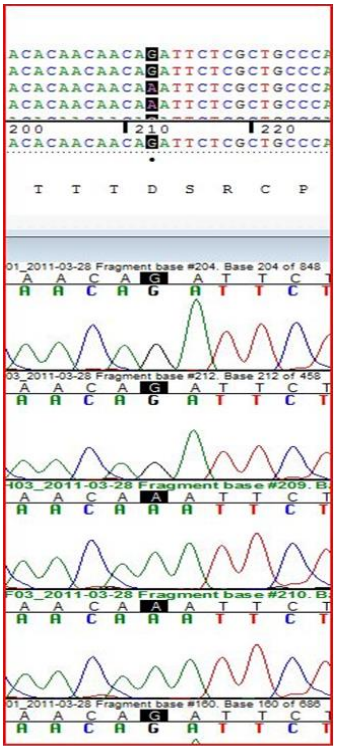

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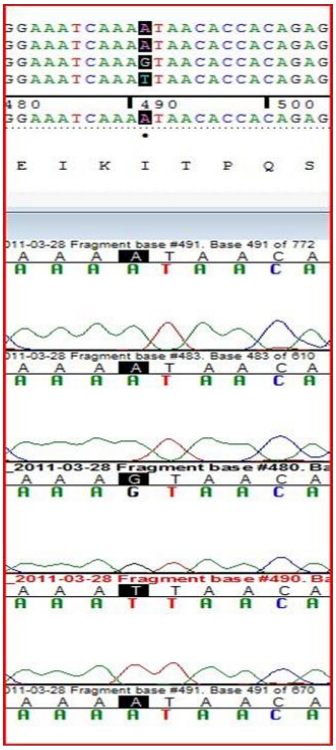
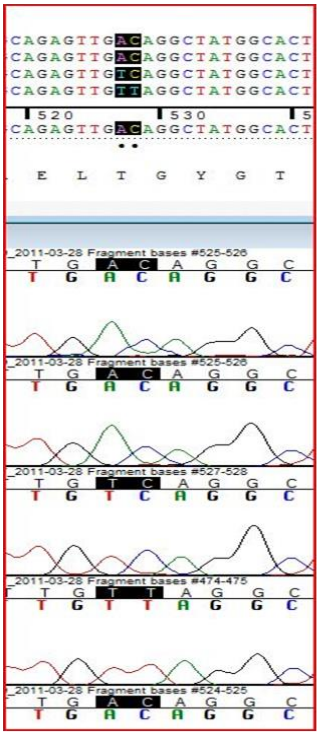


E164



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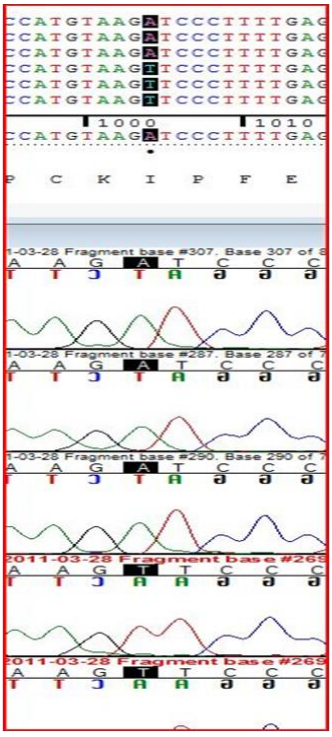

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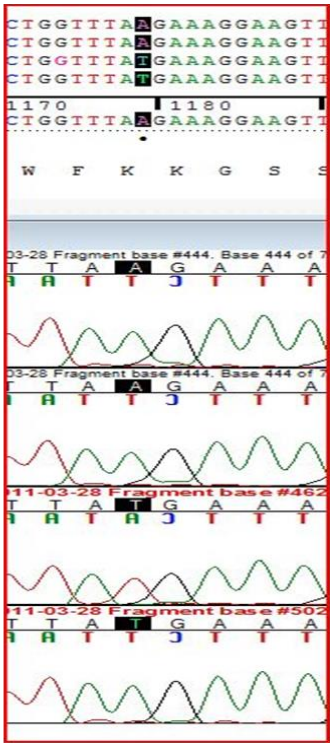

E393

Appendix F: **résumé**

Résumé

Mohammed Bashar Danlami *Ph.D.*
House No. 82 Wara, Opposite Wara I Model Primary School
Ngaski Local Government
Wara, Kebbi State
Nigeria
Tel: +2348130000095, +2348104245366, +60149715435
E-mail: mr.bash@gmail.com

Areas of Expertise

1. Virology 2, Immunology and 3, Molecular Biology

Areas of Interest

1. Medical Virology and Molecular Biology – understanding antigenic evolution of emerging viruses

2. Immunology of Infectious Diseases- understanding of the pathogenesis of disease

3. Public health microbiology

Working Experience

Graduate Research Assistant **March 2008 - October 2013**
University of Malaya (Malaysia)
Award: University Malaya Post-graduate Fellowship Scheme 2008-2010

Principal Food Scientific Officer **January 2010 - January 2013**
Kebbi State Local Government Service Commission (Department of Primary Health Care Ngaski Local Government, Nigeria)

Food Scientific Officer **January 2007 - January 2009**
Kebbi State Local Government Service Commission (Department of Primary Health Care Ngaski Local Government, Nigeria)

Graduate Assistant **May 2005 - May 2006**
International Islamic University Malaysia (Malaysia)
Award: Regional Emerging Disease Intervention Travel Scholarship September 2005

Scientific Officer **May 2003 - January 2007**
Kebbi State Local Government Service Commission (Department of Primary Health

Care Ngaski Local Government, Nigeria)

Quality Control Officer **February 2002 - February 2003**
Savannah Sugar Company (Numan Adamawa State Nigeria)

Laboratory Technician **July 1999 - December 1999**
Sir Yahaya Memorial University (Birnin Kebbi, Kebbi State Nigeria)

Academic Qualification

Doctor of Philosophy (virology and molecular biology) **August 2009 – February 2014**
University of Malaya Malaysia

Master of Science Biomedical Sciences (virology and molecular biology) **February 2005 - February 2007**
International Islamic University Malaysia

Bachelor of Science Microbiology (microbiology) **July 1996 - November 2000**
Usmanu Danfodiyo University Sokoto Nigeria

Secondary School Certificate (sciences) **February 1992 - May 2005**
Government Science College Birnin Kebbi Nigeria

Junior Secondary School Certificate **January 1989 - November 1991**
Government Science Secondary School Gusau Nigeria

Primary School Certificate **January 1983 - May 1989**
Danturai Model Primary School Gusau Nigeria

Key Skills and Abilities

- A high level of ability and interest in medical science and biocomputing
- High ethical standards
- The ability to take responsibility for decision making
- An enquiring mind and good problem solving skills
- The ability to lead a research and development team
- Writing and verbal communicating skills
- Good knowledge of recent advances in infectious diseases

Research Experience

1. Advance Virology

- Basic virology (research and diagnostic)
- Virus infection (cells and animals)
- Virus titration
- Up-scaled propagation

2. Biocomputing offline/online

- Competent in using standard web-based nucleotide and protein blast-search engines
- Competent in sequence manipulation software, e.g. GeneDoc and Sequencher
- Competent in sequence alignment and phylogeny analysis program, e.g. ClustalX, MEGA5 and BEAST
- Knowledgeable in statistical analysis programs, e.g. SPSS, Sigma Plot and Prism GraphPad

3. Bacteriology

- Basic bacteriology (research and diagnostic)

4. Proteomics

- Proteins extraction methods (bacterial and cell culture samples)
- Protein separation, detection and visualization techniques
- Crystallization

5. Genome Sequencing

- DNA sequencing using ABI 3730xl sequencer
- DNA sequencing using ion torrent
- Sequencing data analysis

6. Immunological Techniques

- Immuno Blot techniques
- ELISA and Immunofluorescence
- Microneutralization
- Generation of polyclonal antisera in mice

7. Laboratory Maintenance

- Proposal writing, grant application
- Report preparation and presentations
- Daily maintenance and management of laboratory
- Training and supervision of post-graduate (Masters) and undergraduate students

8. Laboratory Animal Work

- Intra-cerebral inoculation of suckling mice
- Intra-peritoneal and subcutaneous inoculation of adult mice
- Tail and heart puncture bleeding

9. Molecular Biology

- DNA, RNA extraction and purification techniques
- PCR, RT-PCR and Long-PCR recombination technique
- Quantitative PCR
- Primer design
- Cloning techniques and plasmid extractions
- Electroporation and other transfection methods
- Site-directed mutagenesis

- Phage display

10. Tissue Culture Techniques

- Media preparation and sterilization techniques
- Maintenance of primary and secondary cell lines
- Cell cytotoxicity and proliferation assays

Publications

Papers

1. Predicting the emergence of predominant DENV-1 with potential to cause major outbreak in Malaysia (2012). **M.B. Danlami**, B.T. Teoh, J. Abd-Jamil, N.M. Mahadi, S. Abubakar. International journal of infectious diseases: volume 16 issue Page e252 DOI: 10.1016/j.ijid.2012.05.887 (ISI/SCOPUS Cited Publication)

2. Purifying selection in the evolution of dengue virus type 1 in Malaysia (2012). B.T. Teoh, S.S. Sam, K. -K. Tan, **M.B. Danlami**, J. Johari, M. -H. Shu, S. -P. Pang, N.A. Nor-Amdan, S.H. Hashim, V. Tiong, J. Abd-Jamil, N. Mat Rahim, S. Misbah, P.F. Wong, K. Zandi, N.M. Mahadi, S. Abubakar. International journal of infectious diseases: volume 16 issue Page e273 doi: 10.1016/j.ijid.2012.05.930 (ISI/SCOPUS Cited Publication)

3. Extract of *Scutellaria baicalensis* inhibits dengue virus replication (2013) Keivan Zandi, Tong-Hye Lim, Nor-Aziyah Rahim, Meng-Hooi Shu, Boon-Teong Teoh, Sing-Sin Sam, **Mohammed-Bashar Danlami**, Kim-Kee Tan and Sazaly Abubakar. BMC Complementary and Alternative Medicine 2013, 13:91 doi: 10.1186/1472-6882-13-91 (ISI/SCOPUS Cited Publication)

4. Rapid detection of dengue viruses from clinical samples using the single-tube reverse transcription-loop-mediated isothermal amplification assay (2013). Boon-Teong Teoh, Sing-Sin Sam, Kim-Kee Tan, Jefree Johari, **Mohammed Bashar Danlami**, Poh-Sim Hooi, Rafi Md-Esa and Sazaly AbuBakar. BMC Infectious Diseases 2013, 13:387 doi: 10.1186/1471-2334- 13-387 (ISI/SCOPUS Cited Publication)

5. Dengue virus type 1 clade replacement in recurring homogenotypic outbreak: possible association with oscillation of homogenotypic human herd immunity (2013). Boon-Teong Teoh, Sing-Sin Sam, Kim-Kee Tan, Jefree Johari, Meng-Hooi Shu, **Mohammed Bashar Danlami**, Juraina Abd-Jamil, Nor Aziyah Mat Rahim, Nor Muhammad Mahadi and Sazaly AbuBakar. BMC Evolutionary Biology 2013, **13**:213 doi: 10.1186/1471-2148-13-21 (ISI/SCOPUS Cited Publication)

6. Sazaly AbuBakar, Boon-Teong Teoh, Sing-Sin Sam, Kim-Kee Tan, Jefree Johari, **Mohammed Bashar Danlami**, Meng-Hooi Shu, David Brooks,

Piepenburg Olaf, Nentwich Oliver, Annelies Wilder-Smith 2013. Reverse transcription-recombinase polymerase amplification assay for rapid detection of dengue virus. The American journal of tropical medicine and hygiene, 2013; 89(5):321 (ISI/SCOPUS Cited Publication)

Proceedings

1. Sazaly AbuBakar, Boon-Teong Teoh, Sing-Sin Sam, Kim-Kee Tan, Jefree Johari, Mohammed Bashar Danlami, Meng-Hooi Shu, David Brooks, Piepenburg Olaf, Nentwich Oliver, Annelies Wilder-Smith 2013. Reverse transcription-recombinase polymerase amplification assay for rapid detection of dengue virus. 62nd Annual meeting of American society of tropical medicine and hygiene, Washington, USA. (Non-ISI/Non-SCOPUS Cited Publication)

2. Boon-Teong Teoh, Sing-Sin Sam, Kim-Kee Tan, Johari Jefree, **Mohamed Bashar Danlami,** Sazaly AbuBakar 2013. Development of a simple single-tube reverse transcription-loop-mediated isothermal amplification assay for detection of dengue virus from clinical samples. 8th European Congress on Tropical Medicine & International Health, Copenhagen, Denmark. (Non-ISI/Non-SCOPUS Cited Publication)

3. Hasliana Azrah Ab-Rahman, Nurhafiza Zainal, Pui-Fong Jee, Kim-Kee Tan, Boon-Teong Teoh, Sing-sin Sam, **Mohammed Bashar Danlami**, Asyura Amdan, Meng-Hooi Shu, Sazaly AbuBakar (2012). Optimization of *Burkholderia pseudomallei* Antigenic Protein Expression in Escherichia coli. 31st Symposium of the Malaysian Society for Microbiology (Non-ISI/Non-SCOPUS Cited Publication)

4. Sulaiman S, Zulkifle NI, Zainal N, Tan KK, Jee PF, Ab- Rahman HA, Teoh BT, Sam SS, **Bashar MD**, Nor-Amdan NA, Abubakar S. (2012). One-step purification and on column refolding of *Burkholderia pseudomallei* recombinant protein expressed in Escherichia coli. 31st Symposium of the Malaysia Society for Microbiology, Sabah, Malaysia, p. 136. (Non-ISI/Non-SCOPUS Cited Publication)

5. **M. B. Danlami**, B. T. Teoh, J. Abd-Jamil, N. M. Mahadi, S. Abubakar; Predicting the emergence of predominant DENV-1 with potential to cause major outbreak in Malaysia. 15th International Conference on Infectious Diseases, Bangkok 2012 (ISI/SCOPUS Cited Publication)

6. B. T. Teoh, S. S. Sam, K. -K. Tan, **M. B. Danlami**, J. Johari, M. -H. Shu, S. -P. Pang, N. A. Nor-Amdan, S. H. Hashim, V. Tiong, J. Abd-Jamil, N. Mat Rahim, S. Misbah, P. F. Wong, K. Zandi, N. M. Mahadi, S. Abubakar; Purifying selection in the evolution of dengue virus type 1 in Malaysia. 15th International Conference on Infectious Diseases, Bangkok 2012 (ISI/SCOPUS Cited Publication)

7. **Bashar M. D**. and AbuBakar S. Phylogenetic Analysis of the NS5 Gene of Dengue Virus Type 4 Isolated In Malaysia. Colloquium on Updates on Dengue and Arbovirus research in Malaysia, University of Malaya, 12-13 April 2010 (Non-ISI/Non-SCOPUS Cited Publication)

8. **Bashar M. D**. and AbuBakar S. Genetic Analysis of Malaysian Dengue Virus 4 Isolated from 1989-2007. 4th Asia Dengue Research Network Meeting, Duke-NUS Graduate Medical School, Singapore, 8-11 December 2009 (Non-ISI/Non-SCOPUS Cited Publication)

9. **Bashar M. D.** and AbuBakar S. Phylogenetic Analysis and Prediction of Antigenic Hotspots of Malaysian DENV- 4 Isolates. 3rd Regional Conference on Molecular Medicine, Kelantan, 2-4 May 2009 (Non-ISI/Non-SCOPUS Cited Publication)

10. **Bashar M. D**., Othman A., and AbuBakar S. Comparative Study of Mouse Brain Adapted Dengue Virus on BALB/c Suckling Mice. 2nd Asian Regional Dengue Research Network Meeting Singapore 2005 (Non-ISI/Non-SCOPUS Cited Publication)

Research Projects

1. Antigenic Evolution of Dengue Virus in Malaysia- Thesis submitted in fulfillment of requirement of Doctorate. Completed (Investigator)

   Key task: to determine antigenic evolution of dengue virus type 1 and type 2 in Malaysia

2. Dengue Genome: Dynamics of dengue virus antigenic epitopes in recurring dengue outbreaks, MOSTI-2008-2011, completed (Investigator)

   Key task: Sequencing of dengue virus genome

3. Malaysian Dengue Virus Genome Data Base, with Malaysia Genome Institute, University Kebangsaan Malaysia and Quantum Beez SDN BHD, MDeC, 2009-2011, completed (Investigator)

   Key task: Consultant on management and maintenance of dengue virus genome database

4. Innovative tools and strategies for surveillance and control of dengue (Novel diagnostic assay for resource-limited settings), EU-Dengue tools 2011-2014, in progress (Investigator)

   Key task: Evaluating dengue virus screening method in resource limited environment

5. Space crystallization of industrially important proteins on board the Japanese experimental module (JEM), MOSTI-2011-2013, in progress (Investigator)

   Key task: Designing, cloning and screening of the antigenic protein

6. Design and construction of DENV epitope driven vaccine, IPPP-2008-2009, completed (Co-Investigator)

   Key task: Construction of gene cassettes

Workshop and Seminars

1. **Workshop on the Laboratory Diagnosis** of Dengue: Department of Medical Microbiology Faculty of Medicine University of Malaya Malaysia. 9th-13th May 2005

2. **Basic SPSS: Data Analysis Workshop**: Information Technology Department International Islamic University Malaysia. 26th July 2007

3. **Discovery Studio Workshop**: Malaysian Genome Institute Malaysia. 21st – 25th July 2008

4. **Workshop on Protein Modelling**: Division of Bioinformatics and Biocomputing Institute of Biological Sciences University of Malaya Malaysia. 19th – 21st August 2008

5. **Confocal and Live Cell Imaging Seminar:** NIKON and micro Lambda Malaysia. 28th August 2008

6. **Pathway Studio Enterprises Workshop**: Institute of Biological Sciences University of Science Malaysia.3rd – 7th November 2008

7. **Genome Informatics**: Malaysian Genome Institute Malaysia. 15th – 19th December 2008

8. **Applied Biosystems 3730xl Genetic Analyzer Operational Training:** Annalisa Resources and Applied Biosystems. 6th – 9th January 2009

9. **UK-Malaysia Symposium on Current Strategies in Antimicrobial Therapeutics**: British High Commission Malaysia and Tropical Infectious Disease Research and Education Centre University of Malaya Malaysia. 18th – 19th March 2009

10. **3rd Regional Conference on Molecular Medicine**: Genomics, Proteomics and the Omics; Reality vs Dreams. Institute for Research in Molecular Medicine University of Science Malaysia. 2nd – 4th May 2009

11. **Colloquium on Dengue and Arbovirus Research in Malaysia**: Tropical Infectious Disease Research and Education Centre University of Malaya

Malaysia. 12th – 13th April 2010

12. **ESCO Biotech Training Workshop:** Laminar Flow and Biosafety Cabinet Technology and Fume Hood Awareness. ESCO Biotechnology Equipment Division and MATRIOUX. 12th May 2010

13. **25-Hours Molecular Research Laboratory Biosafety Training Program**: Tropical Infectious Disease Research and Education Centre University of Malaya Malaysia. 14th – 25thSeptemeber 2010

14. **15th International Congress on Infectious Diseases**: Internal Society of Infectious Diseases. 13th – 16th June 2012

Referees

Prof. Dr. Sazaly Abubakar Ph.D.
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