DENGUE PATHOGENESIS IN VIET NAM: AN EXPLORATION OF TRANSMISSION BLOCKING ANTIBODIES AND THE HOST RESPONSE TO DENGUE IN PREGNANT WOMEN

by

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Abstract

Dengue is the most prevalent arboviral disease that affects humans. Only a small proportion of patients develop severe disease and death is rare, but no specific therapeutics are available and there is only one licensed vaccine, which has limited efficacy and can only be administered to certain groups. The pathogenesis of dengue is also not fully understood. Studies on mAbs from dengue immune donors have provided insights into antibody-mediated protection and disease pathogenesis. Human mAbs neutralize DENVs propagated in highly-controlled cell-culture systems, but there is limited information on whether any of these mAbs neutralize DENVs in viremic blood from patients. I led the development of a ViBNA and characterized the potency of 12 human-derived mAbs (Chapter 2). I found five mAbs failed to neutralize DENV serotypes using the ViBNA, in contrast to the effects observed in cell-culture systems, highlighting the requirement for a more robust DENV neutralization assay. Seven mAbs neutralized at least one DENV serotype, and may have potential as therapeutic antiviral agents for dengue. In Chapter 3, I established 50% effective concentrations for three neutralizing mAbs, ranging from 0.013 μg/mL to 1.289 μg/mL, which could inform the dosing strategy for therapeutic trials of these mAbs. In Chapter 4 I examined both antibodies and other immune response components in pregnant and non-pregnant women with dengue, comparing a range of immunological and virological factors between these two groups. I found the percentage of CD8+ CD27- CD45RA- T cells, and the concentrations of eotaxin, monocyte chemoattractant protein, and macrophage-derived chemokine were lower in pregnant patients. These results contribute to the understanding of dengue pathogenesis in pregnant patients, and support further studies on immunological and virological aspects of dengue during pregnancy.

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achieve the vision which is to have local, regional and global impact on heath by leading a locally driven research program on infectious diseases in Southeast Asia.

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Authorship

I am responsible for the content of this thesis, of which I wrote every section with support from my supervisors (Dr. Lauren Carrington, Prof. Cameron Simmons, and Prof. Bridget Wills). I performed the majority of the laboratory experiments (except as indicated below) including homogenization and RT-PCR of mosquitoes, preparation of human immune globulin, culture of C6/36 cells and dengue viruses, BCA protein assay, phenotyping of lymphocytes, and genotyping of HLA. I also collated a range of data from patients and mosquitoes, screened the data, and conducted all statistical analyses with help from Dr. Lam Phung Khanh in the biostatistics group.

Nevertheless, I could not have completed this thesis without enormous contributions from my colleagues. Specifically, the molecular team of the Dengue group performed RNA extraction and RT-PCR on plasma samples collected from patients, sequencing, NS1 ELISA, IgM capture ELISA, and IgG indirect and capture ELISA. The insectary team conducted mosquito preparation, blood feeding, intrathoracic inoculation and mosquito collection. The enrolment, clinical management and follow-up of patients was carried out by the doctors and nurses of the Hospital for Tropical Diseases and the OUCRU. Finally, collaborators at the Singapore Immunology Network measured cytokines in plasma of dengue patients for me, and a member of the OUCRU Zoonosis group performed the SDS-PAGE for monoclonal antibodies.

This thesis has not been submitted for a degree or other qualification to this or any other university.

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Abbreviations

ADCC	Antibody dependent cell mediated cytotoxicity
ADE	Antibody dependent enhancement
ALT	Alanine aminotransaminase
AST	Aspartate aminotransaminase
BCA	Bicinchoninic acid
Cap	Capsid protein
cDNA	complementary DNA
CI	Confidence interval
CM	Central memory
CNS	Central nervous system
DI	Domain 1
DII	Domain 2
DIII	Domain 3
DENV-1, 2, 3, 4	Dengue virus serotype 1, 2, 3, 4
DENV(s)	Dengue virus(es)
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DNA(s)	Deoxyribonucleic acid(s)
DSS	Dengue shock syndrome
E	Envelope
EC_{50}	50% effective concentrations
EGF	Epithelial growth factor
ELISA	Enzyme linked immunosorbent assay
EM	Effector memory

EMRA	Effector memory re-expressing CD45RA
Fab	Antibody binding fragment
Fc	Crystalization fragment
FGF-2	Fibroblast growth factor 2
Flt-3L	FMS-like tyrosine kinase 3 ligand
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GRO	Growth regulated oncogene
GSK	GlaxoSmithKline
HCT	Hematocrit
HIG	Human immunoglobulin
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTD	Hospital for Tropical Diseases
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
IP-10	Interferon gamma induced protein 10
IQR	Interquartile range
JEV	Japanese encephalitis virus
KIR(s)	Killer cell immunoglobulin-like receptor(s)
LCs	Langerhans cells
M	Membrane
mAb(s)	Monoclonal antibodies

MCP-1	Monocyte chemoattractant protein
MDC	Macrophage-derived chemoattractant
MHC	Major Histocompatibility complex
MICB	MHC class I chain-related protein B
MID	Multiplex identifier
MIP	Macrophage inflammatory protein
NA	Not applicable
NK	Natural killer
NKG	Natural killer cell group
NS	Nonstructural protein
OFI	Other febrile illness
OR(s)	Odds ratio(s)
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethics Committee
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
prM	pre-membrane
PRNTs	Plaque reduction neutralization test(s)
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA(s)	Ribonucleic acid(s)
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase polymerase chain reaction

sCD40L	Soluble cluster of differentiation 40 ligand
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sIL2A	Soluble interleukin 2A
sTNFR	Soluble tumor necrosis factor receptor
sVEGFR	Soluble vascular endothelial growth factor receptor
TCR(s)	T cell receptor(s)
TGF-α	Transforming growth factor *alpha*
TGF-β	Transforming growth factor *beta*
TNF	Tumor necrosis factor
VCD	Virologically confirmed dengue
VEGF	Vascular epithelial growth factor
WHO	World Health Organization
YFV	Yellow fever virus
ZIKV	Zika virus

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Chapter 1

Introduction

1.1 Dengue

Dengue is the most prevalent arboviral disease to affect humans. It is an illness of major global health significance that is particularly prevalent across Asia and Latin America [1]. DENVs, the causative agent, are transmitted by Aedine mosquitoes. There are four antigenically distinct but related viral serotypes that may all cause disease. Dengue is an acute systemic disease with a wide spectrum of clinical of manifestations, although the majority of DENV infections are actually asymptomatic [2]. When symptomatic, infected individuals typically progress through three clinical phases known as the febrile, critical and recovery phases. Most people recover without complications, but severe dengue may develop in a small number of patients. There are several case reports of pregnant patients with severe dengue [3, 4]. The epidemiological and clinical characteristics of dengue are described below with a focus on clinical aspects of dengue during pregnancy.

1.1.1 Dengue epidemiology

The first epidemics of what is now thought to have been dengue occurred in 1779 and 1780 on three continents - Asia, Africa and North America [5]. Over the next couple of hundred years a gradual expansion of disease occurred across the tropics and subtropics until the mid-twentieth century. However, during the last 50-60 years the incidence of the disease has increased dramatically, particularly in Southeast Asia, with estimates now ranging from 58 to 96 million symptomatic cases occurring worldwide each year [1, 6]. In line with

the expanding footprint of dengue, the number of countries reporting dengue has increased globally, rising from 9 countries reporting disease in 1970 [7] to 128 countries in 2012 [8]. Mortality is generally low, with estimates varying from 9,000 [6] to 20,000 [1] cases per year globally, but the overall burden on public health systems in endemic countries is considerable. Recent estimates suggest a cost of 1.14 million disability-adjusted-life years [9] and US $8.9 billion annually [6].

In tropical and subtropical regions, seasonality of peak transmission varies by country according to the timing of the rainy season, which provides optimal conditions for mosquitoes to breed. Rapid urbanization and climate change are other factors that have influenced the spread and persistence of *Aedes aegypti* in new locations [10]. International travel and the expansion of the global shipping industry have also allowed inadvertent introduction of both DENVs and *Aedes* mosquitoes to non-endemic regions. For example, the major European DENV-1 outbreak that occurred in Madeira, Portugal, in 2012 [11] is thought to have originated from Venezuela [12], with the virus potentially carried by tourists on cruise ships and then introduced to the local mosquito population [13].

The distribution of the four DENV serotypes varies considerably between geographical locations and over time. For example, DENV-2 was prevalent in Ho Chi Minh City (Vietnam) in 2006 while DENV-1 and DENV-4 were dominant in Bangkok (Thailand) in the same year [14]. The same article describes how DENV-1 gradually replaced DENV-2 and DENV-4 in Ho Chi Minh City between 2002 and 2010. The introduction of a different DENV serotype to a population with previous exposure to another serotype creates the

potential for repeat dengue episodes in the same individual. Infection with one serotype provides lifelong immunity to that serotype, but only short-term protection against the remaining serotypes, and there is a complex interplay between immune status, repeat infections with different serotypes, and clinical severity (discussed in Section 1.4.3.2)

Age is also a factor in the epidemiology of clinical disease. Dengue used to be considered primarily as a disease of children, and in hyperendemic settings almost all individuals have already experienced at least one DENV infection by the time they reach adulthood. However, with the expansion into previously non-endemic countries an increase in the age of symptomatic dengue patients has been noted in some studies, thought to be related to immune status. In a recent meta-analysis summarizing information from publications describing dengue outbreaks between 1990 and 2015, the mean ages of patients reported before and after 2010 were 27 and 34, respectively [15]. However, given that outbreak reports rarely originate from hyperendemic settings (where large dengue caseloads are not considered remarkable), the data are likely biased towards locations with lower endemicity. In the same report, the proportion of patients who had DHF significantly decreased after 2010 [15].

In Vietnam, dengue is a major public health issue, especially in the southern provinces during the rainy season from around June to October. Weekly reported cases usually increase progressively from May/June onwards, as shown for 2019 in

Figure 1.1, peaking at around 8,000 cases per week in mid-August [16]. An overview of annual estimates for the country from 2007 to 2016 showed an average of 90,884 cases reported each year [17]. However, given that only hospitalized cases are reported, the actual number of dengue cases must be much higher. Estimates indicate more than 2 million cases occur annually [1, 6], including those treated outside formal health care sectors, in outpatient settings, and as inpatients. In 2013 dengue was estimated to be responsible for 39,884 disability-adjusted life years in Vietnam [9], but it is clear that notable differences exist in the methodology used by different groups for estimating case numbers and for calculating disability-adjusted life years.

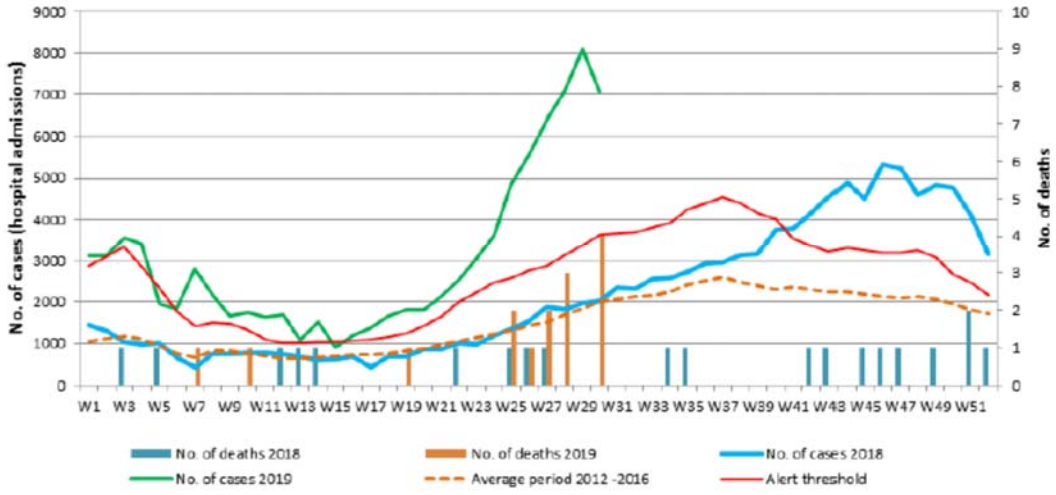


Figure 1.1: Reported dengue cases and mortality in Vietnam

The data were reported weekly to the WHO in 2018 and 2019. X-axis indicates time in weeks. (The figure was copied from the regional update for dengue [16].)

1.1.2 Dengue symptoms and progression

Conventionally symptomatic illness is considered to have three phases (Figure 1.2) [18]. Common symptoms during the febrile phase include fever, headache, general malaise, nausea, vomiting, myalgia, joint pain, and abdominal pain [19]. Laboratory tests carried out at this time often demonstrate thrombocytopenia, leukopenia, a decrease in neutrophils, and elevated hepatic transaminases [19]. Virus is also usually detectable in blood during this phase, with plasma viremia decreasing gradually to undetectable levels over 4-5 days from fever onset [18]. In most symptomatic patients, the febrile phase resolves without complications, but in a small number of cases complications develop as the fever settles and the patient enters the critical phase. This normally coincides with the time of viral clearance and the onset of detectable immune activation, indicating that patients' immune responses play a role in dengue pathogenesis.

The complications that can occur during the critical phase include a systemic vascular leak syndrome, coagulation abnormalities that may be associated with bleeding, and organ involvement, typically hepatic or neurological [20]. Mild vascular leakage is often undetectable, but moderate leakage can result in clinically detectable signs (such as pleural effusions and ascites) often accompanied by increasing hemoconcentration. When severe, leakage may lead to development of hypovolemic shock – i.e. dengue shock syndrome (DSS). Very severe cases, usually those with profound or prolonged shock, may go on to develop metabolic acidosis, disseminated intravascular coagulation, organ impairment and severe hemorrhage [18].

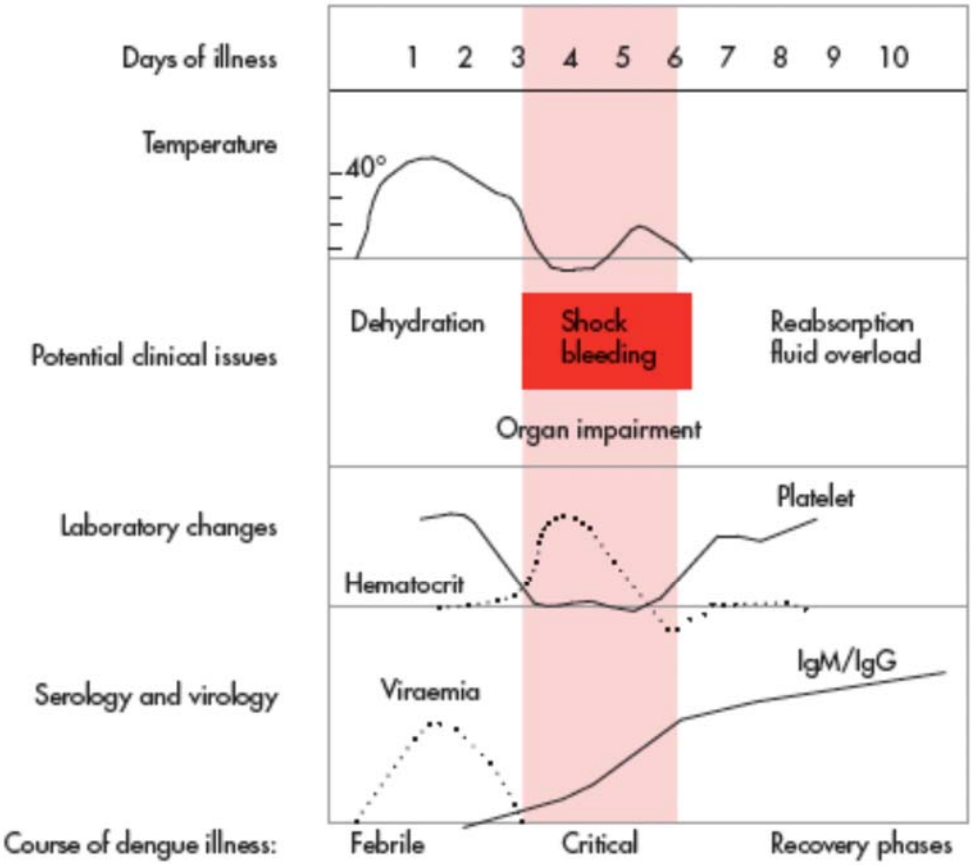


Figure 1.2: The course of dengue

Dengue typically progresses through three different phases: febrile, critical and recovery phases. The changes of clinical and laboratory parameters, over a period of 10 days of illness, are shown. (The figure was copied from the WHO guidelines for dengue [18].)

Fortunately, even among patients with severe disease, with good supportive care most patients recover. During the recovery phase (from around day 6-7 of illness), the altered vascular permeability returns to normal and leaked fluid is spontaneously reabsorbed, causing a decrease in the hematocrit. Both the white blood cell count and platelet count

usually recover promptly after fever resolves. In some cases a florid “convalescent” rash may persist for several weeks [19]. In some adults recovery can be prolonged, with profound tiredness and depression lasting for weeks to months after the acute illness has resolved [21].

1.1.3 Dengue classification

WHO developed guidelines and a classification system to help with diagnosis and management of dengue. Originally proposed in the 1960s, the classical guidelines separated symptomatic dengue into two main clinical syndromes, DF and DHF, plus the important subcategory of DHF called DSS. According to these guidelines DF is characterized by fever and systemic symptoms (headache, myalgia, arthralgia etc.) that last for around 5-7 days and may be disabling, while patients with DHF experience plasma leakage, thrombocytopenia, and bleeding in addition to the DF syndrome. DSS is diagnosed when individuals with DHF develop hypovolemic shock due to plasma leakage. However, clinical observations over time and in different countries have shown that some of the symptoms thought to be specific for DHF (for example, plasma leakage, and hemorrhage), also occur in apparently uncomplicated DF cases. Conversely, some patients with DSS do not fulfill the four necessary criteria for DHF (including specific lab parameters), or else these cannot be demonstrated until after the patient has recovered, making the system unsuitable to guide clinical management [22].

To overcome these difficulties, WHO developed a revised system in 2009 [18]. The revised system is intended to be simpler and to be used in real time, with dengue classified into one of two categories at each clinical assessment: dengue or severe dengue (Figure 1.3). The criteria for dengue remain similar to those for DF in the original classification system, while severe dengue is diagnosed as soon as a patient develops serious plasma leakage, hemorrhage or organ impairment. Reassessment should be performed regularly, and management is determined by the classification status at the time of each assessment. There is no requirement for specific laboratory values.

Being able to reliably predict who will develop severe dengue could be very useful to facilitate triage and optimal use of limited resources in endemic areas. As shown in Figure 1.3 a number of clinical warning signs have been highlighted by WHO as potential signals of impending deterioration. It is recommended that patients who develop any of these warning signs should be hospitalized for close monitoring. However, the revised WHO classification also highlights the unpredictable progression of the infection by acknowledging that severe dengue can occur without these warning signs. In addition, some warning signs, for example, lethargy and restlessness, are difficult to define and can be common in patients with uncomplicated dengue. Thus, while the revised classification has increased the capability for early diagnosis of severe cases, if all patients who develop those common warning signs are hospitalized during dengue epidemics, the resulting public health and economic burden in resource limited countries can be significant. Therefore, some countries have not adopted the revised classification, but continue to use the original DF/DHF system.

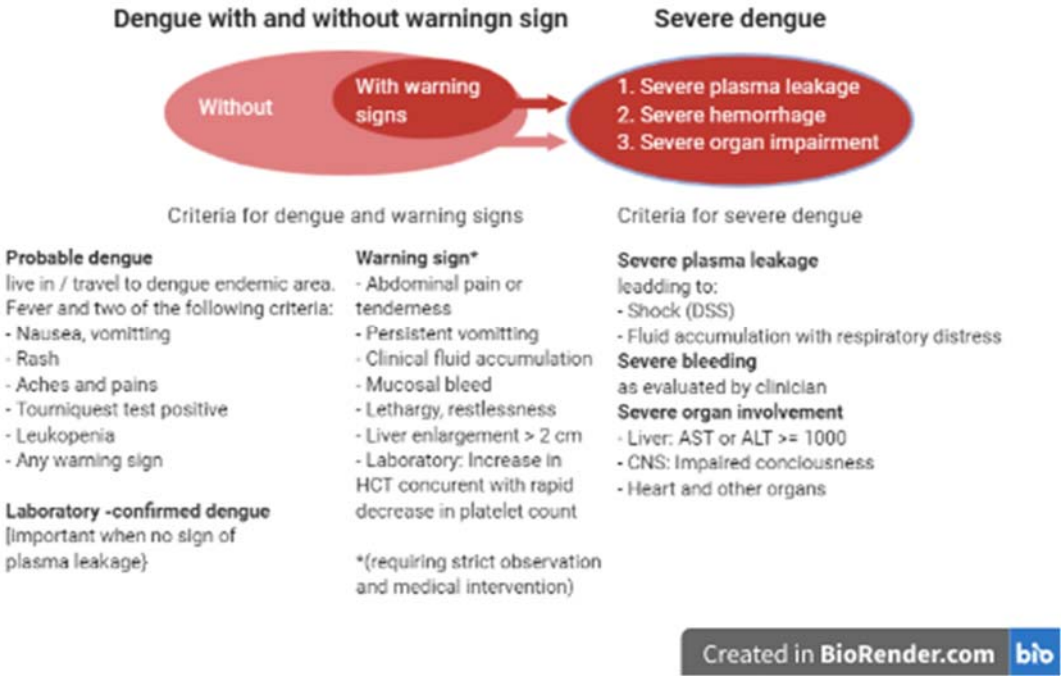


Figure 1.3: Dengue case classification and severity

Dengue is classified into dengue (± warning signs) and severe dengue. Criteria for all forms of the illness are detailed. Note that severe dengue can develop with or without any observable warning signs. (The figure was redrawn from the WHO guidelines for dengue [18].)

1.1.4 Dengue diagnosis

In endemic areas, for acute case management diagnosis of dengue is mainly based on clinical signs and symptoms. However, it is also important that efficient and accurate diagnosis is available for outbreak control, surveillance activities, and academic research. Laboratory diagnosis can be carried out using a range of assays, with the three main laboratory-based approaches being a) molecular virology to detect viral antigens, b) direct

virus culture, and c) serology. The best use of these different diagnostic methods is influenced by the setting and the timing of sample collection. Generally, for samples collected in the early days of the acute period, detection of viral particles is preferred. From the end of the acute period onward, serological methods which target DENV-reactive immune responses are preferable. Advantages and disadvantages of the three approaches are discussed below.

Virological methods, which target DENV biomolecules, are the most robust approach for laboratory diagnosis of dengue. The RT-PCR detects the presence of viral RNA with high sensitivity and specificity [23]. However, RT-PCR is expensive, and requires trained staff and specialized facilities which are not often available in low-resource settings. As an alternative, there are antigen-based rapid diagnostic tests available, which detect one of the DENV non-structural proteins called NS1 (see Section 1.3.1). These NS1 rapid assays are often preferred by clinicians due to the technical simplicity and short turn-around time. However, the sensitivity of the NS1 rapid assays varies by DENV serotypes, being generally lower for DENV-2 infections [24].

Another diagnostic method is virus culture, which is often used for disease surveillance and research. This is considered the gold-standard method for dengue diagnosis and allows the infectious DENVs to be maintained for downstream research including sequencing. However, the method is not used in clinical settings because of the requirement for specialized facilities and highly trained staff. Furthermore, this technique provides no

benefit for clinical management because the clinical outcomes of dengue are characterized before results are available (after 3-4 weeks).

Serological methods that target anti-DENV IgM (either formal ELISA tests or rapid tests) can be helpful for clinical management, since these generally become positive from around day 5 of illness. Anti-DENV IgG assays can be used for dengue surveillance and research at a population level. There are two types of IgG assays - indirect and capture ELISAs. Indirect IgG assays are very sensitive and can detect low levels of dengue specific IgG; they are usually used on single specimens to assess prior infection status for surveillance/epidemiological purposes rather than for diagnosis of acute DENV infection. Conversely anti-DENV capture IgG assays are used to confirm acute DENV infection based on changes in antibody titers over time, indicating seroconversion; two consecutive samples, taken several days or weeks apart, are required.

One limitation of serological methods is that the antibodies detected are often cross-reactive, i.e. they may also bind to other flaviviruses [25]. For this reason, results of serological assays must be interpreted with caution, especially in areas where other flaviviruses, such as JEV, ZIKV or YFV, are in co-circulation.

Another serological method is the PRNT. PRNTs employ DENV reference strains and cultured cells to quantify neutralizing antibodies (described in detail in Section 1.4.3.3). Compared to other serological methods, the use of PRNTs can enable serological identification of the particular DENV serotypes an individual has experienced, potentially

providing information on immune status (i.e. whether an infection is a primary or secondary exposure). However, PRNTs are typically only applied in research and for surveillance, since the test requires a specialized laboratory, well-trained staff, and the materials are not standardized, i.e. different laboratories employ different DENV strains and cell lines.

1.1.5 Management of dengue

Dengue is managed primarily by careful observation for warning signs together with provision of supportive care, for example the use of anti-pyretic drugs and pain killers, aiming to reduce the magnitude and duration of symptoms. Intravenous fluids are sometimes needed during the febrile phase for persistent vomiting or dehydration. If the patient develops shock, prompt fluid resuscitation is required, which can be very effective and result in low mortality if applied carefully [26]. The principle of intravenous fluid therapy is to compensate for the volume of leaked plasma. For initial resuscitation, isotonic crystalloid fluids (e.g. 0.9% normal saline and ringer lactate) can be used. For patients with profound shock or for those who do not respond to crystalloid fluid, colloid solutions (e.g. hydroxyethyl starch and gelofusine) may be alternatives [27]. The resuscitation must be very carefully monitored to avoid causing fluid overload, which can lead to respiratory difficulties so that the patient may need artificial ventilation.

At present there are no effective antivirals against DENVs despite clinical trials involving a number of potential candidates, including chloroquine [28], balapiravir [29], lovastatin [30], and celgosivir [31]. Although these drugs were shown to be safe in these studies,

none demonstrated a reduction in viremia during the febrile phase, or mitigated the risk of progression to severe disease. An alternative approach that has also been considered is to use immunosuppressive therapy. Since the major complications of dengue usually occur during the critical phase as fever and viremia are resolving, an augmented immune response is believed to contribute to pathogenesis (see Section 1.3, and 1.4). However, a randomized controlled trial showed that early use of prednisolone during the febrile phase did not result in clinical benefits [32], and also did not alter plasma cytokine levels [33].

1.1.6 Dengue during pregnancy

Dengue during pregnancy is a theme that has gained attention over the last few years because of the recent emergence of a range of complications, notably microcephaly and other serious neurological abnormalities, among babies born to women who had experienced ZIKV infections (i.e. a closely related flavivirus) during pregnancy [34, 35]. Although it is known that DENV infections occurring close to delivery may be vertically transmitted from pregnant patients to their infants [36], clinical evidence for the general consequences of dengue on pregnant women and their babies is limited.

When pregnant women have dengue, these two health-related events can have an effect on each other. While dengue predisposes pregnant women to obstetric complications, such as maternal death [37], preterm labor and bleeding [3], there is little published literature about potential complications for the fetus. In one recent systematic review and meta-analysis for fetal outcomes of dengue during pregnancy, the authors found increased risks for miscarriage, preterm birth, and low birth weight [38]. The association between preterm

birth and dengue during pregnancy was also identified in a retrospective cohort analysis using large-scale passive surveillance datasets based on the Brazilian national reporting systems for dengue and livebirths [39]. Although stillbirth could not be analyzed in these two studies, the risk of stillbirth was found to be increased in a retrospective case-control study that analyzed data from other formal Brazilian public health information systems [40]. In one small prospective matched cohort study about dengue during pregnancy, a borderline association between stillbirth and dengue was reported [41].

Conversely, pregnancy may affect the clinical presentation and management of dengue. In particular, dengue can be confused with obstetric syndromes such as the gestational HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), due to similarity in clinical manifestations [42]. In this situation, incorrect diagnosis may lead to a delay in appropriate clinical management of dengue. Another potential effect of pregnancy on dengue management occurs if a patient develops severe dengue and fluid management is required; resuscitation must be handled with assiduous attention to avoid complications resulting from fluid overload since pregnancy itself alters vascular permeability and plasma volume.

1.2 Prevention of DENV infections

There are two major strategies to prevent dengue. The first strategy targets mosquitoes while the second strategy relies on preventing infection in humans through vaccination. Up to now conventional vector control strategies have failed to mitigate the risk of DENV infections, reflected by continuing seasonality of dengue in endemic regions. To overcome

this challenge, novel vector-based strategies have been developed and are being trialed. Although vector control and vaccine strategies have separate targets, an integrated approach will likely result in the greatest reduction of DENV transmission [43]. The sections below summarize vector-control strategies, as well as describing the current situation with dengue vaccines.

1.2.1 Vector control

Global prevention of DENV infection has largely relied on controlling the mosquito vector, in particular, the primary vector, *Ae. aegypti*. Because the vector lays eggs in containers of stagnant water, for example flower pots and used tires, managing breeding sites can be an effective method to control the vector population. Other classical measures include the application of insecticides and the use of biological agents which suppress the development of larva and/or adult mosquitoes [44]. Vector control was shown to be successful in suppressing transmission of DENVs in the 1950s and 1960s. A regional program implemented across much of the Caribbean, Africa, Central and South America reduced mosquito abundance and decreased the transmission not only of DENVs but also of other mosquito-borne pathogens such as malaria parasites [45]. Similarly, in the last decade, Singapore has conducted successful programs which targeted larval development [46].

However, these classical vector control approaches require intense effort and tend to be difficult to sustain, indicated by the geographically expanding global distribution of *Ae. aegypti*. As a result, several novel control strategies are now being explored in the hope that these will be more effective [47]. One approach involves introduction of Wolbachia

species into *Ae. aegypti* mosquitoes - Wolbachia are symbiotic bacteria that suppress viral replication in infected mosquitoes [48]. Once Wolbachia carrying mosquitoes are released, they breed with wild mosquitoes and gradually become established in the population due to cytoplasmic incompatibility [49]. Field trials to assess whether this intervention strategy reduces transmission of DENVs (and other arboviruses carried by *Aedes* mosquitoes) are ongoing in several countries.

Another novel approach is to modify the genes of male mosquitoes. The sterile insect technique (SIT) uses radiation to generate sterile male mosquitoes, which are then released into the field and reduce the reproductive potential of the target population [50]. However, sterile male mosquitoes have reduced ability to compete for wild-type female insects for breeding, so an alternative technique called "release of insects carrying a dominant lethal" (RIDL), has been developed [51].

1.2.2 Vaccines

Development of dengue vaccines has been an area of study for almost a century [43]. Currently one vaccine (Dengvaxia) is licensed for use in several endemic countries, while two other vaccine candidates are in phase 3 clinical trials and a number of others are at different stages of research and development.

Dengvaxia, developed by Sanofi Pasteur, consists of four monovalent live-attenuated recombinant viruses, with the structural genes of the four DENV serotypes replaced in a YFV backbone [52]. Results of two large phase 3 trials, published within the last 5 years,

showed moderate efficacy against virologically-confirmed dengue [53-55], but with greater protective efficacy against severe dengue [43]. However, in these trials the efficacy of Dengvaxia depended on the age of the vaccine recipient although a possible effect of the individual's immune status at the time of vaccination could not initially be excluded. Post-hoc analysis of the data confirmed that, irrespective of age, participants who had not been exposed to DENVs before the first vaccination were more likely to develop dengue and severe dengue during the later years of study follow up, compared with participants who received placebo [56]. In light of this, a WHO position paper was published in 2018 stating that Dengvaxia was not recommended for individuals who had not already been exposed to a DENV, and recommending a pre-vaccination screening strategy based on either prior documented lab-confirmed dengue or a positive serology test indicating a prior exposure [57]. However, PRNTs for neutralizing antibodies, the method conventionally used to identify people with prior exposure, require special expertise and equipment that are not available in limited resource settings. Moreover, the new guideline from WHO has come rather late because Dengvaxia has already been licensed in several endemic countries and has been used without serology testing, raising substantial concerns regarding the risk of developing dengue and severe dengue in seronegative vaccine recipients. The clinical trials of Dengvaxia have also highlighted the discrepancy between PRNT data and vaccine efficacy results, suggesting that PRNT results are not a very robust correlate of protective immunity for dengue. While vaccine-induced immunogenicity (as assessed using the PRNT) was high, the vaccine efficacy was moderate. One possible reason is that the PRNT results include measurement of immune-dominant but poorly-neutralizing antibodies that may not be effective in the context of natural DENV infection (see Section 1.4.3.3).

Another important point is that Dengvaxia contains the non-structural genes of YFV rather than DENV, and this appears to elicit an incomplete T-cell immune response to DENVs (see Section 1.4.3.1). Both the structural and non-structural genes of DENVs are included in the other two vaccine candidates currently undergoing phase 3 clinical trials, and these vaccines are believed to elicit relevant and more complete immune responses to DENVs. The first candidate, named TV003/TV005, was developed by the American National Institute of Allergy and Infectious Diseases, and includes live attenuated viruses of DENV-1, DENV-3, DENV-4 with a chimeric DENV-2 [58-60]. The other vaccine candidate, TDV, was developed by Takeda and is composed of an attenuated DENV-2 strain and three chimeric viruses, DENV-1, DENV-3 and DENV4 [61].

1.3 Dengue virology

DENVs, along with other viruses such as JEV, West Nile virus, YFV and ZIKV, belong to the genus *Flavivirus*, family *Flaviviridae*. The four genetically distinct DENV serotypes are named DENV-1 to DENV-4. The following sections outline the viral structure, genome and proteins of DENVs, and describe the life cycle and maturation process, with particular reference to the immunological responses induced.

1.3.1 Genome and proteins

DENVs are a group of positive-sense RNA viruses. The genome contains a long open reading frame that encodes a single large polyprotein that is post-translationally processed into seven non-structural proteins and three structural proteins. While the non-structural

proteins are involved in the viral replication process, the structural proteins form the protective shell that covers the viral RNA. Table 1.1 indicates roles of structural and non-structural proteins.

The structural proteins include the envelope (E), membrane (M) and Capsid (Cap) proteins. The Cap protein is located at the core of the virus and interacts with the viral genome, while the E and M proteins are present on the surface of DENVs. Therefore, the E and M proteins are viral antigens that trigger major immune responses.

There are 180 E protein molecules, arranged as dimers, on the surface of each DENV particle. Each E protein monomer is composed of three domains; domain I (DI), domain II (DII) and domain III (DIII) (Figure 1.4). All of these domains are important for DENV infection of host cells – DIII contains residues that bind directly to host cell receptors, while DI and II are important for rotation of the E protein, a process that is required to infect cells. Furthermore, at the tip of DII, there is a fusion loop, which plays an important role in the entry of DENVs to the cell. Taken together, DI, DII, DIII and the fusion loop are the major targets for the immune components that disrupt DENV infection of cells.

M proteins exist initially in the form of pre-membrane (prM) molecules, which are cleaved into pr and M peptides during the maturation of DENVs. PrM protein binds to E protein, and is essential for mediating proper folding of the E protein. Each DENV particle contains 180 prM molecules, equal to the number of E protein monomers. Each prM molecule is composed of three domains, a pr domain at the amine terminus, followed by an M domain

and a transmembrane region. Protein prM is highly conserved across the four DENV serotypes, and as a result, prM-specific immune responses are cross-reactive among the four DENV serotypes [62].

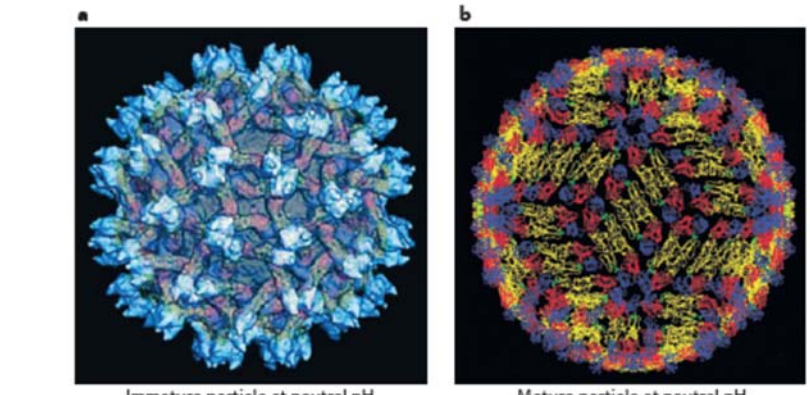


Figure 1.4: Virus particles with two maturation states

A) A spiky, immature virus. E proteins are shown in pink, while prM proteins are in blue. B) The smooth, mature virus with E dimers: domains I, II and III are shown in red, yellow and blue respectively. The fusion loop is in green. (Permission for use of this figure was obtained from the journal [63].)

Table 1.1: Roles of DENV proteins in pathogenesis

DENV proteins	Role
E	Binds to cell receptors Involved in the fusion between DENVs and endosomes [64]
prM	Mediates proper folding of E proteins [65]
C	Compacts viral RNA [5]
NS1	Participates in viral RNA replication [66] Contributes to plasma leakage [67] Impairs complement activation [68]
NS2A	Participates in viral RNA replication and virion assembly [69]
NS2B	Binds to NS3 and forms a protease [70] Binds to NS3 and inhibits type-I interferon responses [71] Prevents mitochondrial DNA sensing [72]
NS3	Binds to NS2B to form a protease [70] Binds to NS2B and inhibits type-I interferon responses [71] Unfolds viral RNA [73]
NS4A	Participates in viral RNA replication [74] Induces alterations in membranes [75]
NS4B	Participates in viral RNA replication [74] Dissociates NS3 from single-stranded RNA [75, 76]
NS5	An RNA-dependent RNA polymerase, which synthesis new RNA [77] Methylates viral RNA [78] Inhibits IFNγ signaling [79]

1.3.2 Viral life cycle and maturation

The DENV life cycle is the sequence of stages that the viruses undergo within cells of both humans and *Ae. aegypti* mosquitoes (Figure 1.5). There is little evidence for a difference in DENV life cycle between the two species, so the following description applies to both.

The life cycle commences when DIII of the E protein binds to cellular receptors, for example dendritic cell-specific ICAM-grabbing non-integrin. This binding facilitates viral entry through endosomes. The acidic environment of endosomes induces a change in the conformation of the E protein, so that DI and DII of the E protein rotate to expose the fusion loop. This loop then merges with the endosomal membrane to release viral RNA into the cytoplasm. The positive-sense RNA is translated into a continuous polypeptide by cellular ribosomes, located in the rough endoplasmic reticulum. The polypeptide is then post-translationally processed into viral proteins which assist the replication of the viral genome. The newly synthesized genome is packaged by Cap, and the nucleocapsid travels into the lumen of endoplasmic reticulum. Here, the viral components are assembled to generate a DENV virion, which is transported in the acidic trans-Golgi network where a cellular protease, named furin, cleaves the linkage between pr and M domains while they still remain attached. The viral particle is then secreted out of the cells. Due to the neutral pH of the extracellular environment, the pre domain detaches from the M domain, and E monomers rotate to form dimers. After this, the DENV particle is mature and capable of infecting other susceptible cells.

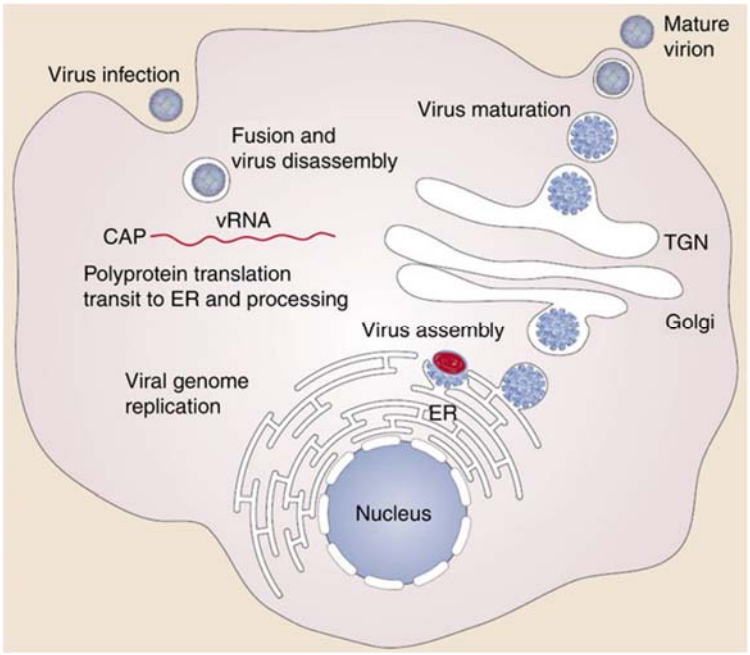


Figure 1.5: Key steps in the life cycle of DENVs

The virus enters susceptible cells through endosomes. DENVs then fuse with endosomes to release viral RNA (in red and abbreviated as vRNA). Next, translation of viral RNA occurs in the endoplasmic reticulum (ER), in association with ribosomes on the surface of ER membranes, to produce a polypeptide which assists with replication of the viral genome. The genome and other structural components are assembled in the ER lumen, and the viral particle is then transported through the trans-Golgi network (TGN). Here, furin-mediated cleavage occurs allowing the viral particle to mature. Next, the mature virion is released and is capable of infecting another susceptible cell. (Permission for use of this figure was obtained from the journal [80].)

Because the activity of furin is often incomplete, DENVs secreted from the host cell can be a mix of completely-mature, completely-immature, and partially-mature viruses [81-83]. This variation in maturation states allows DENVs to infect susceptible cells with different mechanisms, i.e. mature DENVs infect cells after direct binding to cell receptors, while immature DENVs enter cells through a process mediated by the immune system [63].

While both immature and mature DENVs can be infectious, there is a major structural difference between the two maturation states. The structure of immature DENVs is spiky, in contrast to the smooth surface of mature viruses. This significant difference in structure is attributed to the arrangement of the viral E protein molecules. A completely-mature DENV has 90 E-protein dimers, while a wholly-immature DENV contains 60 spikes, each including three E monomers (Figure 1.4).

1.4 Dengue immunology

Immunity, classified into innate and adaptive responses, is the ability of humans to combat unwanted infections or other invasions by foreign material. Innate immunity is the first line of immune responses and comprises a series of strategies, involving both biomolecules and cellular components, which are intrinsic to each individual. By contrast adaptive immunity develops following exposure to a particular antigen, and then plays an essential role in subsequent attacks by similar antigens [84]. Cytokines mediate the recruitment of both innate and adaptive immune cells to the site of infection. In the sections below, details

about the structure, functions, and interactive mechanisms of several immune components that are important in the context of DENV infection are described.

1.4.1 Cytokines

Cytokines are a broad category of small innate proteins important for signal transduction between cells. Based on their functions, cytokines can be classified into five groups, including pro-inflammatory cytokines such as IL-1 and TNF, anti-inflammatory cytokines (IL-10), mixed pro-inflammatory and anti-inflammatory cytokines (IL-6), growth factors such as EGF, and chemokines (IL-8) [85].

Cytokines are thought to contribute to dengue pathogenesis, as evidenced by changes in cytokine responses in the acute phase of the disease. In the clinical trial of Dengvaxia, plasma cytokine levels in the 207 breakthrough patients were measured by a Luminex assay and compared with levels measured on pre-infection samples. Among the 38 tested cytokines, concentrations of 12 cytokines, including eotaxin, FGF-2, IFNγ, IL-10, IL-1Ra, IL-1α, IL-8, IP-10, MCP-1, MDC, TNFα, and sCD40L were increased in the acute phase when compared with levels one-month post the last vaccine dose [86]. In an observational study on 134 children with dengue in Rio de Janeiro in 2008, plasma levels of TNF, IL-10, IP-10, and IFNγ were increased during the acute phase of dengue, compared with the convalescent (recovery) phase [87]. Another observational study on 44 dengue patients in Kuala Lumpur from 2005 to 2009 found the plasma level of IP-10 in dengue patients was higher than healthy donors [88]. The use of blood from healthy donors as the comparison group in this study (rather than the patients themselves acting as controls either before the

illness episode or after recovery) may be a drawback of this study because of the variability in “normal” cytokine levels between individuals.

While cytokine levels increased in dengue patients at the acute phase, for those who develop more serious clinical manifestations (i.e. DHF or severe dengue), the magnitude of the change in cytokine levels is typically more profound than among individuals with uncomplicated dengue. A prospective study on children in 1994 indicated levels of sTNFR80, sCD8, and sIL2R in DHF cases were higher than in DF cases across all days of illness [89]. Another prospective study measuring plasma levels of cytokines in Vietnamese infants found IL-12p70, TNFα, and IP-10 at enrolment were higher in patients who later developed DSS than patients who later developed DHF. In contrast, the level of RANTES was lower [90]. Heterogeneity in cytokine responses was also observed in a retrospective study that focused on VEGF, using ELISA. At the time of plasma leakage, there was a rise in plasma levels of free VEGF-A in DHF patients, compared with DF. In contrast, a decline was observed with levels of sVEGFR2 [91]. When the new WHO 2009 classification was applied to the Dengvaxia clinical trials, levels of IL-1Ra and IP-10 were over-expressed in severe breakthrough patients, compared with non-severe cases [86]. A meta-analysis of 53 cytokines studied in 56 publications shows IL-8, IL-10, and IL-18 levels in DHF were higher than DF. In contrast, levels of RANTES, IL-7, TGF-β, and VEGFR2 were lower in DHF [92]. However, a limitation of the meta-analysis is that the results of the original publications were not always consistent, i.e. cytokines found to be associated with dengue and severe dengue in one study may not correlate with disease severity in another study. This may be caused by differences in study design, study

population, timing of sample collection, methodology etc. Indeed, more studies are required to better understand the involvement of cytokines in dengue.

Several cytokines such as IFNγ, TNFα, and MCP-1, which have been consistently shown to correlate with clinical aspects of the disease, merit more detailed examination in future clinical studies. IFNγ plays a central role in controlling viral infections. Specifically, IFNγ has been shown to correlate with DENV infection in a couple of clinical studies that examine the response in patients [86, 87]. To overcome the activity of IFNγ, DENVs evolved to block IFNγ signaling pathways by inhibiting IFNγ regulatory factors and signal transducers [93]. TNFα is an inflammatory cytokine with higher levels in DHF than DF, as revealed by a study in Brazil [94]. In another study exploring the involvement of TNFα, sTNFR and cytokines that are produced downstream of TNFα, sTNFR showed a positive relationship with dengue severity. Moreover, the study found IL-5 and sIL-1 were lower in DSS, likely reflecting the general loss of protein from the circulation due to capillary leakage. The authors propose a hypothesis that sTNFR may be an early and specific marker of the endothelial changes that cause DSS [95]. MCP-1 is a chemoattractant protein that has been associated with dengue [86]. Interestingly, the levels of MCP-1, derived from PBMC collected before natural infections of humans, were higher in individuals who later developed symptomatic dengue than those who developed subclinical manifestations [96]. In patients with symptomatic dengue, the concentration of MCP-1 in patients without warning signs were lower than those with warning signs [97], also suggesting a role for MCP-1 in dengue pathogenesis. Besides IFNy, TNF-alpha, and MCP-1, a number of other cytokines that are thought to be involved in dengue and severe dengue (Table 1.2) should

be further investigated. It is also worth considering the stability of such cytokines in blood specimens because blood IL-1RA seems to be unstable, even when the blood is stored at 4-8˚C. On the other hand, other investigated cytokines such as IFNy, sIL-2Ra, sTNF-RII, IL-6, TNF-alpha, MIP-1B, IL-12, and RANTES were stable at this storage temperature [98].

Table 1.2: Summary of available cytokine data for dengue and severe dengue

Cytokines	Relevance[a]	Reference
IL-1α	Elevation with dengue	[86]
IL-1ra	Elevation with dengue, and severe dengue	[86]
IL-6	Elevation with dengue	[86]
IL-8	Elevation with dengue, and DHF	[86, 87] [92]
IL-10	Elevation with dengue, and DHF	[92]
IL-12p70	Elevation with DSS	[90]
IL-18	Elevation with DHF	[92]
Eotaxin	Elevation with dengue	[86]
FGF-2	Elevation with dengue	[86]
IFNγ	Elevation with dengue	[86, 87]
IP-10 (CXCL10)	Elevation with dengue, DHF, DSS and severe dengue	[86-88] [90]
MCP-1 (CCL2)	Elevation with dengue	[86] [97]
MDC	Elevation with dengue	[86]
RANTES	Decrease with DHF, DSS	[90] [92]
sCD40L	Elevation with dengue	[86]
sTNFR80	Elevation with dengue, and DHF	[89]
sCD8	Elevation with dengue, and DHF	[89]
sIL2R	Elevation with dengue, and with DHF	[89]
sVEGFR2	Decrease with DHF	[91, 92]
TNFα	Elevation with dengue, and DSS	[86, 87] [90]
VEGF	Elevation with DHF	[91]

[a]The classification of dengue is based on the references.

1.4.2 Innate immune response and the role for NK cells in dengue

Innate immune responses generate an immediate response against pathogens or injury. They consist of biomolecules (such as complement) and cells (macrophages, monocytes, neutrophils, NK cells) acting together in a coordinated manner. Among the cellular components of the innate immune system, NK cells are known to play an important role in the response to viral infections such as DENV infection; these cells are able to secrete cytotoxic granules and function in the first line of defense to kill DENV-infected cells directly. The cytopathic effect is mediated by NK cells with the phenotype of CD16+ CD56dim, while CD16- CD56high NK cells produce inflammatory cytokines [99].

In addition to CD16 and CD56, effector functions of NK cells are determined by a network of surface makers which are classified into activating and inhibitory receptors. The overall balance of these receptors on the cell surface determines the activity of NK cells. For example, if activating markers predominate, the NK cell is activated and may cause virus-infected cells to undergo apoptosis, a process in which proteases in cytotoxic granules disrupt the membrane of targeted cells causing them to die. By contrast, if expression of inhibitory receptors prevails, the NK cell activity is suppressed. Examples of activating receptors are CD57, NKG2D, NKp30, NKp44 and NKp46 while inhibitory NK receptors include the CD159a/NKG2A complex and KIRs.

Receptors on NK cells bind to those of virus-infected cells in a specific manner; for example, KIRs binds to HLA-I, NKG2A binds to HLA-E, while CD16 binds to DENV-specific antibodies. Binding between CD16 and DENV-specific antibodies induces a

cytopathic process known as ADCC. This ADCC activity of NK cells seems to be protective when DENV-specific antibodies exist in the blood of secondary dengue patients. When viremia was assessed in plasma samples obtained before a secondary infection in Thai schoolchildren participating in a prospective cohort study, the ADCC activity of the pre-dengue plasma samples correlated inversely with DENV-3 viremia levels [100]. Although the study only examined patients with secondary dengue caused by two DENV serotypes (DENV-2 and DENV-3), and the key finding was limited to DENV-3, this suggests a protective role for NK cells in early control of secondary DENV replication.

However conversely, DENVs may also compromise the killing activity of NK cells. A co-culture of NK cells and endothelial cells showed that DENV infection upregulated soluble HLA-E (an major histocompatibility complex 1 (MHC-1 protein) that interacted with and activated the inhibitory NKG2A, suggesting that DENVs can inhibit the cytopathic activity of NK cells [101]. Although the interaction did not dampen the killing process of activated NK cells *in vitro*, studies that investigate the relationship between the severity of dengue and the proportion of NKG2A-bearing NK cells *in vivo* would be interesting.

A clinical study has been performed in relation to KIRs, another group of NK inhibitory markers. In this case:control study involving a population in southern Brazil, the genotypes of several KIR genes were assessed in 104 dengue-confirmed cases and 172 heathy controls. Several KIR genes were found to be present with greater frequency in the dengue patients than the controls [102]. This is in line with studies where DENV induced inhibitory

KIRs on the surface of NK cells [103], and upregulated HLA-I across multiple cell lineages [104].

In addition to the proposed role for NK cells in influencing DENV susceptibility, NK cells may also influence disease severity. In a study of Thai children with confirmed dengue presenting within 72 hours of fever onset, the absolute number of NK cells measured during the febrile phase was lower in children who later developed DHF as compared to DF [105]. In contrast, a prospective descriptive study of infants with confirmed dengue showed a positive correlation between the proportion of activated NK cells and the severity of dengue [90]. The different results from these two studies may relate to the fact that the authors combined CD16+ and CD56+ NK cells, i.e. NK subsets with different roles, in their analysis. In a more recent study that characterized the two subsets of NK cells separately, the authors did not find any associations between CD56+ or CD16+ NK cells and the severity of dengue [106]. Further studies are required to understand the disparate data.

The association between increased NK cell responses and severe dengue may also be explained by the expression of different genotypes of MICB. MICB is a gene located within the broad MHC region on chromosome 6; its proteins interact with NKG2d (an activating receptor) and regulate the activity of NK cells. In a genome-wide association study which assessed samples from 2,008 pediatric DSS cases and 2,018 cord blood controls, a genotype for the susceptibility to severe dengue was found on MICB [107]. In

a longitudinal birth cohort, an increase in the level of MICB proteins was found in infants with acute dengue, compared with baseline values [108].

1.4.3 Adaptive immune responses to DENVs

The adaptive immune response involves specific and specialized mechanisms to fight invading antigens [84]. Unlike the immediate response of the innate immune system (which commences within hours), the development of adaptive immunity to antigens may take several days to be activated. Moreover, adaptive immune responses incorporate a memory response, which is important for subsequent attacks by similar antigens. Adaptive immunity includes but is not limited to components such as antibodies, T cells and B cells.

Adaptive immune responses against DENVs can be both protective and pathogenic. After infection with one DENV serotype, a person develops immunological memory which is long-lived against that viral serotype, but only short-lived (1-2 years) against the other heterotypic serotypes. Later, stimulation of this adaptive immune response during a subsequent heterotypic DENV infection, may mediate enhancement of viral replication (a phenomenon known as ADE), potentially leading to severe dengue. The protective and pathogenic effects of the adaptive immune response in the context of DENV infection will be detailed below, with a focus on antibody-mediated immunity.

1.4.3.1 T cells

T cells are a group of lymphocytes that play a central role in cell-mediated adaptive immunity. The name "T" is derived from their specialized maturation site, which is the thymus. Here, naïve T cells are generated, later traveling through blood vessels to arrive at secondary lymphoid organs. They are exposed to antigens in the secondary lymphoid organs, and develop into one of the range of T cell types with different effector functions such as killer T cells, helper T cells, and regulatory T cells. Killer T cells act to directly kill pathogen-infected cells while most helper T cells assist other white-blood cells to eliminate pathogens. Helper and killer T cells are known as CD4+ and CD8+ T cells, respectively.

T cells can develop memory to respond more quickly and effectively to subsequent exposures to similar antigens. In this process, naive T cells, after antigenic exposure and stimulation, differentiate first into CM, then EM, and finally EMRA. The more differentiated the cells are, the more effector capacity they are endowed with. Both CD4+ T cells and CD8+ T cells can progress through these stages of differentiation.

CD4+ T cells support the capacity of B cells to secrete DENV-specific antibodies, and help CD8+ T cells to kill DENV-infected cells directly. DENV-specific CD4+ T cells are also believed to have the direct *ex vivo* cytotoxic activity, as revealed by a study phenotyping HLA-restricted CD4+ T cells derived from natural DENV infection [109]. The same study also found CD4+ EMRA T cells (CD45RA+ CCR7-) expressed an increased in several cytotoxic molecules such as CD107a, perforin, granzyme B, suggesting that CD4+ EMRA

T cells play a key role in mediating the direct cytotoxic activity [109, 110]. However, CD4+ T cells may also be pathogenic in dengue, with some studies showing that both the proportion and absolute numbers of CD4+ T cells correlate with severity of disease [90, 111].

CD4+ T cells recognize a greater variety of DENV encoded antigens compared with CD8+ T cells. When scanning their epitopes across the DENV-2 proteome, Cap, E, and NS3 proteins were identified as major binding to receptors on CD4+ T cells while NS3, Cap, and NS5 are the major targets of CD8+ T cells [110, 112, 113]. Interestingly, CD4+ T cells recognize HLA-restricted peptides derived from the E protein. Those same peptides may also be recognized by DENV-specific B cells, suggesting the possibility that some of the anti-DENV CD4+ T cell immune population stems from B cells recognizing these peptide through their B cell receptor and presenting, through MHC class II, these peptides to CD4 T cells. On the other hand, CD8+ T cells mainly target NS3, capsid and NS5 epitopes. Peptides of these non-structural proteins are presented on the surface of DENV infected cells for CD8+ T cells to kill directly.

CD8+ T cells are activated by antigen presenting cells. Langerhans cells (LCs) are a specialized type of antigen presenting cells present in skin tissues. As such, LCs are considered the sentinel cells for DENV infection [114]. After inoculation by an infected mosquito bite, LCs ingest DENVs present in the skin tissue and break them down into viral peptides. These viral peptides are then loaded onto MHC-I proteins, a group of cell surface proteins. The DENV-presenting LCs then migrates to secondary lymphoid organs such as

the spleen and lymph nodes. Here, the LC introduces DENV peptides in complex with MHC-I (for example, NS3 peptide in complex with HLA-A*1101 [115]) to receptors on CD8+ T cells (TCR). This presentation of DENV antigens to TCRs on naïve CD8+ T cells activates them to differentiate and acquire cytopathic effector functions.

A pathogenic role for DENV-specific CD8+ T cells has been suggested in several studies. Among infants and children with acute dengue, the proportion of activated CD8+ T cells positively correlated with overall disease severity [90, 105]. CD8+ T cells from DHF patients have a less marked cytopathic effect but produce more cytokines, compared with those from DF cases [116]. Interestingly, these DENV-reactive cytokine-producing CD8+ T cells are also cross-reactive and have been shown to bind to more than one DENV serotype [117]. Given this cross-reactive characteristic, these T cells may function not only in a primary DENV infection but also in subsequent infections caused by different serotypes. In one study that examined the phenotypes of DENV-specific CD8+ T cells from secondary dengue patients [118], the affinity of DENV-specific T cells was low for the current infecting DENV, while it was higher for previously encountered DENV serotypes. This study supports the theory of original antigenic sin, which underscores the role for cross-reactive CD8+ T cells in the pathogenesis of secondary dengue.

However, the timing of DENV-specific CD8+ T cell responses seems to lag behind the period when replication of DENVs peaks. In one study analyzing the presence of CD8+ T cells in blood samples obtained during the febrile and critical illness phases, DENV-specific CD8+ T cells were found to increase only after viremia had already begun to

decline [119]. This is also the time when severe manifestations such as shock due to vascular leakage become apparent, which goes against a directly pathogenic role for CD8+ T cells and suggests that other mechanisms may be responsible for triggering leakage earlier in the illness course.

1.4.3.2 B cells and antibody-mediated immunity

B cells are a group of lymphocytes specialized in antibody secretion. Naïve B cells are generated in bone marrow and subsequently migrate to secondary lymphoid organs such as the spleen and lymph nodes, where they may be exposed to antigens. After exposure and activation, the B cells proliferate and differentiate into short-lived plasma cells, also known as plasmablasts, that produce low-affinity antibodies. The potential for a role for plasmablasts in dengue pathogenesis is indicated by the finding that in secondary dengue numbers of these cells correlate positively with severe disease [120].

B cells can also differentiate into memory B cells, becoming long-lived plasma cells that secrete antibodies of high affinity. These memory cells are generated in germinal centers inside secondary lymphoid organs.

B cells produce antibodies, which are immunoglobulin proteins with a Y-shaped structure. Antibodies have two physical forms: a soluble form secreted into blood and tissue fluids, and a membrane-bound form that binds to the surface of B cells. Antibodies function to kill extracellular microbes through four mechanisms: neutralization, complement fixation, antibody-dependent cell-mediated cytotoxicity, and phagocytosis. Structurally, an

antibody comprises two light chains and two heavy chains connected via a disulfide bridge. While the two light chains consist only of Fab, the two heavy chains have both Fab and an Fc. The Fab element is the region that binds to antigens; each one is specific to a particular epitope and there is tremendous diversity to cover the range of possible antigens that might be encountered. In contrast the Fc elements are highly conserved in order to facilitate communication with other components of the immune system. The Fc element is required for all the antibody-mediated mechanisms described above, except neutralization where only Fabs are required. There are five Fc structures, corresponding to the five isotypes of antibodies, i.e. IgA, IgD, IgE, IgG, IgM. These isotypes are directed differently against microbial pathogens; for example, IgA is specialized against mucosal (gut, respiratory) infections, IgE relates to allergy and helminth infections, while IgM and IgG are present in blood for microbial clearance.

Detection of DENV-specific IgM and IgG can provide serological evidence of an acute/recent dengue infection [18]. During a primary DENV infection, specific IgM is the first class of antibody to be elicited, followed by IgG. In a secondary DENV infection, DENV-reactive IgGs are often detected earlier and with greater magnitude than in a primary infection, while the IgM profile remains the same [19]. As a result, IgM/IgG antibody ratios have been used to distinguish primary and secondary DENV infections. Another difference is that while DENV-specific IgM is usually undetectable in blood by about two months after DENV infection, DENV-specific IgGs are long-lived [18]. DENV-specific IgG, with high neutralization capacity [121], is important for life-long protection against subsequent DENV infections.

However, antibodies may also play a role in dengue pathogenesis, by way of ADE. ADE is a theoretical concept, described initially in the 1970s following a series of *in vitro* experiments [122], that aims to explain both the increase in viremia and the risk of severe dengue that have been observed in association with secondary infections. While intrinsic ADE refers to an increase in the intracellular production of DENVs, extrinsic ADE revolves around an increase in the number of infected cells [123, 124]. Extrinsic ADE postulates that at low concentrations serotype-cross-reactive antibodies contribute to dengue pathogenesis. When individuals encounter their first (i.e. primary) DENV infection, B cells induce antibodies that are specific to the infecting serotype, but are also cross-reactive to the other three DENV serotypes. Blood levels of these cross-reactive antibodies decline over time. Upon infection with a different serotype from the first one (i.e. during a secondary infection), Fabs elicited during the primary infection bind to the heterologous virus, while the Fc regions facilitate entry of the new infecting virus into Fc-bearing cells such as monocytes [125]. This leads to an increase in viral replication at a time when the immune system is still trying to generate antibodies that are capable of neutralizing the virus, potentially leading to more severe clinical manifestations of dengue.

Numerous studies have highlighted this potential role for low levels of cross-reactive antibodies in dengue pathogenesis. A cohort study has been running in Nicaragua for more than 10 years in which serological surveillance for evidence of DENV infection is carried out periodically, in addition to observation of acute symptomatic infections in the study participants. When quantifying the titers of neutralizing antibodies in serum samples

collected at baseline (i.e. before a DENV infection occurred), they found that the risk of severe dengue was highest within a narrow range of pre-existing DENV-Ab titers. Specifically, in children with baseline DENV-reactive antibody titers of 1:21-1:80, the risk of developing severe dengue was 7.64-fold higher than among those with higher titers of pre-existing DENV-reactive antibodies [126]. Results from a similar study in a Thai cohort, in which pre-infection neutralizing titers <1:100 were associated with severe dengue, provide additional evidence for the role for low concentrations of pre-existing DENV-reactive antibodies in secondary dengue [127]. By contrast, high concentrations of antibodies protected people from subsequent DENV infections in the Nicaraguan cohort study, with titers of pre-infection neutralizing antibodies showing a negative correlation with the probability of symptomatic dengue occurring thereafter [128].

The phenomenon of ADE occurring in sequential DENV infections has important implications for dengue vaccination programs. In seronegative individuals, vaccination can be considered as the first exposure to DENVs, ideally eliciting a balanced response to all four serotypes at the same time. However, if this does not occur, a post-vaccination natural infection with a serotype not covered adequately by the vaccine could lead to enhanced viral replication and might predispose the vaccine recipient to severe dengue – i.e. a serious adverse event.

ADE may also occur after sequential infection not only with heterologous DENV serotypes, but also with other viruses in the flavivirus genus. For example, in *in vitro* studies DENV-reactive antibodies derived from samples obtained from acute dengue

patients are able to bind to ZIKV, enabling neutralization and enhancement of both DENVs and ZIKV [129]. This adds another layer of complexity to the potential pathogenesis of DENVs, and also to the performance and interpretation of immunological studies in the context of co-circulation of DENVs and other flaviviruses such as JEV and ZIKV.

1.4.3.3 Correlates of protective immunity

Correlates of protective immunity are measurable immune markers that are associated with protection from a particular infection in an individual [130]. These immune makers can be derived either from prior exposure to the infectious agent or following vaccination against the infection, and are usually detected using serological methods. In immunological studies, these markers are typically used to determine the level of individual protection from an infectious disease, or to assess other important features such as the force of infection or the microbial virulence in a particular epidemiological setting. In vaccine research, such markers can be used to optimize the antigens selected during the initial process of vaccine development, as well as later on in formal trials designed to assess vaccine efficacy and potentially support licensure.

Immunological markers that can be used as correlates of protective immunity against infection include cytokines, T cells, B cells and antibodies. For example, in influenza research, antibodies are typically used as the immune marker indicating protection, usually assessed with hemagglutinin inhibition assays (HI) or micro-neutralization assays [131]. For dengue, antibodies are also commonly employed as immune markers, often measured by PRNTs.

The principle of the PRNT is as follows: serial dilutions of plasma containing antibodies, or saline as a negative control, are incubated with individual strains of a cultured virus. After allowing 30 minutes for the antibodies (if present) to neutralize the virus, the mixture is inoculated onto monolayers of susceptible cells. If the virus has not been neutralized, cellular infection can be visualized by plaque formation, provided the virus exerts cytopathic effects, and this forms the endpoint of the PRNT. Alternatively, the degree of cellular infection can be assessed by immunological staining with fluorescent-conjugated antibodies. The antibody titer is defined as the geometric mean value that renders a 50% reduction ($PRNT_{50}$) or 90% reduction ($PRNT_{90}$) in infection of the monolayer compared with the negative controls. Different laboratories define specific cut-off values that they consider indicative of exposure to each serotype; if the antibody titer to a particular serotype is higher than this cut-off value, then the individual is considered to be seropositive for that DENV serotype.

A major advantage of PRNTs is that the test targets antibodies that neutralize infectious DENVs, as opposed to ELISA or HI where antibodies bind to viral particles (E or prM proteins). Also PRNTs are said to differentiate between antibodies specific to the DENV serotypes to which an individual has been exposed; depending on the PRNT cutoff values used for a serum sample incubated with each of the four serotypes, it is theoretically possible to determine whether an individual's immune status is seronegative or seropositive, whether this is primary versus secondary dengue, and to determine which serotypes the individual has experienced. These determinations of immune status and

serotype exposure have been applied in research and surveillance [52-54, 132], and also in assessing the immunogenicity of dengue vaccines, i.e. whether vaccinated individuals develop protective immunity against all four DENVs.

However, PRNT results have failed to predict dengue vaccine efficacy in clinical trials. For example, in the Dengvaxia trials there was notable discordance between the number of seropositive vaccine recipients, as assessed using PRNT data, and clinical measures of vaccine efficacy, particularly for DENV-2 [52-54]. There are many possible explanations for the failure of the PRNT in these circumstances. First, a $PRNT_{50}$ cut-off value of 10 was set to define protective immunity to all four DENVs. However, in cluster studies in Thailand, protective neutralizing titers appeared to be higher for DENV-2 than other serotypes [133]. When measuring vaccine-elicited immunogenicity, if a higher cut-off value had been used for DENV-2, the percentage of seropositive individuals in the Dengvaxia trial population would have been lower, and likely closer to the efficacy estimates.

Second, the use of cultured DENVs, which express higher levels of prM molecules on the virion surface [63], and seem to be less mature than circulating wild-type DENVs [82, 134], may influence the PRNT results. Anti-prM antibodies that neutralize cultured DENVs may not bind to circulating DENVs, and this may result in mischaracterization as neutralizing antibodies using PRNTs.

The third potential explanation revolves around the use of non-human cell lines, such as

Vero cells which were originally derived from a monkey. Such cell lines may not be suitable for PRNTs because the natural infection and transmission cycle of DENVs mostly does not involve mammals other than humans. Moreover, these cells do not possess receptors important for DENV infection, such as Fc receptors or dendritic cell-specific ICAM-grabbing non-integrin. If the wrong cells are used, the antibodies that are targeted may differ from those that neutralize DENVs in nature.

Another consideration is that, in addition to neutralization, the other characteristics of the antibodies measured in the PRNT remain unknown. Studies indicate that the detailed structure of the DENV epitopes targeted is crucial in determining whether particular antibodies neutralize virus effectively. For example, antibodies that bind to quaternary epitopes of E proteins are thought to be very important for neutralization *in vivo* [135]; a quaternary epitope is a structural arrangement generated by the specific configuration of more than one E protein molecule on the surface of the virus. In contrast, antibodies that bind to the fusion loop or to prM proteins are not as effective in neutralizing DENVs. Moreover, prM and fusion loop binding antibodies are typically cross-reactive and thus they may play a role in ADE [62, 82]. In PRNTs however, all of these antibodies contribute to neutralizing the DENVs [82, 136], which may lead to overestimation of the true neutralization capacity and thus the results may not predict vaccine efficacy correctly.

Despite those limitations, PRNTs remains the gold standard to examine DENV-reactive neutralizing antibodies. Other second-generation neutralization assays are being developed, which utilize more biologically relevant cells and substitute immune markers

such as T cells or NK cells for antibodies [130], but these novel assays are not yet available for practical application.

1.4.4 DENV-specific monoclonal antibodies

Monoclonal antibodies (mAbs) are laboratory-derived molecules secreted by a single B-cell clone. These antibodies are specific to a particular antigen and have become a valuable tool in medicine. For example, mAbs are widely used to treat certain autoimmune or non-communicable diseases. For infectious diseases, the applications of mAbs are still under development [137].

In dengue, a large number of human DENV-reactive mAbs have been generated and characterized. These not only provide insights into the neutralization process against DENVs, but also have potential to be used as antiviral therapeutic agents.

For example, DENV-reactive mAbs have revealed epitopes which are important for the neutralization of DENVs. It is known that all ten proteins of DENVs present epitopes to mAbs [136], but the binding of mAbs to some epitopes does not neutralize the virus. For example, cross-reactive mAbs bind to prM, which are highly conserved across the four DENV serotypes. The prM-specific antibodies, generated in response to an infection with one DENV serotype, can bind to another DENV serotype acquired later and mediate ADE - instead of neutralizing the later virus [62].

In contrast, most of the available E-specific mAbs have been characterized as potent neutralizers of DENV infectivity for host cells [82, 136, 138-142]. Major binding sites of these mAbs are the three domains of the viral E protein. In particular, DIII contains receptor-binding residues where the binding mAbs can block infection of the cells early [139, 143]. Along with DIII, the DI-II hinge is also a potential target for neutralizing mAbs, since binding at this site may disrupt the conformational change required for fusion between the DENV and host cells [138].

The neutralization capacity of mAbs depends not only on the viral proteins to which they bind, but also on the arrangement of those molecules on the surface of DENVs. Specifically, when E dimers are assembled on the intact virion, quaternary epitopes are formed which are recognized by neutralizing mAbs [135, 144-146]. Unlike quaternary epitopes, linear epitopes consist of individual, separate E proteins that are binding sites for both neutralizing and non-neutralizing mAbs *in vitro* [135]. However, a common limitation of *in vitro* studies is that they are performed using cultured DENVs. Cultured DENVs are maintained in passage, allowing small evolutionary changes to occur *in vitro,* which may diverge from *in vivo* DENVs. As such, a mAb may differ in antiviral potency against *in vitro* and *in vivo* DENVs.

At present no effective antiviral drugs are available against DENVs (Section 1.1.5), and DENV-specific mAbs are being considered for both therapeutic and prophylactic indications. In principle, mAbs could be used as therapeutic agents in the febrile phase

when viremia is high; the mAbs could bind to the infecting DENVs, inhibit viral entry into host cells, and thus suppress viral replication.

1.5 Immune responses during pregnancy

The maternal immune system is inherently modified during pregnancy. These modifications help achieve a balance between tolerating the development of an allogenic fetus while still protecting pregnant women from microbial infections. If the balance is lost, this may predispose the women to higher susceptibility and severity of infections.

1.5.1 Pregnancy and the involvement of maternal immune responses

A human normal pregnancy passes through three distinct stages, including implantation, fetal growth and development, and parturition [147]. During implantation, the fertilized egg becomes a blastocyst and adheres to the uterine wall. There are two cell types in the blastocyst: inner cell clumps which develop into the embryo and the outer cell layer which gives rise to the placenta. This outer cell layer consists of trophoblasts that bind to the maternal decidua, the pregnancy-linked uterine lining [148].

Immune components are present in the maternal decidua from the very first stages of gestation. They include immune cells such as dendritic cells, macrophages, and NK cells (Figure 1.6), and immune biomolecules such as IgG, a class of antibody that can travel through the placental barrier to be present in the fetal blood.

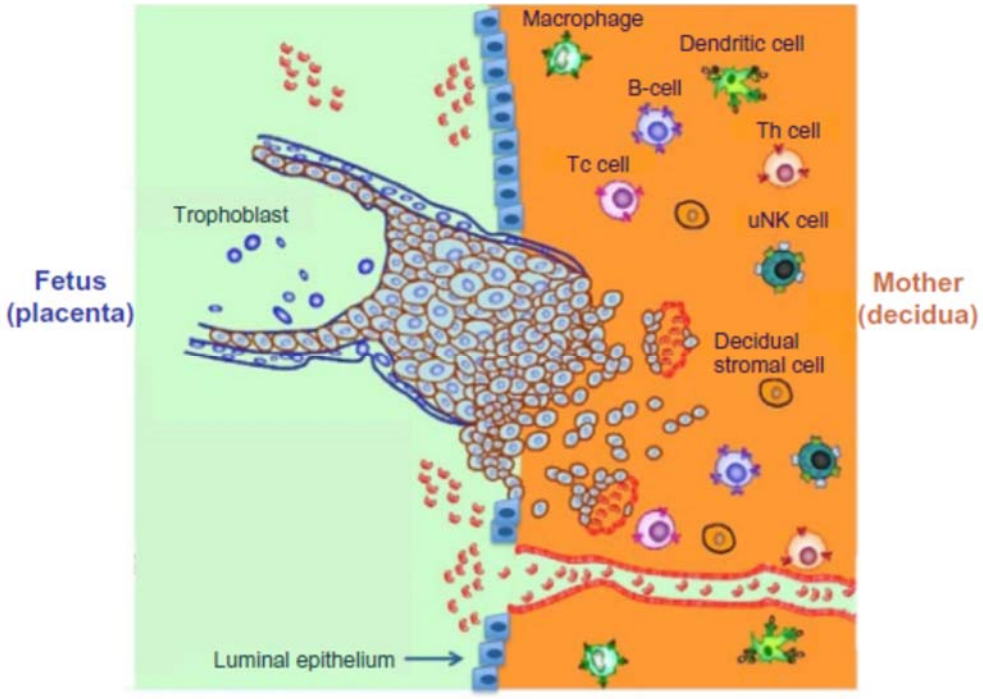


Figure 1.6: Immune components present in the maternal decidua

Immune cells such as macrophages, dendritic cells, B cells, and T cells are present at the maternal-fetal interface. (This figure was copied from an open-access article [148].)

The maternal immune system is modified during pregnancy to enable the mother's body to tolerate a fetus that contains paternal antigens. These modifications to the maternal immune system were traditionally thought to be suppressive, but more recent empirical evidence suggests a more complex situation. The classical theory holds that fetuses are subject to rejection mediated by maternal immune responses against paternal antigens [149], and that successful pregnancy is associated with constant suppression of maternal immunity, while obstetric complications such as miscarriage result from failure to induce continual suppression of the maternal immune system.

However, this immune-suppressive theory is not supported by recent studies that highlight the supportive role for the maternal immune response in fetal development. For example, decidual NK cells regulate the binding of trophoblasts to the uterus and secrete factors that induce vascular growth [150]. Moreover, placental type-I IFN has been shown to protect pregnant mice from viral infections [151]. In humans, the immunological profile of maternal immunity is indicated in two studies below. In the first study, differences in immune cells were shown between healthy women in the third trimester of pregnancy and non-pregnant individuals [152]. Specifically, pregnancy reduced the percentage of B cells but increased that of plasmablasts, although differences between individuals were not controlled for in this study. The second study controlled for intrinsic differences between individuals by comparing cytokine levels during gestation with post-partum cytokine levels in the same participants [153]. During each of the three trimesters, a peripheral blood sample was collected and cytokine levels were quantified. The results showed higher levels of IL12p70, TNFα, IFNγ, eotaxin, G-CSF, GM-CSF and IL-15 during pregnancy compared to the postpartum period, with no apparent difference in results between the three trimesters. In contrast, the concentrations of IP-10, MCP-1 and VEGF were lower during pregnancy.

Thus the view of pregnancy being associated only with immune suppression has recently evolved. The modifications to the immune system are now seen as more complex [154], and appear to be driven by the different stages of pregnancy.

1.5.2 Correlation between maternal immune responses and pregnancy stages

The maternal immune response is pro-inflammatory during implantation, then dominated by anti-inflammatory responses while the fetus is growing, and at the last stage of gestation returns to a pro-inflammatory profile in order to facilitate parturition [147].

During implantation the invasive blastocyst causes a breakdown and re-structuring of both of the uterine lining and the trophoblast layer. When decidual cells, derived from pregnant women who were at the first trimester, were cultured, the cells secreted pro-inflammatory cytokines which activated macrophages *in vitro* [155].

Following implantation, during the phase of fetal growth and development, a reduction in expression of pro-inflammatory cytokines and an elevation in that of anti-inflammatory cytokines has been demonstrated in pregnant women who are at the second or third trimesters. This anti-inflammatory profile was compared with their own baseline status, as assessed after delivery [156].

Before parturition, a switch back to a pro-inflammatory response is necessary to facilitate delivery of the baby. When epithelial cells, derived from the amnions of women who had undergone caesarean section, were cultured, the cells exhibited a pro-inflammatory response, indicated by the induction of nuclear factor kappa B (a protein that plays a role in regulating immune responses to infections). Moreover, genes encoding inflammatory cytokines such as IL-1, IL-8 were also up-regulated in this *in vitro* study [157].

Although changes in the inflammatory response profile occur during the three gestational stages [153, 154], the middle stage, when immune responses are suppressed, is the longest period of pregnancy [147]. As such, overall the immune response during pregnancy is suppressed to achieve a balance between tolerating the development of the fetus while providing protection from infection to the pregnant women. The immunosuppression puts pregnant women at risk of acquiring infections and developing severe diseases caused by viruses such as influenza, hepatitis E, and herpes simplex virus [158].

While the maternal immune response against these viruses has been studied extensively, immunity against flaviviruses in general is not well understood as yet. Due to the recent emergence of ZIKV infection, and the recognition of the severe complication that can occur in children born to mothers who are infected with this virus during pregnancy, the role for maternal immune responses against flavivirus infections has been brought sharply to the attention of the scientific and public health communities in endemic countries.

1.5.3 Maternal immunity against flavivirus

There is some evidence that the maternal immune response is impaired when pregnant women are infected with ZIKV. When levels of cytokines were compared between pregnant women with ZIKV disease and healthy pregnant women, an increase in immunosuppressive cytokines such as IL-10, CD163 were observed in the patients [159]. This immunosuppression may allow increased ZIKV replication, potentially increasing the risk of transmission across the placental barrier and the likelihood of developing microcephaly and other congenital abnormalities. In another study examining levels of 45

immune mediators in six ZIKV-infected pregnant women, higher levels of IL-22, MCP-1, TNFα, IP-10 were found in the one woman whose fetus had congenital anomalies than the other five women with normal fetuses [160]. These two studies highlight the relevance of immune components to ZIKV infections during pregnancy. In contrast, maternal immune responses against DENVs remain unknown.

The majority of women living in dengue-endemic areas will have been exposed to dengue prior to becoming pregnant, and some individuals experience dengue during pregnancy. In a study enrolling healthy pregnant women in Thailand, the seroprevalance for DENVs at delivery (indicating infection at some point in the past) was at 94.7% [161]. This seroprevalence is much higher than the value obtained from another study in India, where the value was 6.9% [162]. Both of the studies used a cross-sectional design, so the timing of the DENV infection remains unknown. Another study examined the serological responses of pregnant women with dengue within approximately two weeks of delivery [163], aiming to estimate the risk of vertical transmission of dengue. However, as yet no groups have examined immunological responses among pregnant women experiencing dengue in any detail, and the effects of infection at different times during gestation remain unknown. Yet if the immune profile of pregnant women with dengue is characterized, this might provide insight into the risk for adverse outcomes such as pre-term birth and still-birth, consequences for which there is some evidence for an association with dengue during pregnancy [39, 40, 164].

1.6 Summary, knowledge gaps and aims of my thesis

In summary, there is a considerable progress in clarifying and understanding dengue pathogeneses, but still many aspects remain poorly understood. For my thesis, I focus on two particular areas related to human mAbs against DENVs and immune responses of pregnant patients.

Studies on human-derived DENV-reactive mAbs have revealed some of the characteristics of antibodies that are desirable to elicit via immunization. One key feature of these DENV-reactive mAbs is their ability to neutralize cultured DENVs in mammalian cells. However, this feature is often assessed in highly controlled conditions using DENVs propagated in cultured cells, whereas in reality neutralization of DENVs in viremic blood is likely to be a much more complex process [134]. It remains unknown which mAbs neutralize DENVs *in vitro* but are not effective against DENVs *in vivo*. To this end, I measured antiviral capacities of individual mAbs using a viremic blood neutralization assay (Chapter 2). All the mAbs I tested had been previously characterized as neutralizing antibodies against DENVs *in vitro,* but I hypothesized that they would fail to neutralize DENVs in viremic blood.

For dengue no licensed antivirals exist as yet, and treatment is limited to supportive care. Although therapeutic administration of human DENV-reactive mAbs has potential as a treatment, the functional antiviral features of the various neutralizing mAbs (such as 50% effective concentrations, the potency of mAb variants etc.) remain unknown. In Chapter 3,

I aimed to explore these features of several of the neutralizing mAbs I evaluated in Chapter 2, selecting the three mAbs that were most suitable for detailed investigation.

Antibody-mediated neutralization is a critical feature of the immune response to fight against DENV infections of humans. In special circumstances, such as pregnancy, the immune response may be compromised, allowing greater replication of infecting DENVs and a higher risk of severe dengue. However, how the host response to dengue alters during pregnancy is poorly understood. The aim of my Chapter 4 was to investigate both immunological and virological factors in a cohort of women who were hospitalized with symptomatic dengue, comparing the findings between pregnant and non-pregnant patitents. I hypothesized that pregnancy may be associated with a less pronounced immune responses to DENVs. In contrast, pregnancy might be a risk factor for increases in virological factors.

Chapter 2

A novel viremic blood neutralization assay to measure the anti-viral potency of human mAbs against DENVs

Abstract:

A number of human mAbs has been characterized as being able to neutralize the infectivity of DENVs for mammalian cells in cell-culture systems. However, DENV-reactive mAbs may not be effective in neutralizing DENVs in the complex matrix of human blood. Here, I led the development of a ViBNA which uses DENVs in viremic blood samples drawn from acutely infected patients. The endpoint of the ViBNA is DENV infection of mosquitoes that feed upon the mAb-spiked blood meal. I employed the ViBNA to characterize the antiviral potency of 12 human mAbs, previously characterized as neutralizing antibodies using cell-culture systems. Results show that five out of the 12 mAbs failed to neutralize tested DENV serotypes, unlike when used in cell-culture systems. Seven mAbs were characterized as neutralizing antibodies using the ViBNA. The neutralization capacity of one of the seven mAbs was reduced by a substitution (N52D) in the amino acid sequence of E proteins. Results of this Chapter advance the understanding of types of antibodies that would be desirable to elicit using a dengue vaccine, and highlight the requirement for an alternative DENV neutralization assay.

2.1 Introduction

DENV infections are highly prevalent in the tropical and subtropical world [1]. Following a primary DENV infection it is widely accepted that an individual develops long-lived clinical immunity to the infecting serotype but not to other serotypes. DENV infection can be subclinical, or result in a febrile syndrome that in a small percentage of patients is complicated by vascular leakage, thrombocytopenia and altered hemostasis, usually between the fourth and sixth days of illness [19]. The risk of clinically important complications is higher when an individual is infected for a second time with a different DENV serotype from the first [165]. In this situation, cross-reactive antibodies are believed to enhance the viral infection [125].

Antibodies are central to the concepts of dengue pathogenesis and naturally-acquired or vaccine-elicited immunity. For example, the induction of virus neutralizing antibodies by vaccination is the minimum goal of most candidate dengue vaccines [166]. The only-licensed dengue vaccine, Dengvaxia, was developed on the basis that it could elicit antibodies that neutralized all four serotypes of DENVs *in vitro* [52]. Unfortunately, the vaccine mediated an increased risk of symptomatic dengue in vaccine recipients who were seronegative at baseline [56]. Despite the agreed centrality of antibodies to dengue pathogenesis, laboratory correlates of immunity (or disease enhancement risk) have not been tightly defined or standardized. There is; however, a general consensus that increasingly higher serum concentrations of neutralizing antibodies are associated with reduced risk of dengue [128, 133].

The ability to generate and characterize human mAbs from dengue immune donors has provided insights into the type of antibodies that might be desirable to elicit via immunization, or alternatively, to use as therapeutic agents. Most prominent are those human mAbs identified as being able to neutralize DENV infectivity for mammalian cells [82, 136, 138-142]. Some of these neutralizing mAbs bind to viral envelope proteins that have quaternary structures [135, 144-146].

However, DENV-reactive mAbs were tested for neutralization capacity in a highly controlled condition of cell-culture systems. Whether these mAbs fail to neutralize DENVs in a more complex system remains poorly understood. Here, I developed the ViBNA using viremic blood from dengue patients, and considered DENV infection of *Aedes aegypti* as the assay endpoint. The primary aim is to measure the antiviral potency of 12 human mAbs against DENVs. These mAbs were previously characterized as neutralizing antibodies using cell-culture systems (Table 2.6). Because the ViBNA differs from other cell-culture assays in terms of viral strains, host cells, technical procedures etc., I hypothesize that tested mAbs would fail to neutralize DENVs in the ViBNA.

2.2 Materials and methods

2.2.1 Schematic overview of experiments

In brief, we enrolled consenting patients who were laboratory confirmed to have dengue. From these patients, the viremic blood samples were collected and divided into two aliquots. The first aliquot was used to conduct laboratory diagnosis tests such as serotyping qRT-PCR while the second aliquot was used to conduct the ViBNA. Here, I spiked the second aliquot with given concentrations of mAbs, and exposed them to *Ae. aegypti*. DENV infection of mosquitoes, assessed with RT-PCR, is the endpoint of the ViBNA. The antiviral potency of tested mAbs was then analyzed using a statistical model.

HIG, collected from a cohort of DENV-infected patients unrelated to this study, and PBS were considered as positive and negative controls in the ViBNA, respectively. The performance of HIG and PBS across ViBNA experiments was investigated (Figure 2.1).

I received 12 human mAbs from collaborators. All of the mAbs were previously characterized as neutralizing antibodies using various cell-culture systems. Before testing the mAbs with the ViBNA, I examined their quality and purification using gel electrophoresis. Gel electrophoresis also helped estimate concentrations of mAbs which were quantified previously by collaborators. However, I re-measured the concentrations for several mAbs using a BCA protein assay (Figure 2.1).

Next, I examined the antiviral potency of 12 human mAbs using the ViBNA. I expected that several mAbs would not neutralize DENVs and thus highlight the discrepancy between

the ViBNA and cell-based assays. We also performed the sequencing of RNAs encoding E proteins in a subset of viremic blood samples, spiked with a mAb. Then I investigated whether any changes in the epitopes of the mAb existed, compared with published residues. I also examined the influence of patient-derived DENV-reactive IgG on the neutralization capacity of two mAbs. To this end, we conducted the Panbio indirect IgG ELISA (Figure 2.1).

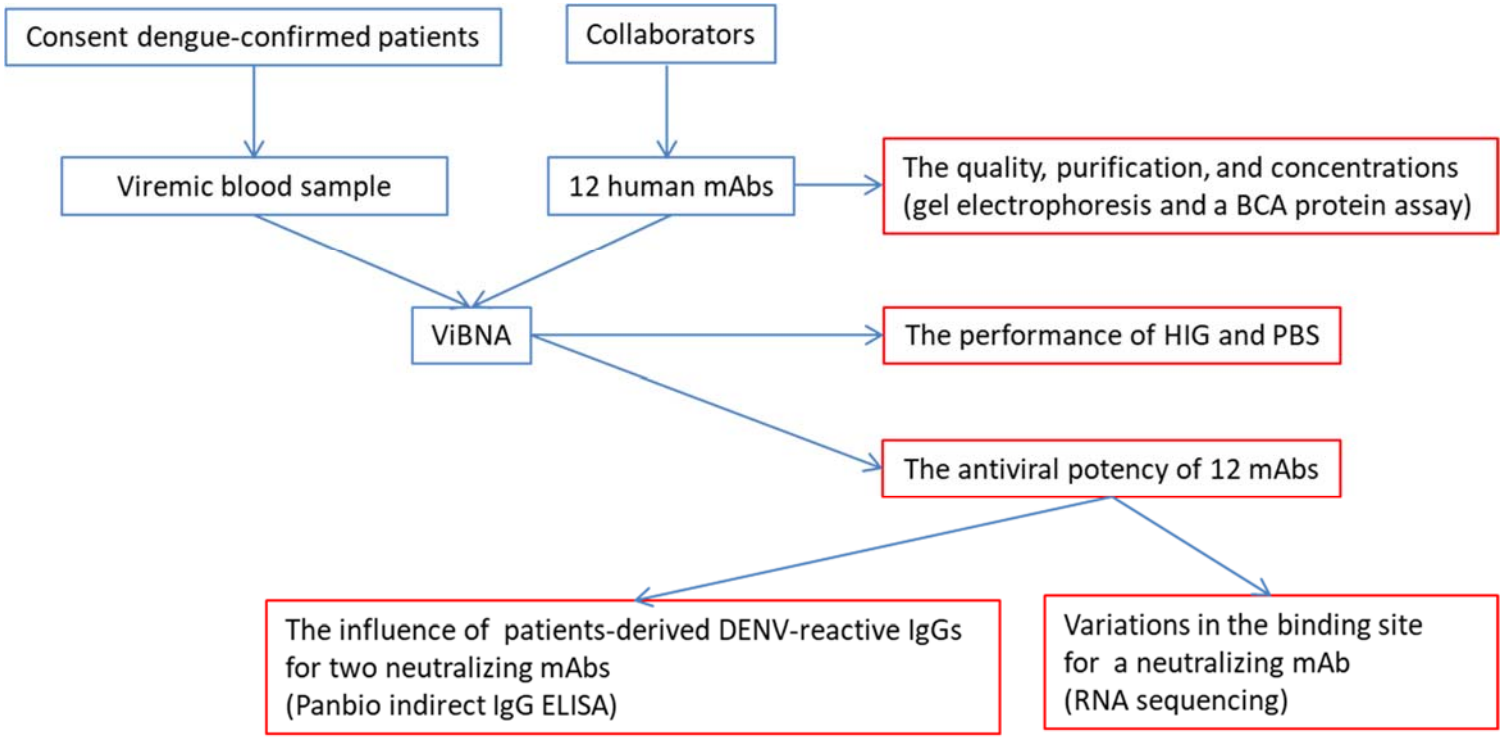


Figure 2.1: Schematic overview of experiments

Topics investigated in this Chapter are outlined in red.

2.2.2 Dengue patient cohort

Study doctors and nurses enrolled dengue patients and collected venous blood samples, respectively. Members of the Dengue group carried out NS1 rapid test, RNA extraction and serotyping RT-PCR.

2.2.2.1 Ethical statement

The patient samples used in this study were obtained via the approved protocols of two studies. These studies were approved by the Ethics Committee of Hospital for Tropical Diseases, Vietnam (CS/ND/12/16, CS/ND/16/27) and the Oxford University Tropical Research Ethics Committee, UK (OxTREC 30-12, OxTREC 45-16). All patients provided written informed consent.

2.2.2.2 Patient enrolment

Eligible patients, who were admitted to inpatient wards at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, were enrolled between April 2013 and September 2014. The inclusion criteria were: age ≥15 years; <96 hours of fever history; and a positive NS1 rapid test to confirm DENV infection. Patients were excluded if they were unconscious, or severely ill, or pregnant. A single 3-5 mL venous blood sample was drawn from each participant on the day of enrolment, or occasionally, the following day. A plasma sample was collected, and subsequent procedures (RNA extraction and serotyping RT-PCR) was performed on the plasma sample to determine the DENV serotype infecting each of the patients, and to quantify levels of viremia.

2.2.2.3 NS1 rapid test

The BioRad Dengue NS1 strip test is used as a diagnostic for dengue virus infection in humans. It uses a sample of human serum or plasma to rapidly and qualitatively detect the dengue virus NS1 antigen. The standard operation procedure for NS1 rapid test is described in Appendix, entitled "performing dengue NS1 Ag strip".

2.2.2.4 RNA extraction and serotyping RT-PCR

Viremia was determined as the number of viral RNAs in the plasma sample [23]. First, viral RNAs were extracted, and qRT-PCR was performed. In these specific procedures, positive controls were serotypes of cultured DENVs. Internal controls were Equine Arteritis Virus while PBS was negative controls.

MagNA pure 96 DNA &viral NA small volume kit was used for RNA extraction. The procedures followed the instruction from Roche. Briefly, the extraction of RNAs was based on the MagNA pure magnetic glass particle (MGP) technology. By binding to the MGP, nucleic acid was separated from other components of plasma samples. The key steps of a MagNA Pure 96 NA isolation procedure were:

1. Sample material was lysed, nucleic acids were released, and nucleases were denatured.
2. Nucleic acids bound to the silica surface of the added MGPs due to the chao-tropic salt conditions, and the high ionic strength of the lysis/binding buffer.
3. MGPs with bound nucleic acids were magnetically separated from the residual lysed samples.
4. Unbound substances, such as proteins, cell debris, and PCR inhibitors were removed with several washing steps.

5. Purified nucleic acids were eluted from the MGPs.

The nucleic acid was then used to identify DENV serotypes and to quantify viremia with multiplex qRT-PCR.

A single sample was run in two independent tests. With NS5-specific primers, the first test amplified DENV-1 and DENV-3 while the second test amplified both DENV-2 and DENV-4. The results of the two assays combined informed the infected DENV serotype, and viremia. Paired primers of the four DENV serotypes targeted viral NS5 RNA sequence. The addition of serotype-specific fluorogenic probes to the master mix (DNA Master Hydrolysis Probes, Roche) allowed the detection of the amplified targets. Standard curves were also included for quantification of DENV RNAs.

Roche Light Cycler II PCR machines were used as thermal cyclers that report results automatically. Values, lower than the Limit of Detection, were regarded as negative samples. The limit of detections (copies/test) were 5 for DENV-1, 1 for DENV-2, 5 for DENV-3, and 10 for DENV-4 [23]. DENV RNA equivalents per mL were calculated thereafter. Standard operation procedures for RNA extraction and serotyping RT-PCR are described in Appendix, entitled “RNA extraction using MagNA Pure 96 DNA & viral NA small volume kit” and “Serotyping and quantification of DENV by one-step multiplex RT-PCR”, respectively.

2.2.3 DENV-specific monoclonal antibodies

Collaborators produced mAbs and sent them to me. Clone names and characteristics of the 12 human mAbs used in this study are described in Table 2.6.

2.2.4 Viremic blood neutralization assay (ViBNA)

The ViBNA is an assay that measures the antiviral potency of antibodies. Uniquely, the ViBNA uses viremic blood drawn directly from acutely-infected dengue patients, and DENV infection of blood-fed mosquitoes as the assay endpoint.

Members of the Dengue group and I carried out the ViBNA. We reared mosquitoes, homogenized mosquitoes, and carried out mosquito RT-PCR. I performed data screening and statistical analysis. A statistician helped me decide what test to use.

2.2.4.1 Overall procedure

Viremic blood was drawn from NS1-positive dengue patients, spiked with the mAbs. The concentration of mAbs in viremic blood was at 10 μg/mL, except for 747(4)B7 (3.7 μg/mL) and 753(3)C10 (5 μg/mL). The blood-antibody mix was incubated at 37°C for 30 minutes for virus neutralization to occur. The 30-minute duration is a standard time for mAbs to neutralize virus, as with other neutralization assays [131, 167]. PBS (negative controls) and HIG (positive controls) were prepared in parallel. The blood-antibody mix was then maintained at 37°C in artificial membrane feeders, attached to a circulating warm water system (Figure 2.2). Mosquitoes were allowed to feed on the antibody-blood mixture for maximum 1 hr. Between 10-50 wild-type *Ae. aegypti* fed on a viremic blood sample spiked with a mAb, constituting a cohort of mosquitoes. Engorged mosquitoes were then maintained for 7 days, allowing DENVs to be detectable in mosquitoes. Mosquito mortality was recorded daily; those surviving the incubation period were sacrificed and harvested for testing. Whole bodies were collected and homogenized. Individual

mosquito homogenate was then tested for the presence of DENV RNAs using RT-PCR procedures described below. Data were screened and analyzed with a statistical model.

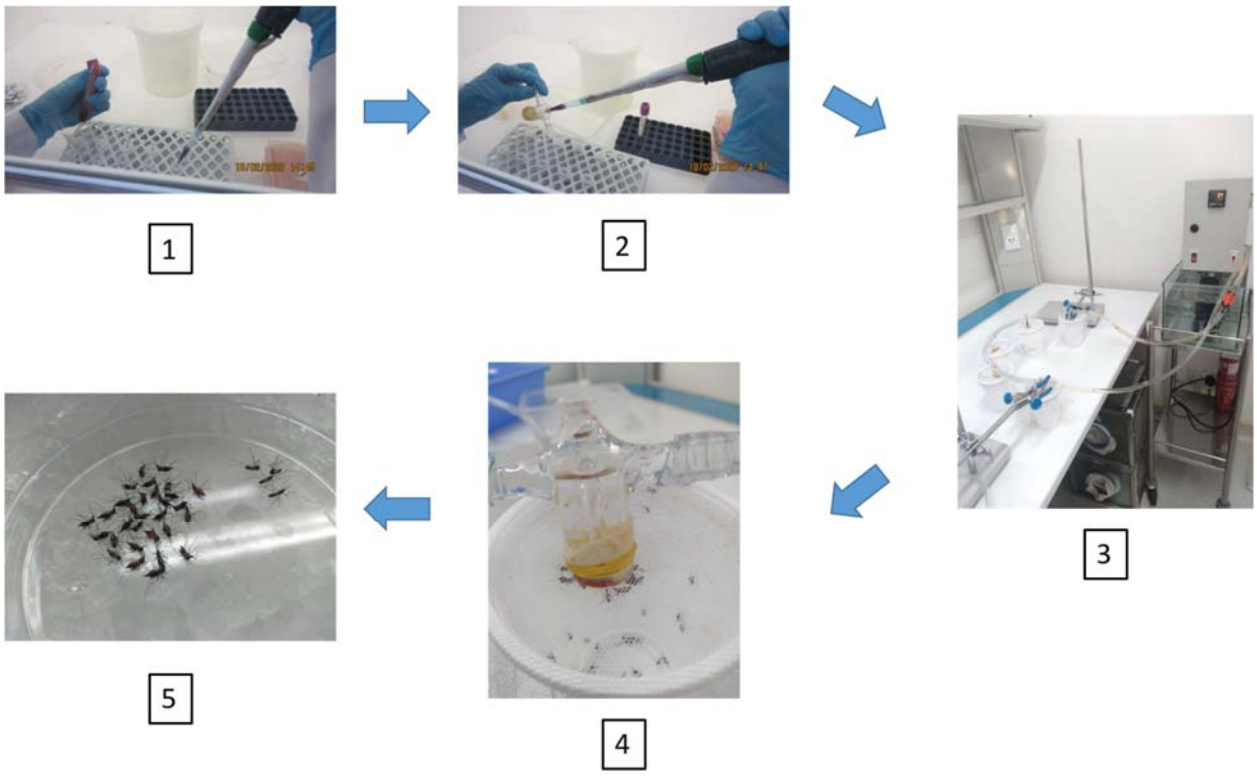


Figure 2.2: Key steps of ViBNA

1. Viremic blood sample is pipetted into the required number of tubes or the number of mAbs treatment groups and controls in the particular experiment, then spiked with individual mAbs or controls. 2. The mix of a mAb and a viremic blood is transferred into a mosquito's feeder. 3. Water-jacketed system with multiple feeders is set up. Each feeder contains a blood sample spiked with a mAb/control. 4. Mosquitoes feed on blood in the feeder. A group of mosquitoes that feed on a viremic blood spiked with a mAb constitutes a cohort of mosquitoes. 5. Engorged mosquitoes are distinguished from unfed mosquitoes (engorged mosquitoes have patient's blood in abdomen).

2.2.4.2 Preparation of mosquitoes

We used 3-5 days-old F_3 laboratory-reared *Ae. aegypti* in the ViBNA. All mosquitoes were derived from field-sourced materials (eggs or larvae) collected in Ho Chi Minh City, Viet Nam. Mosquitoes were maintained at 28°C, with a 12:12 light:dark cycle and 80% relative humidity. Mosquito colonies were blood-fed on healthy human volunteers and had access to 10% sucrose *ad libitum*. The standard operation procedure for preparation of mosquitoes is described in Appendix, entitle "Rearing *Aedes* mosquitoes".

2.2.4.3 Homogenization and mosquito RT-PCR

For homogenization, individual mosquitoes were rinsed into the solution of squash buffer and protein K, made with the ratio of 80:1. Squash buffer consisted of 10 mM Tris pH 8.2, 1 mM EDTA, and 50 mM NaCl. They were then homogenized with beads (size = 1 mm), using a tissue lyser. One-step multiplex RT-PCR was then carried out to target both 3'UTR of pan-serotypes of DENVs and *Rps17* gene of *Ae. aegypti* (internal controls). The RT-PCR used multiple primers and fluorescence probes (specific for each target) included in the master mix. Fluorescently-labelled probes (FAM for the DENV probe, Cy5 for the mosquito probe) each emitted a distinct wavelength of light that were received by a specific channel in the LightCycler™ system.

Positive controls and negative controls were also included for the homogenization and mosquito RT-PCR. Positive controls were DENV-infected *Ae. aegypti* using intrathoracic inoculation while the negative controls were mosquitoes never exposed to DENVs. Standard operation procedures for homogenization and mosquito RT-PCR were described in Appendix, entitled "Mosquito homogenization" and "One-step multiplex RT-PCR targeting DENV + wolbachia + *Aedes aegypti*", respectively.

2.2.4.4 Data screening and statistical analysis

We excluded blood meals (patients' samples) for any of these reasons: 1) PBS cohort (i.e. mosquitoes exposed to PBS) were RT-PCR negative for DENVs at seven days post feeding; 2) all mosquitoes of either PBS cohort or HIG cohort died during the 7-day incubation; 3) the number of harvested mosquitoes from a single exposure was fewer than four. Due to the nested nature of the data, i.e. 10-50 mosquitoes fed on each preparation of blood of the same dengue patient, a marginal logistic regression model was used to evaluate the antiviral potency of any given mAb, in reference to the PBS cohort from within each antibody treatment. Because viremia varied between dengue patients, which might influence the binding of mAbs and DENV infection of mosquitoes [168], the statistical model was adjusted for plasma viremia levels. A p value of less than 0.001, adjusted for multiple-comparisons by the Bonferroni correction method, was considered statistically significant.

For data visualization, the reduction in percentage of mosquitoes with DENV infections was calculated for each blood meal. This allowed us to determine whether there was a negative or

positive influence of the mAb on the mosquito infection frequency. The following equations were used:

$$100 - \left(\frac{\text{The proportion of infected mosquitoes in the test mAb group}}{\text{The proportion of infected mosquitoes in the saline group}} \right) \times 100$$

The results of this equation sometimes lower than –100% which do not reflect the natural actions of mAbs, i.e. the mAbs was unable to enhance the infection by more than 100%. As such, if the results of the equation were lower than 0, the equation will be re-computed as

$$-[100 - \left(\frac{\text{The proportion of infected mosquitoes in the saline group}}{\text{The proportion of infected mosquitoes in the test mAb group}} \right) \times 100]$$

Values ranged from -100 to 0 when the test mAb cohort had higher infection than the saline cohort, and from 0 to +100 when the saline cohort had higher infection than the test mAb cohort.

2.2.5 The performance of HIG and PBS in the ViBNA

I analyzed the performance of the positive and negative controls with all blood meals we employed. The positive controls were HIG, created by pooling convalescent plasma samples of 119 laboratory-confirmed patients with any of the four DENV serotypes (from other approved OUCRU-based studies). Specifically, the proportion of samples creating the pool with DENV-1 and DENV-2 infections was higher than that of DENV-3 and DENV-4 although plasma samples were not viremic. The plasma pool was then heated at 56°C for 30 minutes to inactivate complement, and aliquoted for single use. Negative controls were 0.9% PBS solution. Both

controls were diluted 1:9 in viremic blood for use in all mosquito exposures. I pooled the convalescent plasma samples, heated and aliquoted the pool to obtain HIG.

2.2.6 The quality, purification, and concentrations of tested mAbs.

The quality, purification, and approximate concentrations of mAbs were examined using gel electrophoresis. Because the concentrations of the 12 mAbs were indicated by collaborators at 10 μg/mL, the thickness of gel bands were used to visually compared between tested mAbs. If any tested mAbs had a visually-different band thickness, their concentrations were re-quantified using a BCA protein assay. A member of the OUCRU Zoonosis group conducted SDS-PAGE for mAbs tested in this study while I performed BCA protein assay.

2.2.6.1 Sodium dodecylsulfate polyacrylamide gel electrophoresis

SDS-PAGE was employed to examine the purification of mAbs and to estimate their concentrations. Briefly, the mAbs were denatured by heating at 100°C, then sodium dodecylsulfate was added. These led to the dissociation of all disulfide bonds of polypeptides and negatively-charged complexes of sodium-dodecylsulfate -polypeptides formed a rod with lengths proportional to the molecular weight. As such, the heavy and light chains of mAbs could be separated on the basis of their size only.

The charged polypeptides moved through a polyacrylamide gel under an electrical field. The polyacrylamide gel was made of monomeric acrylamide and bis-acrylamide. The two reagents whose concentrations determined the size of pores were polymerized by tetramethylenediamine and ammonium persulfate.

Two polyacrylamide layers were used in a gel: a narrow layer of stacking gel was above a separating layer (10%). Stacking gel layer had acidic pH while the separating layer had alkaline pH. Another difference is the pore size of stacking layer is larger than that of separating layer (10%). As such, stacking layer concentrated polypeptides before they entered the separating layer. This enhanced the resolution of SDS-PAGE. The standard operation procedure for SDS-PAGE is described in Appendix, entitled "Analysis of expression of samples by SDS-PAGE".

2.2.6.2 Pierce™ BCA Protein Assay

This assay, developed by Thermo Scientific, was based on BCA for the colorimetric detection and quantification of mAbs. This method combined the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of Cu^{+1} using a unique reagent consisting of BCA. The purple-colored reaction product of this assay was formed by the chelation of two molecules of BCA with one Cu^{+1} ion. This water-soluble complex exhibited a strong absorbance at 562 nm that was nearly linear with increasing protein concentrations over a broad working range (20-2000 μg/mL).

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan, and tyrosine) were reported to be responsible for color formation with BCA. Protein concentrations were determined and reported with reference to standards of bovine serum albumin.

I performed the BCA protein assay. The procedure was as follows:

1. Mix 50 parts of BCA reagent A with 1 part of BCA reagent B to obtain working reagent (WR)
2. Pipette 25 µL of each standard or unknown sample replicate into a microplate well
3. Add 200 µL of washing reagent to each well and mix the plate thoroughly on a plate shaker for 30 seconds
4. Cover plate and incubate at 37°C for 30 minutes
5. Cool plate to room temperature and measure the absorbance at or near 562 nm on a plate reader
6. Generate automatically the concentrations of mAbs from the reader and its software

2.2.7 The anti-viral potency of mAbs against DENVs

To address my hypothesis that several tested mAbs would fail to neutralize DENVs in viremic blood, we tested their antiviral potency using the ViBNA. All of the mAbs were previously characterized, by collaborators, as being able to neutralize one or more of DENV serotypes using cell-culture systems (Table 2.6).

2.2.8 Variation at the epitope of a moderately-neutralizing mAb

To examine whether the anti-viral potency of a neutralizing mAb was associated with changes in viral amino acid residues at the epitope, we performed the sequencing of RNAs encoding E proteins of the DENV in viremic blood samples, using Roche 454 system. The viremic blood samples were spiked with the mAb. The sequencing included four major steps: preparation for cDNAs, amplification for targeted nucleic acid, primer adapter amplification for starting-point labelling, and emulsion PCR for sequencing. Members of the Dengue group conducted all steps

within the 454 sequencing procedure while I used the software Mega7 to generate amino acid sequences, and performed the statistical analysis.

Briefly, cDNAs were synthesized from viral RNAs, then we added a set of primers that was designed to amplify template specific sequences, or amplicons (Figure 2.3 in green). The next step of 454 sequencing was the use of primer-adapters. While primers amplified template specific sequences, adapters functioned in fixing amplified products on a stationary phase in the subsequent emulsion PCR (Figure 2.3 in blue). The adapters also carried "four-nucleotide key" (TCAG) sequence that is, along with MID, defined as the starting point of a sequence read (Figure 2.3). For each template specific sequence, two primer adapters, A and B, were allowed to sequence the target bi-directionally.



Figure 2.3: Components of primer adapters

Forward and reverse primers, which amplify a template specific sequence (in green), contain two different MID. At the 5' end of both primers, there are sequences that is necessary for the binding of PCR product into complementary beads in emulsion PCR. There are also four "key residues" (in red), along with MID, indicates the starting point of sequences.

The residues at the 5' end of adapters immobilized targeted DNAs onto capture beads. The double-stranded amplified DNAs were diluted and compartmentalized as water droplets in a water-in-oil emulsion [169]. The oil helped the droplet of water and DNAs resist changes such as heating and cooling during the emulsion PCR. Here, polymerase and primers helped nucleotides bind to DNAs, fluorescence was emitted and recorded by a camera. The intensity of fluorescence distinguished nucleotide bases. For example, the intensity of A was higher than those of C, G, and T. By plotting fluorescence intensity on a graph, the sequence of target RNAs could be decoded.

Amino acid sequences were generated from RNA sequences using the software Mega7. The Wilcoxon rank sum test, where a p-value < 0.05 denotes statistical significance, was used to analyze the association between a change in amino acid residues and the antiviral potency of the moderately neutralizing mAb. Standard operation procedures of 454 sequencing are described in Appendix, entitled "cDNA synthesis from RNA", "Amplicon library preparation for 454 sequencing", and "emPCR amplification and GS junior sequencing".

2.2.9 The impact of patients' DENV-specific IgG on the antiviral potency of mAbs

To examine whether patient-derived DENV-reactive antibodies compete with mAbs for epitope binding, and reduce the antiviral potency of mAbs, members of the Dengue group conducted the Panbio indirect IgG ELISA to assess DENV-reactive IgG in the viremic blood. The procedures followed instruction from the manufacturer (Panbio). Briefly, plasma samples of patients were diluted (normally at 1:100), then were added onto a microplate well pre-coated with antigens of the four serotype of DENVs. Residual patients' antibodies were removed before the addition of peroxidase-conjugated anti-human IgG. When the colorless tetramethylbenzidine chromogen was

added, the enzymatic activity of peroxidase was activated, which change the color of the chromogen to blue. Sulfuric acid 10% was then added to stop the reaction, resulting in a subsequent color change from blue to yellow. The color absorbance then was read at a wavelength of 450 nm with a reference filter of 600-650 nm.

Each microplate included five microwells for controls. These were one positive control, one negative control, and three calibrators. Mean absorbance of calibrators were calculated, and multiplied by 0.62 (calibration factor) to obtain cut-off value specific for that plate. Panbio units were then calculated following the equation below:

$$\text{Panbio units} = \frac{\text{sample absorbance x } 10}{\text{cut}-\text{off value}}$$

Samples with Panbio units of less than 9 meant IgG negative while those of more than 11 meant IgG positive. If the Panbio units fell into a range from 9 to 11, the indirect DENV-reactive IgG results were equivocal.

2.3 Results

Between April 2013 and September 2014, we enrolled 119 acutely-infected laboratory-confirmed adult patients with dengue. DENV-1 and DENV-4 were the most prevalent serotypes in the cohort, followed by DENV-2, and DENV-3. Median viremia levels of DENV-1 and DENV-2 were higher than DENV-3, and DENV-4 (Figure 2.4 Table 2.1).

The 119 viremic blood samples were spiked individually with a median of 10 (IQR 9-12) mAbs. A median of 29 (IQR 26-31) mosquitoes was then allowed to feed on each mix of a viremic blood and a mAb, constituting a mosquito cohort. Approximately 44% of mosquitoes were engorged. After seven days of maintenance, about 23% of mosquitoes died and were thus excluded from the subsequent analysis. I also excluded from statistical analysis data of: 1) blood meals used to optimize the ViBNA, 2) blood meals where mosquitoes of PBS cohort were negative for DENVs, 3) blood meals where all mosquitoes within the HIG or PBS cohorts died before being collected, and therefore could not be assessed. Therefore, 71 blood meals were included into statistical analysis (Figure 2.4).

Table 2.1: Baseline information on patients

Fresh viremic blood samples from these patients were individually spiked with human mAbs.

The patients were enrolled from April 2013 until September 2014.

All Patients	
n	119
Year of age	
n	106
Median (IQR)	28 (22-34)
Sex	
n	108
Percentage of males	43
Log10 viremia (copies/mL)	
n	113
Median (IQR)	8.2 (7.1-9.0)
DENV Serotype	
n	113
DENV-1 n (%)	50 (44)
DENV-2 n (%)	13 (12)
DENV-3 n (%)	13 (12)
DENV-4 n (%)	37 (32)

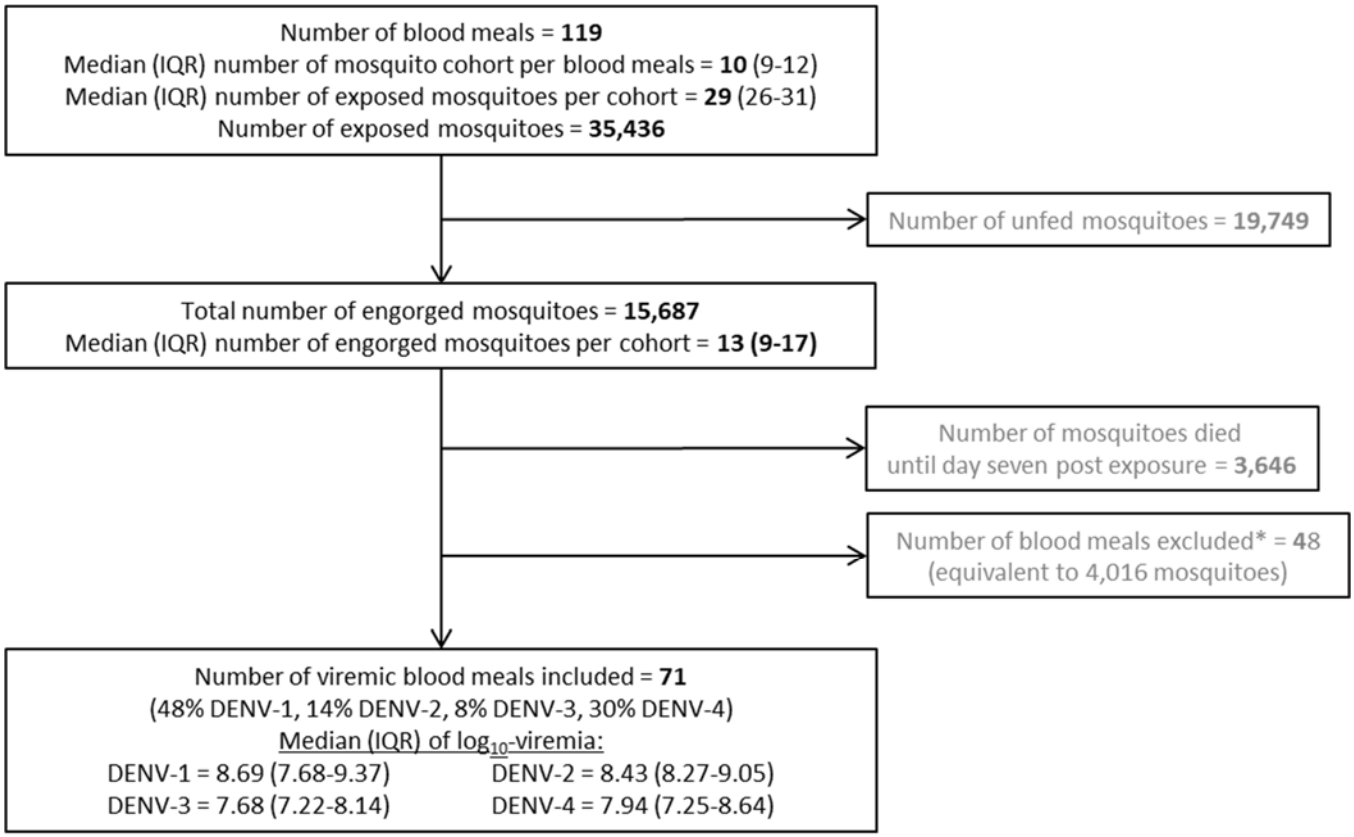


Figure 2.4: Flow chart of the processing of the ViBNA

Mosquitoes that fed on each combination of a viremic blood sample and a mAb constitute a cohort (Figure 2.2). Engorged mosquitoes were then reared until seven days after blood feeding. Only surviving mosquitoes were collected and tested for the presence of DENV RNAs. *Excluded data originated from cases where blood was used to optimize the ViBNA, from cases where mosquitoes of PBS cohort were negative for DENVs, from cases where all mosquitoes within the HIG or PBS cohorts died before being collected, and therefore could not be assessed. Viremic blood samples were independently examined for DENV serotypes and viremia.

2.3.1 The performance of HIG and PBS in the ViBNA

Each of the 71 blood meals was spiked with HIG and PBS as controls. Results show HIG neutralized all four DENV serotypes while PBS did not (Table 2.2) (Figure 2.5). Moreover, ORs for DENV-1 and DENV-2 were lower than those for DENV-3, and DENV-4 (Table 2.2), implying more effective HIG-mediated neutralizations of DENV-1 and DENV-2. In summary, HIG and saline performed efficiently as positive and negative controls of the ViBNA, respectively.

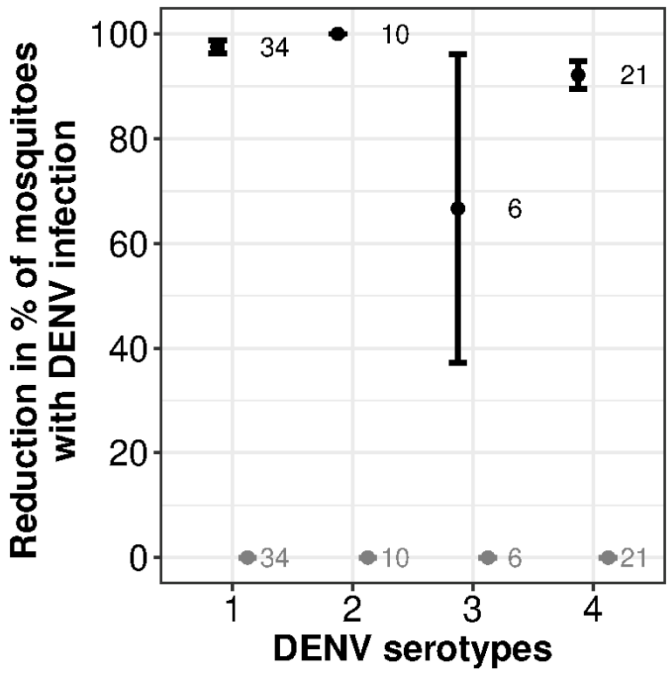


Figure 2.5: The HIG and PBS were satisfactory as controls in all ViBNA experiments. HIG and PBS were added into each and every 71 blood meal. Numbers of blood meals, stratified by DENV serotypes, are shown. Test results for HIG are in black while those for PBS are in grey. The y-axis value of 100 indicates that HIG inhibited DENV infection of mosquitoes completely, relative to PBS cohorts.

Table 2.2: Neutralization capacity of HIG in the ViBNA

Mosquitoes were fed on blood meals containing DENVs spiked with HIG. Infected mosquitoes were then detected with the presence of DENV RNAs.

Viral Serotype	Number of blood meals	Percentage of infected mosquitoes (Total number of mosquitoes tested)		OR[a] (95% CI)	*P*
		HIG	PBS		
DENV-1	34	2 (287)	84 (308)	0.00 (0.00-0.01)	<0.001
DENV-2	10	0 (66)	93 (97)	0.00 (0.00-0.01)	<0.001
DENV-3	6	6 (33)	48 (50)	0.06 (0.01-0.33)	0.001
DENV-4	21	5 (176)	63 (218)	0.02 (0.01-0.06)	<0.001

[a]OR was calculated using data from the PBS cohort as the reference. P values were calculated using marginal logistic regression models, adjusted for the patient's plasma viremia.

2.3.2 The quality, purification, and concentrations of tested mAbs

We obtained 12 mAbs from many collaborators who used heterogenous approaches to generate, purify, and quantify them (Table 2.6). To check for their existence, purification and approximate concentration, we conducted SDS-PAGE before any ViBNA experiment. Gel results show all mAbs had two clear bands, corresponding to the heavy and light chains, respectively (Figure 2.6). Although the concentration of all mAbs were claimed to be at 10 µg/mL, Thee thickness of gel bands varied visually. Specifically, 747(4)B7 and 753(3)C10 generated thinner bands compared with other mAbs (Figure 2.6). Therefore, I performed a BCA protein assay to quantify concentrations of 747(4)B7 and 753(3)C10. Results show the concentration of 747(4)B7 was at 3.7 µg/mL while that of 753(3)C10 was at 5 µg/mL. These concentrations of the two mAbs were subsequently used when I analyzed data from ViBNA experiments.

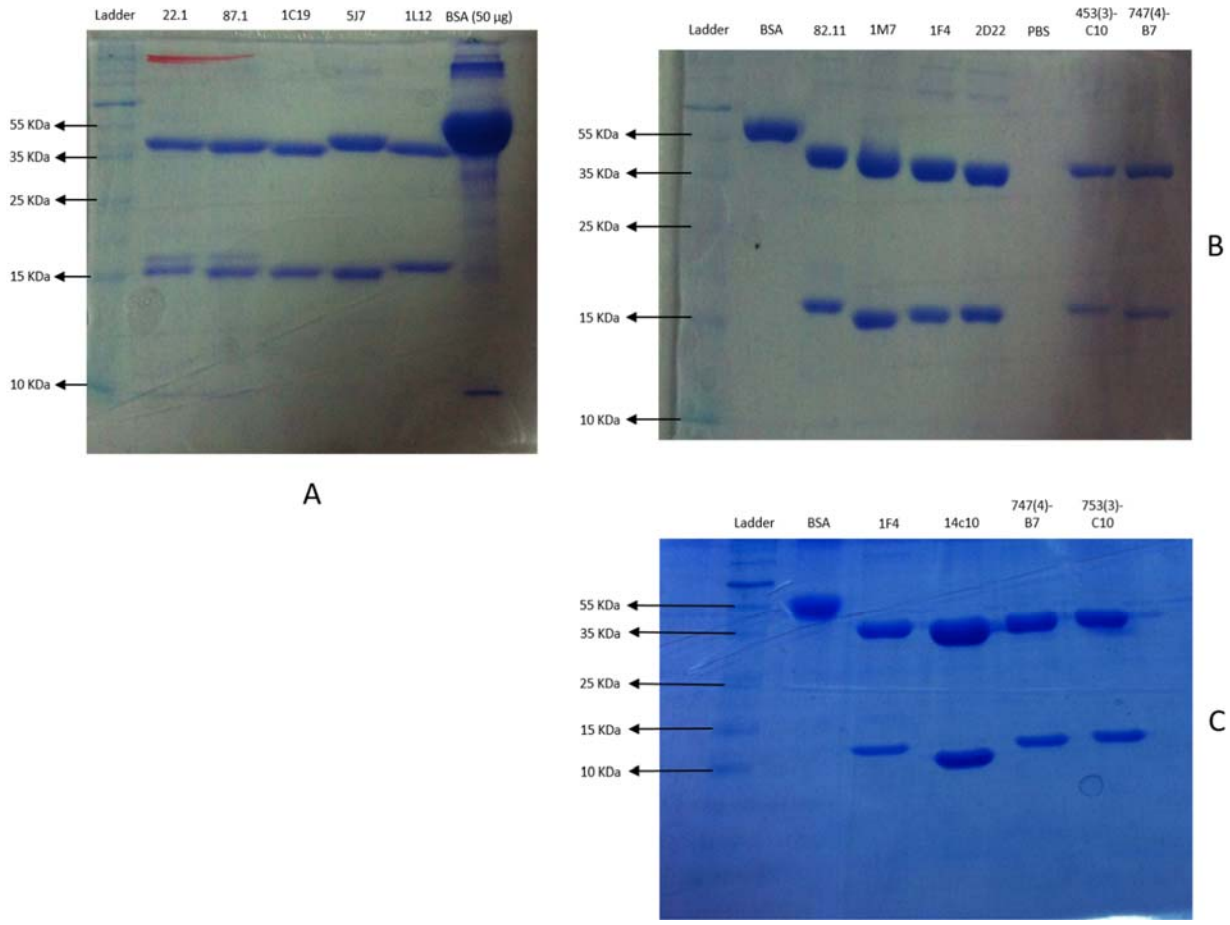


Figure 2.6: The quality of the 12 tested mAbs was high.

After receiving mAbs, we tested them with SDS-PAGE for existence, purification, and approximate concentration. We performed three batches of SDS-PAGE, indicated in panel A, B, and C. The clone names of mAbs are shown at the top of each panel. Each mAbs were added into a corresponding well at the amount of 5 µg/well. Several mAbs such as 1F4, 747(4)B7 and 753(3)C10 appear in duplicate. Bovine serum albumin, and PBS were served as positive and negative controls, respectively. Bovine serum albumin (BSA) was also added at 5 µg/well, except for in the panel A where the amount in the well was 50 µg. The molecular weight marker is shown.

2.3.3 The anti-viral potency of mAbs against DENVs

Using the ViBNA, I assessed the antiviral potency of 12 mAbs by viremia levels (Figure 2.7). Statistical analyses show that five of the 12 mAbs failed to neutralize any of the DENV serotypes, shown by p-values > 0.001 (Figure 2.8, Table 2.3). These non-neutralizing mAbs were 1M7, 1C19, 22.3, 82.11, 87.1.

Seven mAbs inhibited the infectivity of viremic blood for mosquitoes, indicated by p-values ≤ 0.001 (Figure 2.8, Table 2.3). 1F4 and 5J7 were modestly active against DENV-1 and DENV-2, respectively. The mAbs 14c10 (DENV-1 specific), 1L12 and 2D22 (both DENV-2 specific) blocked viral infection of mosquitoes at 10 μg/mL. Two mAbs (747(4)B7 and 753(3)C10) had different potency characteristics at the highest concentration tested (3.7 μg/mL and 5 μg/mL, respectively). 753(3)C10 blocked DENV-1 and DENV-4 but did not prevent mosquitoes from acquiring DENV-2 infection. 747(4)B7 was also highly potent against DENV-1, but only partially blocked DENV-2 and DENV-4 (Figure 2.8, Table 2.3).

Because there were an insufficient number of patients with DENV-3 infection, the antiviral potency of mAbs, assessed with the ViBNA, was inconclusive for DENV-3. For example, 747(4)B7 could be cross-reactive to four serotypes, but these ViBNA experiments did not have enough data to draw the potency of 747(4)B7 against DENV-3,

Notably, several mAbs appeared to enhance DENV infections of mosquitoes because the odds ratios were higher than 1, such as 1M7 and 1C19 against DENV-4 (Figure 2.8, Table 2.3).

Although these odds ratios were not statistically significant, the data suggested the possibility of the antibody-mediated enhancement of DENV infection of mosquitoes, or that there was simply some degree of variation in the mosquito infectivity between blood meals.

In this experiment, viremic blood was spiked with up to 10 μg/mL of mAbs. Based on the molecular weight of monoclonal antibody ($160*10^3$ g/mol, 10 μg/mL of mAbs is equivalent to approximately $3.8*10^{16}$ antibody molecules/mL. If two mAb molecules bind to one E dimer, $3.8*10^{16}$ antibody molecules/mL may neutralize approximately 10^{14} virion/mL. Indeed, the highest viremia level is estimated at around 10^9 RNA copies/mL, so the ratio of mAb:virus is calculated roughly at around 10^5. This calculated ratio means there is a sufficient amount of mAbs to neutralize each virus.

In summary, I characterized seven and five mAbs as neutralizing and non-neutralizing antibodies, respectively, using the ViBNA. Five of the seven mAbs were type specific while the other two mAbs were cross-reactive. Several tested mAbs also appeared to enhance DENV infection of mosquitoes.

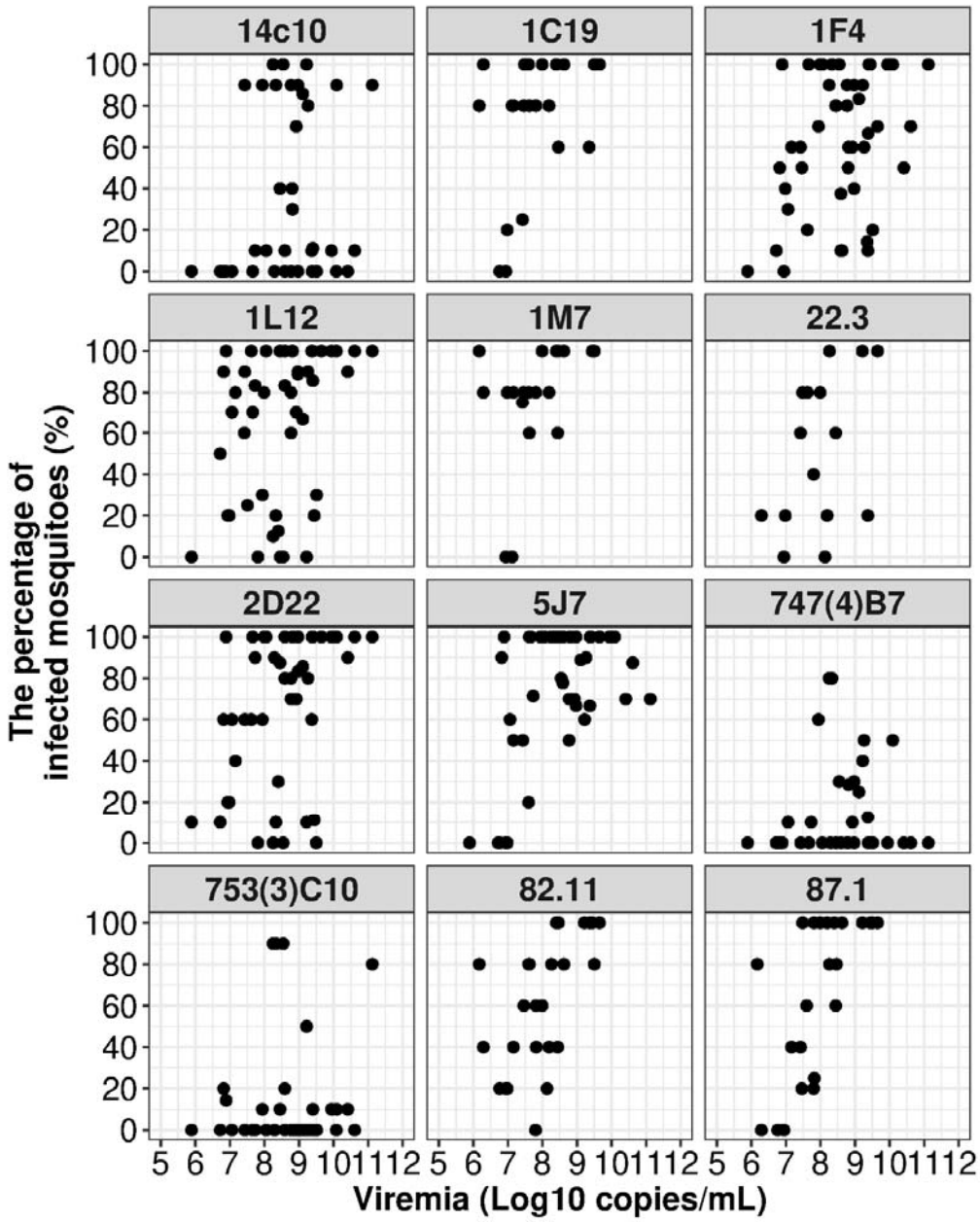


Figure 2.7: Dose-response scatterplot of plasma viremia versus the percentage of infected mosquitoes

Mosquitoes were fed on viremic blood spiked with individual mAbs. Each dot represents a cohort of mosquitoes fed on a mix of a viremic blood sample and a mAb. Clone names of mAbs were indicated in the top of each panel.

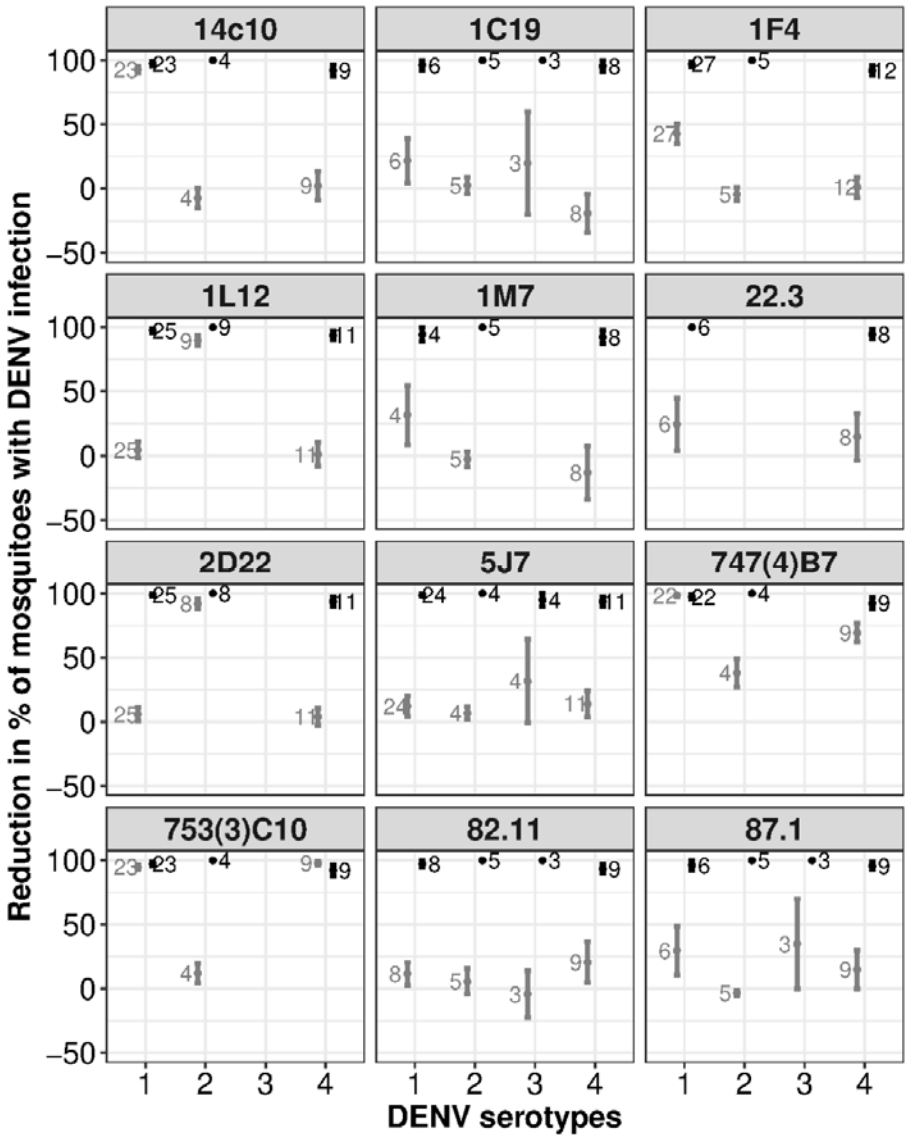


Figure 2.8: Five mAbs failed to neutralize DENVs using the ViBNA.

At the top of each panel are the mAb clone names. Means and standard errors of test results were calculated from three or more independent ViBNA measurements per serotype. Numbers of blood meals, spiked individually with mAbs, are shown. Test results for HIG are in black while tested mAbs are in grey. The y-axis value of 100 indicates that the mAb blocks DENV infection of mosquitoes completely, relative to the PBS cohort.

Table 2.3: Neutralization capacity of all mAbs in the ViBNA

Mosquitoes were fed on viremic blood meals spiked with high concentrations of mAbs. We then tested for the presence of DENV RNAs in mosquitoes. The neutralizing mAbs, highlighted in grey, were effective against at least one DENV serotype ($p < 0.001$).

mAb	Viral Serotype	Number of blood meals	Percentage of infected mosquitoes (Total number of mosquitoes tested)			OR[a] (95% CI)	*P*
			HIG	mAb	PBS		
1C19	DENV-1	6	3 (30)	70 (30)	84 (57)	0.38 (0.13-1.13)	0.08
	DENV-2	5	0 (22)	88 (25)	91 (43)	0.69 (0.20-2.38)	0.56
	DENV-3	3	0 (15)	67 (15)	61 (28)	2.46 (0.51-11.81)	0.26
	DENV-4	8	3 (39)	72 (39)	51 (79)	2.58 (0.89-7.51)	0.08
1F4	DENV-1	27	3 (206)	51 (255)	83 (249)	0.17 (0.09-0.35)	**<0.001**
	DENV-2	5	0 (44)	95 (39)	94 (54)	1.13 (0.28-4.55)	0.86
	DENV-3	2	7 (15)	100 (15)	65 (20)	NA[b]	NA[b]
	DENV-4	12	5 (91)	71 (91)	76 (119)	0.69 (0.31-1.51)	0.36
1M7	DENV-1	4	5 (20)	63 (19)	78 (37)	0.28 (0.10-0.80)	0.02
	DENV-2	5	0 (24)	96 (23)	95 (41)	1.08 (0.19-6.19)	0.93
	DENV-3	2	0 (10)	90 (10)	80 (20)	2.26 (0.48-10.68)	0.30
	DENV-4	8	5 (39)	69 (39)	53 (75)	1.96 (0.55-7.01)	0.30
22.3	DENV-1	6	0 (75)	67 (30)	82 (57)	0.38 (0.10-1.41)	0.15
	DENV-2	0	0 (0)	NA	NA	NA	NA
	DENV-3	1	0 (5)	80 (5)	80 (10)	NA[b]	NA[b]
	DENV-4	8	3 (65)	38 (40)	45 (76)	0.70 (0.28-1.76)	0.45
5J7	DENV-1	24	2 (188)	80 (208)	81 (217)	0.89 (0.50-1.57)	0.68
	DENV-2	4	0 (39)	85 (39)	93 (45)	0.46 (0.38-0.54)	**<0.001**
	DENV-3	4	4 (25)	52 (25)	58 (38)	0.70 (0.18-2.70)	0.60
	DENV-4	11	5 (89)	73 (93)	75 (123)	0.70 (0.39-1.28)	0.25
82.11	DENV-1	8	1 (85)	78 (40)	86 (77)	0.52 (0.23-1.17)	0.12
	DENV-2	5	0 (24)	88 (25)	95 (41)	0.34 (0.07-1.82)	0.21
	DENV-3	3	0 (15)	53 (15)	61 (28)	0.76 (0.38-1.52)	0.44
	DENV-4	9	4 (83)	36 (45)	44 (86)	0.69 (0.35-1.36)	0.28

87.1	DENV-1	6	2 (60)	67 (30)	87 (60)	0.15 (0.03-0.79)	0.03
	DENV-2	5	0 (24)	96 (25)	95 (41)	1.18 (0.36-3.91)	0.79
	DENV-3	3	0 (15)	53 (15)	61 (28)	0.74 (0.17-3.15)	0.68
	DENV-4	9	3 (71)	52 (44)	51 (86)	1.02 (0.52-2.00)	0.94
1L12	DENV-1	25	3 (196)	81 (214)	81 (223)	1.00 (0.55-1.83)	0.99
	DENV-2	9	0 (62)	8 (72)	92 (89)	0.01 (0.00-0.02)	**<0.001**
	DENV-3	2	7 (15)	93 (15)	65 (20)	8.56 (0.46-158)	0.15
	DENV-4	11	5 (85)	71 (91)	72 (125)	0.83 (0.34-1.99)	0.67
2D22	DENV-1	25	2 (196)	79 (218)	81 (221)	0.84 (0.48-1.49)	0.55
	DENV-2	8	0 (58)	8 (73)	95 (82)	0.00 (0.00-0.02)	**<0.001**
	DENV-3	2	7 (15)	100 (15)	65 (20)	NA[b]	NA[b]
	DENV-4	11	4 (89)	74 (92)	75 (123)	0.75 (0.42-1.34)	0.33
747(4)B7	DENV-1	22	3 (181)	2 (197)	82 (190)	0.00 (0.00-0.01)	**<0.001**
	DENV-2	4	0 (39)	58 (40)	93 (45)	0.09 (0.03-0.23)	**<0.001**
	DENV-3	1	10 (10)	0 (10)	50 (10)	NA[b]	NA[b]
	DENV-4	9	5 (79)	28 (83)	82 (103)	0.07 (0.03-0.17)	**<0.001**
753(3)C10	DENV-1	23	3 (189)	4 (204)	83 (200)	0.01 (0.00-0.02)	**<0.001**
	DENV-2	4	0 (39)	80 (40)	93 (45)	0.30 (0.12-0.75)	0.01
	DENV-3	1	10 (10)	80 (10)	50 (10)	NA[b]	NA[b]
	DENV-4	9	5 (79)	2 (89)	82 (103)	0.00 (0.00-0.02)	**<0.001**
14c10	DENV-1	23	3 (189)	7 (214)	83 (200)	0.01 (0.00-0.03)	**<0.001**
	DENV-2	4	0 (39)	98 (40)	93 (45)	3.51 (0.16-74.7)	0.42
	DENV-3	1	10 (10)	90 (10)	50 (10)	NA[b]	NA[b]
	DENV-4	9	5 (79)	79 (87)	82 (103)	0.83 (0.23-3.02)	0.78

[a]OR was calculated using data from each mAb with the PBS cohort as the reference. P values were calculated using marginal logistic regression models, adjusted for the patient's plasma viremia. [b]Numbers of blood meals were too small to have reliable models.

2.3.4 Variation at the epitope of a moderately neutralizing mAb

1F4 and 5J7 modestly neutralized DENV-1 and DENV-2 in ViBNA, respectively. The number of DENV-1 blood meals spiked with 1F4 was high (n = 27), allowing us to dissect the antiviral potency. Indeed, we tested 1F4 at 10 µg/mL in viremic blood for two consecutive years, and found that 1F4 consistently reduced DENV infection of mosquitoes in 2013, but the effect of 1F4 decreased significantly in three consecutive months in 2014 (Figure 2.9). In addition to possible degradation of 1F4, another possible explanation for the reduced potency is that DENVs were evolved in the field to escape antibody-mediated neutralization. Therefore, there were differences in amino acid residues at the epitope, which reduced the potency of 1F4. To this end, we performed the sequencing of E genes of 12 DENV-1 strains against which we have data on the neutralization capacity of 1F4. I then investigated whether there were any changes in amino acid residues at the epitope, compared with the previously-published residues.

I found only N52D (asparagine is substituted for aspartic acid at the residue 52 of E proteins), occurring in eight out of the 12 DENV-1 strains (Figure 2.11). The four remaining strains contained the corresponding amino acid N (asparagine). Interestingly, N52D substitutions were negatively associated with the reduction in percentage of mosquitoes with DENV infection (Wilcoxon Rank Sum Test, $W = 4.5$, $p = 0.06$) (Figure 2.10). In conclusion, along with the degradation of 1F4, the substitution of asparagine with aspartic acid at the residue number 52 of E proteins may contribute to the reduction in the antiviral potency of the mAb.

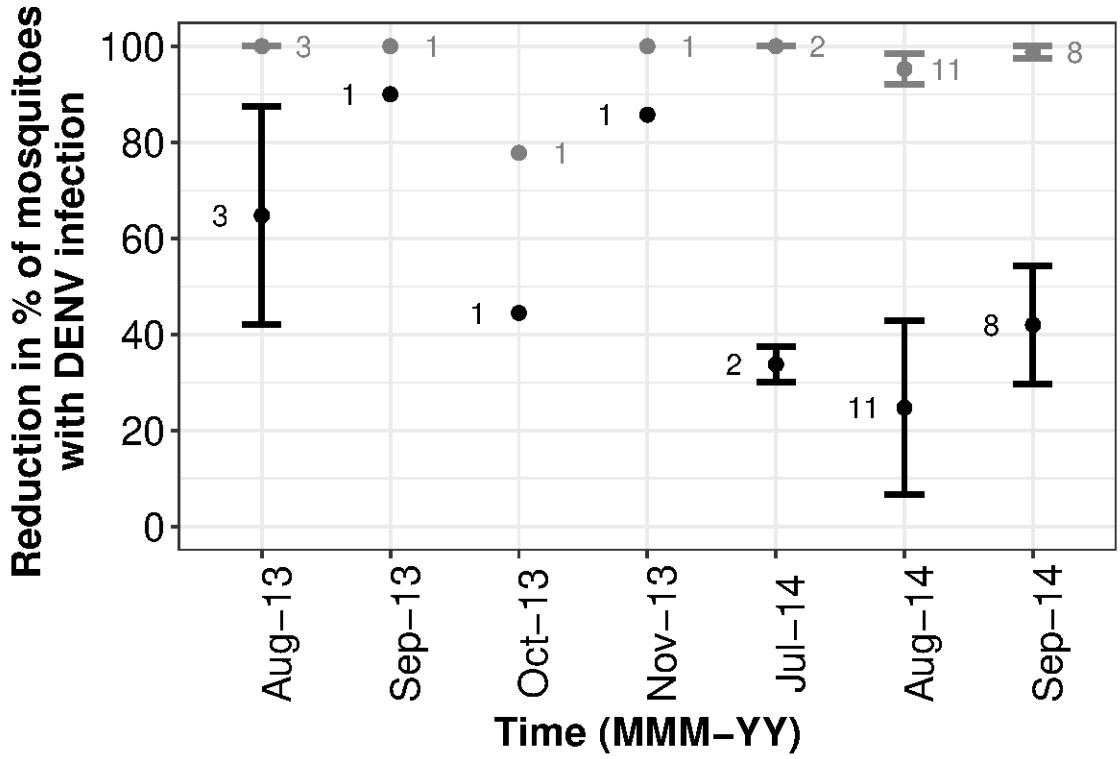


Figure 2.9: Neutralization capacity of 1F4 declined in 2014.

Means and standard errors are shown from more than one blood meal. Numbers of blood meals, spiked individually with 1F4, are shown. The y-axis value of 100 indicates 1F4 blocks DENV-1 infection of mosquitoes completely, relative to the PBS cohort.

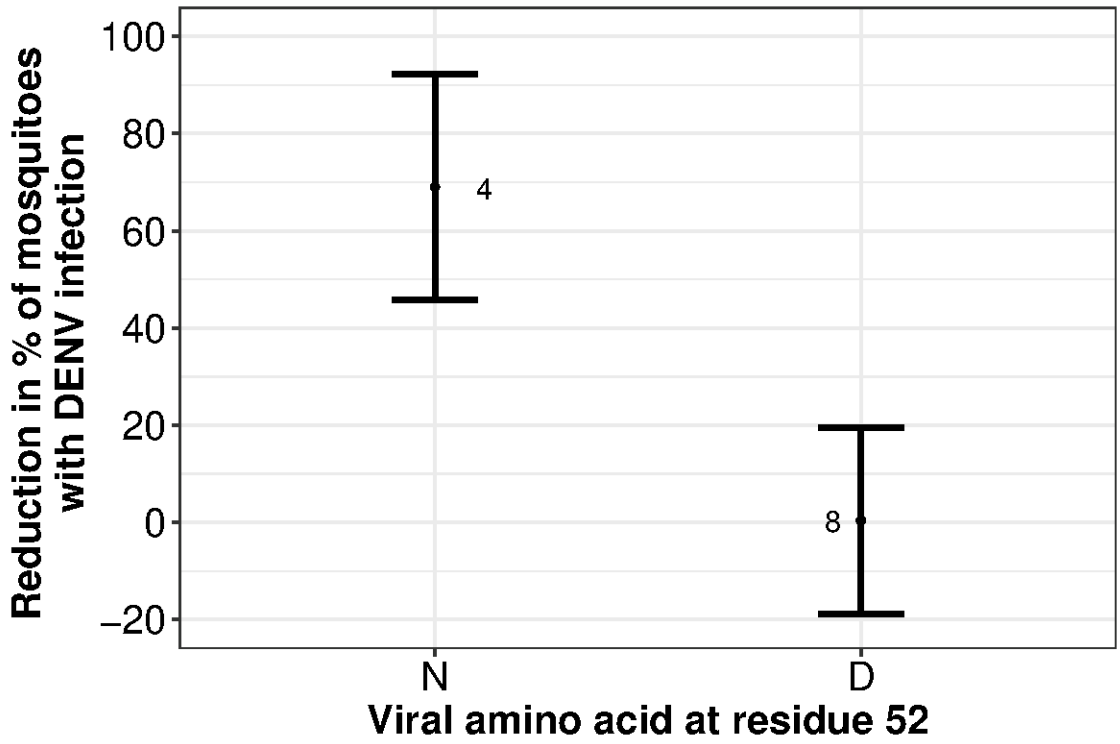


Figure 2.10: N52D substitution in DENV-1 E protein reduced the antiviral potency of 1F4 against DENV-1.

We performed the sequencing of RNAs encoding E proteins of DENV-1 in 12 viremic blood meals where 1F4 was added into. Numbers of blood meals are shown next to the means and standard errors. N stands for asparagine while D stands for aspartic acid. The y-axis value of 100 indicates 1F4 blocks DENV-1 infection of mosquitoes completely, relative to the PBS cohort.

2.3.5 The impact of patients' DENV-specific IgG on the antiviral potency of mAbs

Because the fusion loop is conserved across the four DENV serotypes, patient-derived antibodies that bind to the fusion loop are more likely to be cross-reactive, and poorly neutralizing [82, 136]. It is likely that these antibodies, present in viremic blood, may compete with cross-reactive mAbs for epitope binding, and reduce the antiviral potency of these cross-reactive mAbs. 747(4)B7 and 753(3)C10 were two cross-reactive mAbs (characterized above) that bind to E dimer epitopes overlapping the fusion loop, so their antiviral potency may be influenced by the presence of patient-derived IgG to the DENV virion. To this end, I compared the antiviral potency of 747(4)B7 and 753(3)C10 between IgG-positive and IgG-negative viremic samples. However, I could not distinguish between an IgG competition effect and a serotype effect. Specifically, after taking into account the detection of DENV-reactive IgG (Table 2.4), I could not obtain statistics for DENV-2 (Table 2.4) because no DENV-2 blood meals without DENV-reactive IgG were presented in this study (Table 2.5).

Table 2.4: Indistinguishable effect of patient-derived DENV-reactive IgG and DENV serotypes

Neutralization capacity of 747(4)B7 and 753(3)C10 in the viremic blood neutralization assay, with data for patient-derived DENV-reactive IgG. Mosquitoes were fed on blood meals containing DENV infected blood and 747(4)B7 and 753(3)C10 at 3.7 µg/mL and 5 µg/mL, respectively. Infected mosquitoes were then detected with the presence of DENV RNA. Odds ratios were calculated using data from each mAb with the negative saline control as the reference. P values were calculated using marginal logistic regression models, adjusted for the patient's plasma viremia and the detection of patients' DENV-reactive IgG.

mAb	Viral Serotype	Number of blood meals	Percentage of infected mosquitoes (Total number of mosquitoes tested)			OR (95% CI)	*P*
			HIG	mAb	PBS		
747(4)B7	DENV-1	20	3 (162)	2 (179)	86 (170)	0.00 (0.00-0.01)	**<0.00**
	DENV-2	3	0 (30)	63 (30)	97 (37)	NA[a]	NA[a]
	DENV-3	0	NA	NA	NA	NA	NA
	DENV-4	6	6 (51)	20 (55)	76 (75)	0.07 (0.03-0.20)	**<0.00**
753(3)C10	DENV-1	21	3 (170)	4 (187)	87 (180)	0.00 (0.00-0.01)	**<0.00**
	DENV-2	3	0 (30)	90 (30)	97 (37)	NA[a]	NA[a]
	DENV-3	0	NA	NA	NA	NA	NA
	DENV-4	6	6 (51)	2 (59)	76 (75)	0.00 (0.00-0.03)	**<0.00**

[a]All blood meals were positive with patients' DENV-reactive IgG, so the model failed.

Table 2.5: DENV-2 and DENV-4 viremic blood meals were typically associated with the presence of IgG to the DENV virion.

mAbs	Serotypes	Number of viremic blood meals		
		DENV IgG +	DENV IgG -	DENV IgG unknown[a]
747(4)B7	DENV-1	13	7	2
	DENV-2	3	0	1
	DENV-3	0	0	1
	DENV-4	5	1	3
753(3)C10	DENV-1	13	8	2
	DENV-2	3	0	1
	DENV-3	0	0	1
	DENV-4	5	1	3

[a]Untested or inconclusive-IgG samples

2.4 Discussion

I led the development and employment of the ViBNA to assess the antiviral potency of 12 mAbs. Results highlighted a discrepancy in their antiviral potency between the ViBNA and cell-culture systems. Specifically, five mAbs were characterized as non-neutralizing antibodies using the ViBNA while they had previously inhibited DENV infection of mammalian cells. For a moderately neutralizing mAb (1F4), I found a substitution (N52D), occurring at viral E proteins, was associated with a reduction in percentage of mosquitoes with DENV infection. In contrast, the impact of patients' DENV-specific IgG on the antiviral potency could not be distinguished from DENV serotypes. These results define the potency of these mAbs in the complex matrix of human blood, and against population of DENVs that have replicated in human tissues.

ViBINA is valid in terms of assessing antibody-mediated neutralization of DENVs from patients to mosquitoes *ex vivo.* Blood of dengue patients contains circulating DENVs for mAbs to neutralize. These mAbs also block DENV infection of *Aedes aegypti*, the primary vector for DENVs. Therefore, ViBNA is a particularly unique assay, targeting potent neutralizing antibodies that inhibit the transmission of DENVs from viremic blood to mosquitoes in a natural infection process.

Using the ViBNA, a major finding is that five mAbs were incapable of neutralizing DENVs, while they were previously characterized as neutralizing mAbs using cell-culture systems (Table 2.7 and references therein). This finding may provide an explanation for the suboptimal value of the cell-culture system (PRNT) to predict the vaccine efficacy [52-54]. In particular, the PRNT may measure vaccine-elicited antibodies that are not effective in neutralizing circulating DENVs. A

next hypothesis to test is that the PRNT would be less sensitive than the ViBNA to discriminate between effective and non-effective antibodies against DENVs. To this end, an archive of plasma samples from vaccinated individuals could be used in parallel. Multiple cut-off values can be generated from results of the ViBNA and the PRNT, and can be used to draw the receiver operating characteristic curves (ROC) with the outcome of virologically confirmed dengue. The comparison in ROC curves, generated from results of the ViBNA and the PRNT, will directly highlight the need for another DENV neutralization assay, used to assess correlate of protective immunity in dengue.

Among seven neutralizing mAbs, characterized using the ViBNA, six bind to quaternary epitopes on the virion surface (Table 2.6, Table 2.7). This is in line with other studies indicating human neutralizing antibodies may preferentially recognize quaternary epitopes on the surface of the intact DENV virion [82, 135, 139, 142, 146, 170]. Taken together, vaccine approaches that present intact virions to the immune system, e.g. live attenuated viruses [58] or inactivated whole virion vaccines, are most likely to elicit the kind of quaternary-epitope-specific antibodies that in the ViBNA were most potent.

Moreover, future assays should aim to investigate whether quaternary-binding antibodies are elicited at sufficient levels by vaccination. For example, a competition ELISA can be developed that uses one of the seven neutralizing mAbs, characterized using the ViBNA, and their quaternary epitopes. Here, plasma samples of vaccine recipients can be diluted, and incubated with a fixed amount of the labelled mAb. The dilution that results in the 50% reduction in the intensity of the labelled mAb could translate into the levels of quaternary-binding antibodies in the plasma of the

vaccine recipient. In particular, the higher dilutions of plasma samples, the higher levels of quaternary-epitope binding antibodies may be elicited by the vaccination. Results of the competition ELISA may be used as an alternative correlate of protection from dengue.

I found a paradox that some combinations of mAbs and DENV serotypes resulted in an increase in the proportion of infected mosquitoes, compared with the PBS cohort. This was indicated by odd ratios > 1 (Table 2.3, and Figure 2.8). Although mosquitoes' cells do not express Fc receptor, the enhancement of DENV infection was indicated using a mosquito cell line (C6/36) [82]. Therefore, a possible hypothesis is that ADE would occurs in mosquitoes where mAbs may bind to DENVs in viremic blood and mediate ADE in mosquitoes. To test this hypothesis, mAbs can be diluted and incubated with cultured DENVs, then intrathoracic inoculation can be performed to generate infected mosquitoes. If ADE occurs in mosquitoes, a combination of diluted mAbs and DENVs would result in higher virus titers, or higher percentage of infected mosquitoes than DENVs alone.

An amino acid substitution (N52D) at the epitope of 1F4 was associated with a decrease in the antiviral potency of the mAb. 1F4 binds to quaternary epitope spanning across three domains of an E monomer. 1F4 was also previously characterized as a DENV-1 specific antibody using a cell-culture system. However, the mAb was modestly active against DENV-1 infectivity for mosquitoes. The decreased performance of 1F4 in the ViBNA may be explained by its degradation (Figure 2.6) as well as the N52D substitution. In particular, the residue N52 on E proteins was involved in the binding between 1F4 and DENV-1 (Figure 2.11). When the N52D substitution occurred, the structure of E proteins where 1F4 binds to may be modified, which would lead to the failure of 1F4 to bind to DENV-1. Additional studies, employing electronic microscopy, are necessary to simulate how N52D modifies the affinity and avidity of the binding between 1F4 and its epitope. Results of these structural studies will provide insight into the evolution of DENVs to escape from being targeted by human immune responses.

A drawback of this work is that consensus sequences of DENVs were obtained. If more advanced analysis methods are applied to call other variants of DENVs from an individual patient, it would give more information on the change in amino acid residues of the epitope of 1F4. In particular, appropriate statistical methods and bioinformatics tools should be developed and employed to analyze if there are other changes in the interaction interface between 1F4 and E proteins of DENV-1. Another drawback is that the finding was limited to 1F4 while variations at epitopes of 5J7 and the five non-neutralizing mAbs, characterized by the ViBNA, were not examined. To overcome this, RNAs, encoding E proteins of DENVs in viremic blood spiked with these mAbs, could be sequenced to investigate if amino acid residues of the epitope diverge in reference to the

published data. Results of this study may provide an explanation for the discrepancy in characterizing neutralizing mAbs between the ViBNA and cell-based assays.

The mAbs 747(4)B7 and 753(3)C10 which recognize quaternary epitopes, were also potent neutralizers of virus infectivity, but with the hypothesis that their potency would be lower in patient blood samples that already contained IgG to DENV virions, i.e. blood that contained anti-DENV antibodies elicited by a past infection. A rationale for this hypothesis is that both 747(4)B7 and 753(3)C10 bind to the epitopes near the long-established target of poorly neutralizing antibodies, present in blood of patients with dengue [82, 136]. As such, those patients' DENV-specific IgG may sterically interfere with the binding of 747(4)B7 and 753(3)C10, and reduce the neutralization capacity of the two mAbs. I could not; however, discriminate whether the lower potency was a serotype effect or an antibody competition effect because of extensive co-correlation between DENV-2 and DENV-4 infections and the presence of pre-existing anti-DENV antibodies. If the ViBNA is continued using DENV-2 and DENV-4 blood from patients with primary dengue, the hypothesis that patient-derived DENV-reactive IgG would compete and reduce the antiviral potency of the two mAbs will be tested.

The ViBNA employed HIG and PBS as positive and negative controls, respectively. While HIG neutralized DENV infection of mosquitoes effectively, PBS did not. HIG is composed of plasma from laboratory-confirmed-dengue patients at convalescence when the level of neutralizing DENV-reactive polyclonal antibodies is high. Therefore, HIG was effective in neutralizing DENVs. However, the antiviral potency of HIG was not equally distributed among the four DENV serotypes. In particular, HIG blocked the infectivity of DENV-1 and DENV-2 for mosquitoes more

effectively than DENV-3 and DENV-4 (Table 2.2). The small representation of DENV-3, and DENV-4 in HIG might be an explanation for this observation. Other batches of HIG where the volumes of convalescent plasma were equal among the four DENV serotypes should be tested with the ViBNA. This would result in similar antiviral potency of HIG across four DENV serotypes.

The ViBNA has limitations and three of them are discussed below. First, the concentration of DENVs varied between enrolled patients, which drove variation in the relative antibody-mediated neutralization and subsequent infection of mosquitoes. I could not normalize plasma viremia in this experiment, because of multiple logistical reasons. One of them is that it would have undermined the integrity of the whole blood environment. Alternatively, I used the multivariable logistic regression model to control the confounding factor of viremia. Second, I could not examine neutralization capacity of the 12 mAbs against all four DENV serotypes because access to each serotype was driven by the serotype prevalence in Ho Chi Minh City at the time the study was conducted. In particular, the number of blood meals with DENV-3 was substantially lower than other DENV serotypes. As such, I could not characterize the antiviral potency of tested mAbs against DENV-3. Additional experiments when DENV-3 is prevalent in the cohort of patients should yield the antiviral potency of the 12 tested mAbs against all four DENV serotypes. Third, discrepancies in characterizing neutralizing mAbs, and in specificities of neutralizing mAbs were identified between the ViBNA and previous cell-culture systems (Table 2.7). These discrepancies lack empirical evidence because the ViBNA and cell-based assays were not performed in parallel with the same stocks of antibodies, in the same laboratory. Despite these limitations, the ViBNA extends the understanding of tested mAbs by demonstrating that some of them were also potent at

reducing the infectivity of DENVs in the complex matrix of human viremic blood. Antibodies that neutralized DENVs in cell-culture systems, and in the ViBNA, are the type that might be desirable to elicit with a dengue vaccine or to be used as therapeutic antivirals.

Table 2.6: Previously-defined properties of mAbs used in the ViBNA

Clone name	Specificity and IC_{50}[a]	Epitope binding[a]	Generation method	Origin	Reference
14c10	DENV-1: 1.5 µg/mL	Quaternary	Immortalized B cells	Patient with DENV-1	[142]
1C19	DENV-1: 0.06 µg/mL DENV-2: 0.03 µg/mL DENV-3: 0.04 µg/mL DENV-4: 3 µg/mL	Linear	Immortalized B cells	Patient with DENV-1	[140]
1F4	DENV-1: 0.03 µg/mL	Quaternary	Immortalized B cells	Individuals with DENV-1, 3	[138]
1L12	DENV-2: 0.4 µg/mL	Linear	Immortalized B cells	Individuals with DENV-2	[171]
1M7	DENV-1: 0.55 µg/mL DENV-2: 0.30 µg/mL DENV-3: 0.02 µg/mL DENV-4: 0.50 µg/mL	Linear	Immortalized B cells	Individuals with DENV-2	[140]
22.3	DENV-4: 0.006 µg/mL	Linear	Immortalized B cells	Patient with primary DENV-4	[136]
2D22	DENV-2: 0.08 µg/mL	Quaternary	Immortalized B cells	No information	[139]
5J7	DENV-3: 0.56 µg/mL	Quaternary	Immortalized B cells	No information	[141]
747(4)B7	DENV-1: 0.10 nM DENV-2: 0.11 nM DENV-3: 0.12 nM DENV-4: 93.19 nM	Quaternary	PCR, and cloning	Patient with dengue	[82]
753(3)C10	DENV-1: 0.54 nM DENV-2: 0.18 nM DENV-3: 1.89 nM DENV-4: 0.08 nM	Quaternary	PCR and cloning	Patient with dengue	[82]
82.11	DENV-1: 0.043 µg/mL DENV-2: 0.024 µg/mL DENV-3: 0.090 µg/mL DENV-4: 0.117 µg/mL	Linear	Immortalized B cells	Patient with primary DENV-3	[136]
87.1	DENV-1: 0.004 µg/mL DENV-2: 0.004 µg/mL DENV-3: 0.008 µg/mL	Linear	Immortalized B cells	Patient with secondary DENV-1	[136]

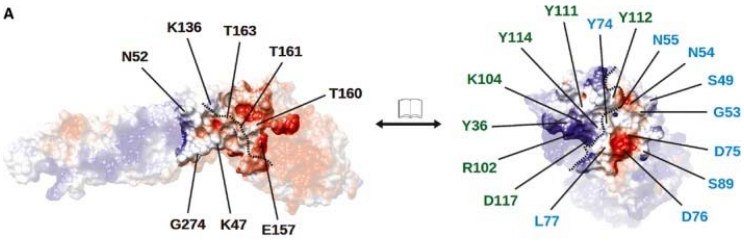
[a]Serotype specificity (assessed using cell-based assays) and types of epitopes to which these mAbs bind were characterized in cited references.

Table 2.7: Summary of the ViBNA and cell-culture systems for antiviral potencies and serotype specificities of the 12 tested mAbs

Tested mAbs are sorted ascending based on the serotype specificity by the ViBNA. While the discrepancy between cell-culture systems and the ViBNA is highlighted in grey, the neutralization against DENV-3 was inconclusive using the ViBNA.

Clone name	Serotype specificity (ViBNA)	Serotype specificity (cell-culture systems)	Reference
14c10	DENV-1	DENV-1	[142]
1F4	DENV-1	DENV-1	[138]
2D22	DENV-2	DENV-2	[139]
5J7	DENV-2	DENV-3	[141]
1L12	DENV-2	DENV-2	[171]
753(3)C10	DENV-1, 4	DENV-1, 2, 3, 4	[82]
747(4)B7	DENV-1, 2, 4	DENV-1, 2, 3, 4	[82]
22.3	No serotypes neutralized	DENV-4	[136]
87.1	No serotypes neutralized	DENV-1, 2, 3	[136]
1M7	No serotypes neutralized	DENV-1, 2, 3, 4	[140]
1C19	No serotypes neutralized	DENV-1, 2, 3, 4	[140]
82.11	No serotypes neutralized	DENV-1, 2, 3, 4	[136]

A



B

DENV-1 E-protein		Fab 1F4	
Segment	Amino acid residue	L-chain	H-chain
46-52	L46	L77	
	K47	Y74, D75, D76, L77	
	E49	D75, K90	
	T51	G53	
	N52	S49, G53	
136-138	K136	N54, N55	Y112
	S138	Y74	
155-165	S155	L78, P79, S80	
	T156	S80	
	E157		Y36, R102, K104, D117
	T160		K104, Y111
	T161	Y74	K104, Y114
	A162		Y111
	T163	Y74	Y111, Y112
	T165		Y112
170-177	T170		K109
	T171		P110, Y111
	E172		T108, Y111
	I173		Y111
	Q174		Y57, N105
	T176		T32, Y36, K104
	D177		T32
272-276	S273	S89, S91	
	G274	S89	
	T275	D75, D76	
	T276	K90, S91	

Figure 2.11: Interaction interface between 1F4 and DENV-1 E protein

A. Molecular structure of the interaction between E protein (left) and 1F4 (right). Positive, negative, and neutral charges were indicated in blue, red, and white respectively. The residue N52 is shown. The dotted line indicate the border between the heavy and light chain. The interacting residues of 1F4 light and heavy chains are indicated in cyan and green, respectively.

B. Table of the list of amino acid residues involved with the interaction between 1F4 and DENV-1 E protein

(The figure and caption were copied from the open-access article [138].)

2.5 Conclusion

Three main results of this Chapter are:

1. Five mAbs failed to neutralize DENVs using the ViBNA although they were previously characterized as neutralizing antibodies using cell-culture systems, highlighting the need for another DENV neutralization assay.
2. Seven mAbs neutralized DENV infectivity not only in cell-culture systems but also in the ViBNA. These antibodies are the type that might be desirable to elicit with a dengue vaccine or to be used as therapeutic antivirals.
3. The antiviral potency of 1F4 was negatively associated with a substitution (N52D) in viral amino acid residue at the epitope of 1F4, indicating the influence of epitope sequence on the potency of mAbs.

These results show the neutralization capacity of various tested mAbs in the ViBNA, which may have implications for studies on correlates of protective immunity and for dengue vaccine.

Chapter 3

The functional anti-viral potency of the human mAbs 747(4)B7, 753(3)C10, and 14c10

Abstract

For dengue, no licensed antivirals exist and treatment is limited to supportive care. Therapeutic administration of DENV-specific antibodies is considered as a potential approach to reduce disease severity, the symptomatology, and the duration of illness. In Chapter 2, I identified seven neutralizing mAbs using the ViBNA. These seven mAbs can be developed as antiviral candidates in dengue, but need additional characterization of EC_{50} first, to support the therapeutic antiviral approach using mAbs. Therefore, in Chapter 3, 747(4)B7, 753(3)C10, and 14c10 were examined for their EC_{50} values. I also showed that the neutralization, mediated by 14c10, was independent of Fc engagement. However, the 14c10-mediated neutralization was incomplete, evidenced by residual infectivity of DENV-1. The mAb 14c10 also failed to neutralize DENV-1 in the saliva of mosquitoes. My results may strengthen antiviral profiles of several DENV-neutralizing mAbs, which have potential to play a role for therapeutic antivirals in dengue.

3.1 Introduction

In humans, dengue is the most prevalent arboviral disease. Viremia increases during the intrinsic incubation period, peaks during the febrile phase, and declines rapidly during the critical phase [19]. Patients with high viremias in the febrile phase are more likely to develop severe dengue [172, 173].

Numerous drugs have been trialed to determine whether they accelerate the decrease in DENV viremia, then mitigate the risk of severe dengue. However, none of them have been effective in clinical trials [28-31]. Due to their high specificity to bind to antigens, mAbs have potential to be developed as antivirals for the disease. In principle, mAbs can be used when symptoms start to develop and when viremia is high. In this situation, mAbs can bind specifically to the DENV, block the viral entry into host cells, inhibit the replication of the virus, and accelerate the decrease in viremia.

However, the mAb therapeutic requires considerations, including but not limited to the following points. First, dosing strategies of mAbs against DENVs is unknown. If it is so little, there will be no effects. If it is excessive, financial cost to buy mAbs will be an issue. Therefore, the first aim of Chapter 3 was to identify EC_{50} values of neutralizing mAbs, including 747(4)B7, 753(3)C10, and 14c10. These three mAbs were characterized as neutralizing mAbs in Chapter 2. Based on previously characterized neutralization titers [135, 142], I hypothesized that these mAbs could neutralize DENVs at EC_{50} of low microgram-per-mL concentrations in the ViBNA.

Second, a potential therapeutic mAb for dengue might carry LALA mutations (a variant of mAbs in which there are two point mutations (L234A, L235A) at Fc) for safety reasons such as mitigating the risk of Fc-mediated pathogenic pathways [174-176] (see Section 1.4.3.2). These common point mutations abrogate mAbs binding to Fc receptors, and inactivate Fc-mediated pathways. However, whether the LALA mutation interferes with DENV neutralization is yet to be investigated. Using the ViBNA, I aimed to investigate the potency of 14c10-LALA variant compared to its wild-type parent. I did not examine LALA variants of the remaining two mAbs (747(4)B7 and 753(3)C10).

Third, the mAbs are believed to neutralize DENVs when they are incubated with viremic blood at 37°C for 30 minutes (see Chapter 2). However, it is unclear whether residual infectious DENVs remain in the blood after the mAb incubation. As such, I examined residual infectivity of DENV-1 after being incubated with 10 µg/mL 14c10. The infection of C6/36 cells was used as the assay's endpoint. Because this endpoint differs from that of the ViBNA where 14c10 was characterized as a neutralizing mAb against DENV-1, and C6/36 cells are highly susceptible to DENV infection, I hypothesized that DENV-1 could be detected in C6/36 cells, meaning infectious DENV-1 would persist after the incubation.

The last point also relates to 14c10, characterized as a neutralizing mAb against DENV-1 in viremic blood of patients with dengue (in Chapter 2). However, we do not know whether the mAb remains effective against DENV-1 in the saliva of mosquitoes. I hypothesized

that 14c10 may not neutralize DENV-1, present in the saliva of mosquitoes. The rationale for this hypothesis is that 14c10 only binds to quaternary epitopes on E proteins of mature DENV-1 [142], and DENVs from human cells seem to be more mature than those from mosquito cells [82, 134].

3.2 Materials and methods

3.2.1 Overview of experiments

A panel of neutralizing mAbs, characterized in Chapter 2, was excluded from Chapter 3 due to the serotype specificity. In particular, because few patients were infected with DENV-2, I excluded three DENV-2 specific mAbs (2D22, 1L12, and 5J7) (previously described in Section 2.3.3). I also excluded 1F4 because its antiviral potency declined in the second half of 2014 (previously described in Section 2.3.4), the time I started ViBNA experiments for this Chapter. As such, tested mAbs here were 747(4)B7, 753(3)C10 and 14c10 (Figure 3.1). EC_{50} was assessed using the ViBNA (previously described in Section 2.2.4).

A focus of Chapter 3 was on DENV-1 specific 14c10. While 14c10 studied in Chapter 2 was obtained from Paul McAry's group in National University of Singapore, the mAb studied in this Chapter was from GSK. As such, a member in the Zoonosis group performed gel electrophoresis again to check the purification and concentration of antibody's fragments. The procedure for gel electrophoresis was previously described in Section 2.2.6.1). I then investigated other functional antiviral potencies of 14c10. The first was the potency of its LALA variant. Second, I investigated the residual DENV-1 infectivity after being incubated with 14c10. Third, I investigated the ability of 14c10 to neutralize DENV-1 in the saliva of mosquitoes (Figure 3.1).

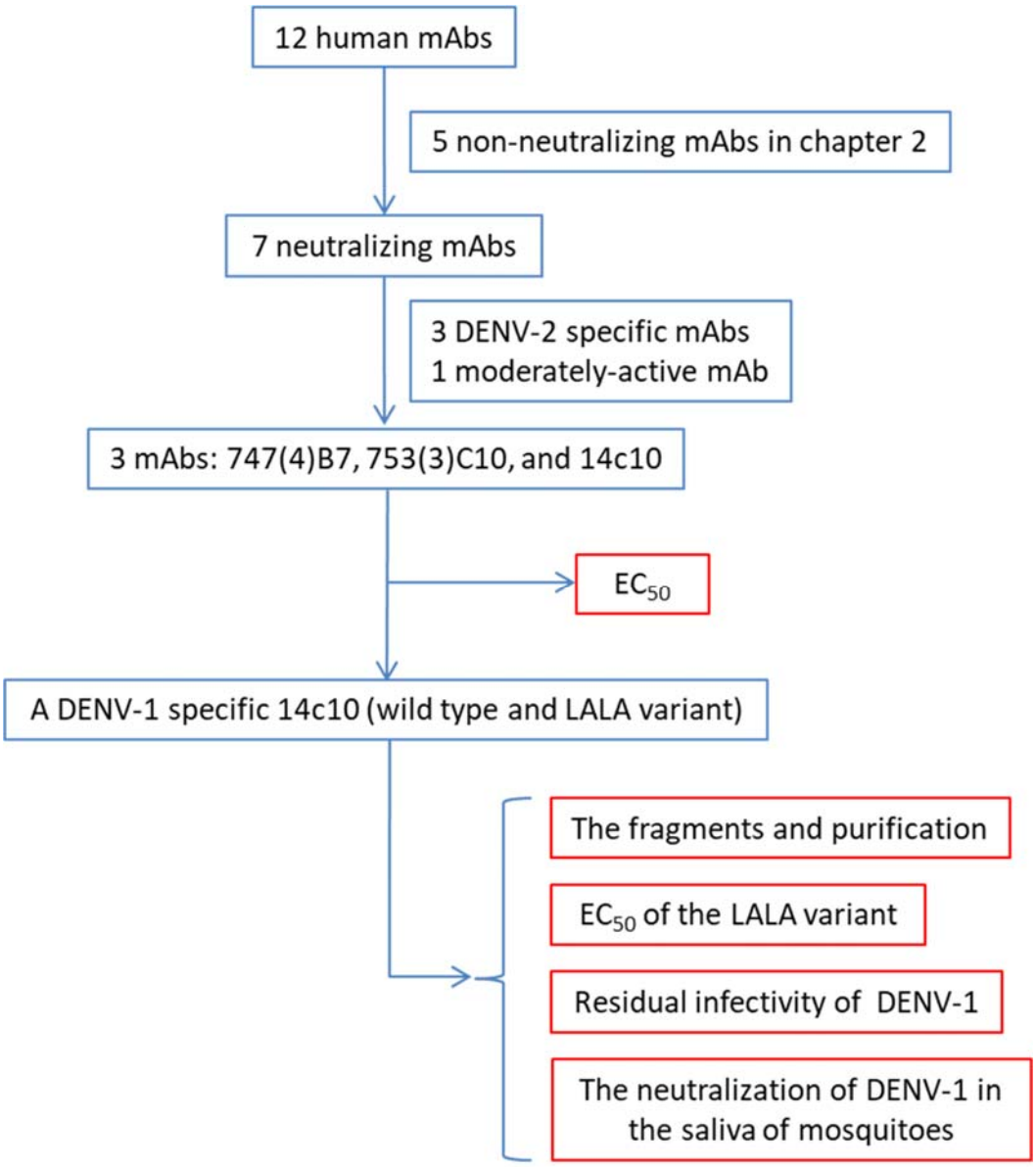


Figure 3.1: Schematic overview of experiments

Topics covered in this Chapter are bordered in red.

3.2.2 EC_{50}

To identify EC_{50} of 747(4)B7, 753(3)C10, and 14c10, I diluted individual mAbs, then performed the ViBNA. Dengue patient cohort, DENV-specific mAbs, ViBNA procedures were previously described in Sections 2.2.2, 2.2.3, 2.2.4, respectively. An exceptional difference was that while I examined one concentration of each mAb in Chapter 2, here I diluted and examined four concentrations of each mAb. The tested concentrations varied between mAbs (Table 3.1), depending on their stock concentrations. The calculation for EC_{50} values was based on dose-response analysis using the three-parameter log-logistic function in the package drc in R [177]. The 95% confidence interval (CI) was derived based on delta method without adjustment for the nested nature of the data, i.e. multiple mosquitoes fed on a single blood meal. I performed the calculation for EC_{50} and 95% CI.

Table 3.1 Tested concentrations[a] of neutralizing mAbs

mAbs	Undiluted stock	10-fold dilution	100-fold dilution	1,000-fold dilution
747(4)B7	3.7	0.37	0.037	0.0037
753(3)C10	5	0.5	0.05	0.005
14c10	10	1	0.1	0.01

[a]The unit of concentrations is μg/mL.

3.2.3 The residual infectivity of DENV-1 after incubating with 14c10

Residual infectivity of DENVs may not be detected using ViBNA. Therefore, to examine residual infectivity of DENV-1 after incubating with 14c10 variants, I added individual mAb variants into viremic blood samples to obtain a concentration of 10 µg/mL. After 30 minutes of incubation at 37°C, I collected plasma, inoculated the plasma onto C6/36 cell lines, and passaged the supernatant for two times to allow un-neutralized DENV-1 to amplify. The virus was then detected in the supernatants of the second passage using RT-PCR. The qualitative assay endpoint was DENV infection of C6/36 cells. If DENV-1 remained un-neutralized by the mAb, the virus would replicate while I passaged it in the cell line, and would be detected in supernatant of the second passage.

I investigated four groups in this experiment, including wildtype 14c10, 14c10-LALA, positive controls and negative controls. The positive control was HIG from patients with dengue (previously described in Section 2.2.5), while the negative control was a mAb that binds specifically to RSV.

As in Chapter 2, the mAbs variants (wildtype 14c10, and 14c10-LALA) were tested in this experiment at the high concentration of 10 µg/mL, where we could expect neutralization to occur after all scenarios. If infectious DENV-1 remains un-neutralized at 10 µg/mL, it will not be neutralized at lower concentrations.

The viremic blood samples were the same as those used to assess the EC_{50} of the two 14c10 variants. Because 14c10 was characterized as DENV-1 specific antibody (Chapter 2), I did not collect passage-2 C6/36 supernatants if they were derived from patients infected with DENV-2, DENV-3, and DENV-4.

The detailed procedure for inoculating blood-mAb mix onto C6/36 cells was as follows. The antibodies were added into the blood samples, then the blood-mAb mix was centrifuged to obtain plasma samples. The plasma samples were diluted 10-fold in a maintenance media (RPMI-1640 supplemented with 2% inactivated fetal bovine serum, 1% L-glutamin, and 1% penicillin-streptomycin) to avoid C6/36 cytotoxicity of plasma. Diluted plasma was inoculated onto C6/36 cells, supplemented with the maintenance media. These mosquito cells were subsequently incubated at 28°C for seven days. Supernatants were then collected, inoculated onto a fresh layer of C6/36 cells, and incubated at 28°C for seven days (passaging). The passaging was repeated once, then DENV-1 was detected using RT-PCR (previously described in Section 2.2.2.3). Here, we excluded standard curves, and positive controls for the other three DENV serotypes. I performed C6/36 cell-culture and DENV-1 inoculation while members of the Dengue group performed RT-PCR. This experiment was carried out with no experimental replicates due to the limited supply of C6/36 cells. Standard operation procedures for C6/36 cell culture and DENV inoculation were described in Appendix, entitled “Cell propagation”, “Isolation of dengue viruses”, and “Amplification of DENV”.

3.2.4 The 14c10-mediated neutralization of DENV-1 in the saliva of mosquitoes

Because 14c10 binds to the quaternary epitope of DENV-1 [142], I asked the question of whether the mAb could mediate neutralization of DENV-1 when present in mosquito saliva. It is possible the neutralization capacity could differ because of variations in the structure of DENV-1, produced by mosquitoes as opposed to those produced by patients. Therefore, members of the Dengue group inoculated a cultured DENV-1 into the thoraxes of index mosquitoes and collected saliva from index mosquitoes. I then added 14c10 into the saliva and members of the Dengue group injected 14c10-saliva mix into secondary mosquitoes. The assay endpoint was DENV infection of secondary mosquitoes. If 14c10 neutralizes DENV-1 in the saliva of index mosquitoes, the secondary mosquitoes would not be infected.

I designed this experiment with four groups, which varied in the antibodies and the concentrations of antibodies in the antibody-saliva mix. Those groups were HIG served as positive controls (described in Section 2.2.5), 14c10 (10 μg/mL), 14c10 (1 μg/mL), and PBS negative controls.

The detailed procedure was as follows. A cultured DENV-1 strain was inoculated into the thoraxes of index mosquitoes, and maintained the mosquitoes for six days at 28°C, with a 12:12 light:dark cycle and 80% relative humidity. Surviving mosquitoes were then anesthetized and allowed to salivate into 1 μL of media containing 0.5 μL of inactivated fetal calf serum (IFCS) and 0.5 μL of 10% sucrose. The mix of saliva and media was

pooled from index mosquitoes, aliquoted and spiked with individual tested groups above (both HIG and PBS were diluted 1:9 in the pool of saliva). Then the mix of antibody and saliva was incubated at 37°C for 30 minutes for antibodies to neutralize DENV-1. I pooled the saliva and added tested antibodies. Next, members of the Dengue group inoculated the mix of each group into the thoraxes of 25 secondary recipient mosquitoes. Those inoculated mosquitoes were incubated for seven days, allowing DENV-1 to replicate. Surviving mosquitoes were sacrificed and pooled with a maximum of five mosquitoes. Members of the Dengue group and I homogenized mosquitoes in the pool, and detected the presence of DENV-1 using the DENV-1 RT-PCR (previously described in Section 2.2.4.3). The experiment was carried out in duplicate in which the numbers of index mosquitoes were 36 and 50. Because the outcome was binary (whether pools of secondary mosquitoes were positive with DENV-1), I performed the chi square test to compare the neutralization capacity between 14c10 and HIG. Standard operation procedures for intrathoracic inoculation and saliva collection were described in Appendix, entitled "Intrathoracic inoculation" and "Collecting mosquito saliva", respectively.

3.3 Results

We performed the ViBNA experiments in this Chapter from December 2014 to July 2017, using a total of 101 viremic blood samples, drawn from patients with NS1-positive dengue. DENV-1 was the most prevalent serotype, followed by DENV-4. DENV-2 and DENV-3 were detected in a small proportion of patients (Table 3.2). We fed a total number of 28,325 mosquitoes; the engorgement frequency was 50.97% (Figure 3.2).

I excluded 59 blood meals due to either of these reasons, including 1) mosquitoes of PBS were negative for DENVs; 2) all mosquitoes within HIG or PBS cohorts died before being collected and therefore could not be assessed; 3) patients were infected with DENV-2, or DENV-3; 4) patients were infected with DENV-4 in experiments with DENV-1-specific 14c10. We included a total of 42 blood meals into data analyses (Figure 3.2).

Table 3.2: Baseline information on patients who blood samples were used in ViBNA in Chapter 3

Fresh viremic blood samples from these patients were individually spiked with dilutions of several human mAbs. The patients were enrolled from Dec 2014 until July 2017.

All Patients	
n	101
Year of age[a]	
n	99
Median (IQR)	27 (22, 33)
Sex[a]	
n	52
Percentage of males	60
Log10 viremia (copies/mL)[b]	
n	95
Median (IQR)	7.8 (6.5, 8.8)
DENV serotypes[b]	
n	95
DENV-1 n (%)	50 (53)
DENV-2 n (%)	9 (10)
DENV-3 n (%)	2 (2)
DENV-4 n (%)	34 (35)

[a]Information on year of age and sex was not available for all patients.
[b]Viremia and serotypes were inconclusive for several patients, using qRT-PCR.

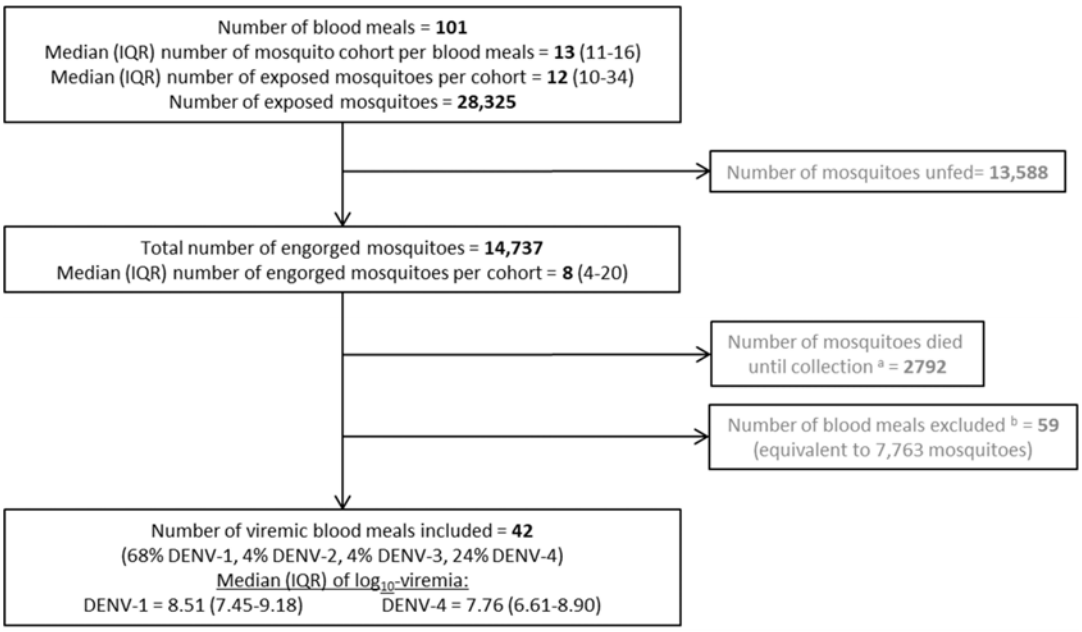


Figure 3.2: Flow chart of the processing of the ViBNA

Cohorts of mosquitoes fed on viremic blood spiked individually with concentrations of mAbs. [a] Engorged mosquitoes were then maintained until five or seven days after blood feeding. Only surviving mosquitoes were collected and tested for the presence of DENV RNAs. [b] Excluded data originated from cases where mosquitoes of PBS cohorts were negative for DENVs; from cases where all mosquitoes within HIG and PBS cohorts died before being collected, and therefore could not be assessed; from cases with DENV-2, DENV-3; and from cases with DENV-4 in 14c10-spiked blood meals. Viremic blood samples were independently assessed for DENV serotypes and viremia.

3.3.1 EC_{50} of 747(4)B7 and 753(3)C10

747(4)B7 and 753(3)C10 were characterized as neutralizing antibodies against DENV-1 and DENV-4 in Chapter 2, so I examined their EC_{50} values against these two DENV serotypes. At the high concentration (3.7 μg/mL), 747(4)B7 reduced DENV-1 and DENV-4 infections of mosquitoes effectively. Diluting 747(4)B7 increased the proportion of infected mosquitoes (

Figure 3.3). EC_{50} values of 747(4)B7 were calculated for DENV-1 at 0.056 μg/mL (95% CI = [not measurable, 0.179]), and for DENV-4 at 1.104 μg/mL (95% CI = [not measurable, 13.921]) (Figure 3.4).

For 753(3)C10, at 5 μg/mL, the mAb reduced most of DENV-1 and DENV-4 infections of mosquitoes. Diluting 753(3)C10 also resulted in an increase in DENV infection of mosquitoes (

Figure 3.5). Required concentrations for 753(3)C10 to neutralize 50% of mosquitoes with DENV-1 and DENV-4 were at 0.643 μg/mL (95% CI = [not measurable, 2.291]) and 1.289 μg/mL (95% CI = [not measurable, 16.433]), respectively (Figure 3.6). In summary, 747(4)B7 and 753(3)C10 were potent in neutralizing DENV-1 and DENV-4 at low microgram per mL concentrations.

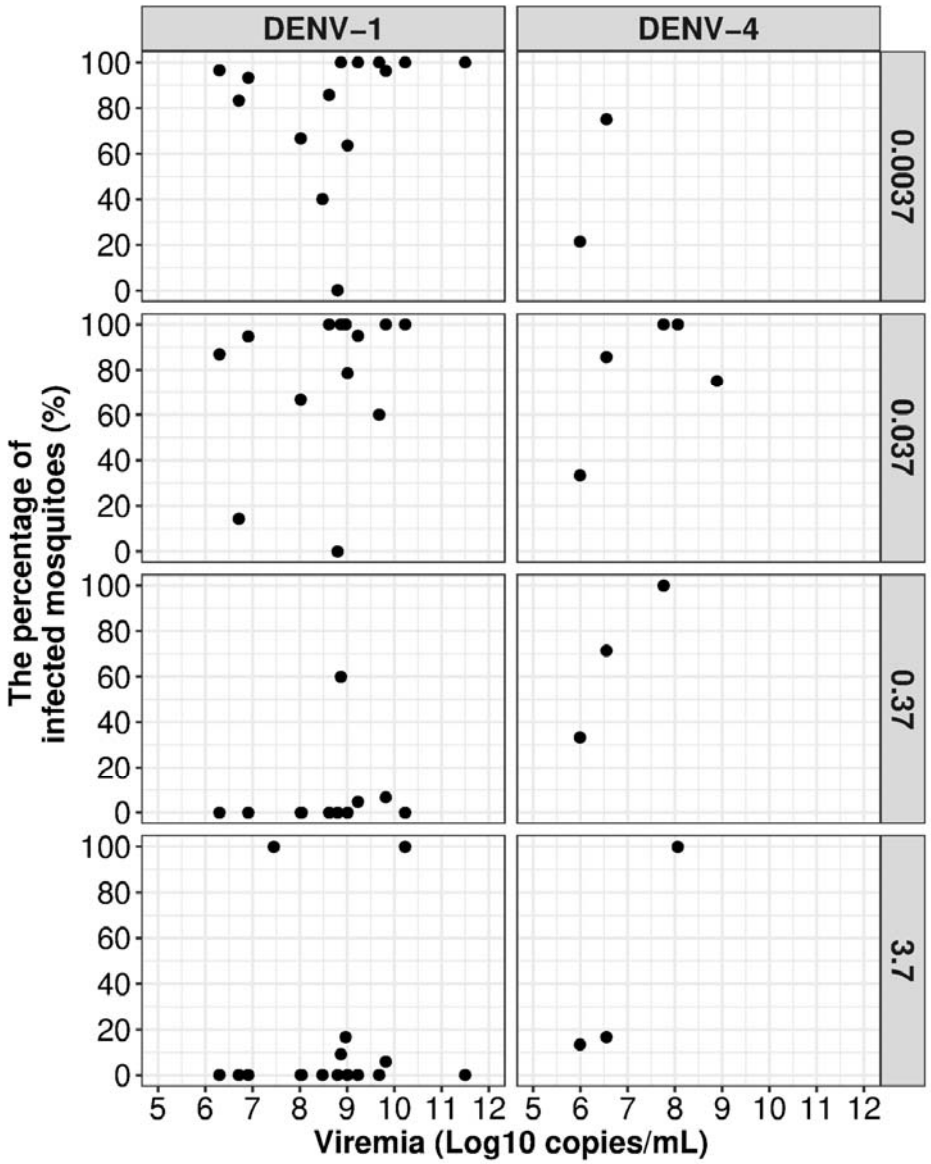


Figure 3.3: Dose response scatterplot of the percentage of infected mosquitoes for 747(4)B7 by plasma viremia

The mAb was diluted to obtain four concentrations (in µg/mL), indicated by rows. Each dot represents a blood meal. The serotype of DENVs in the blood is shown by the column header.

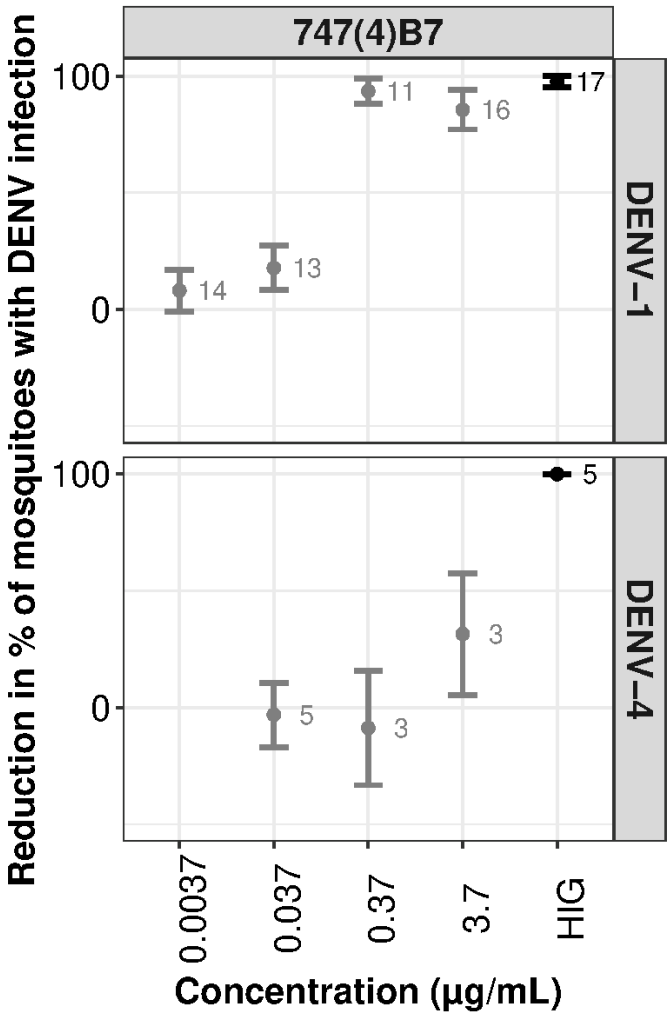


Figure 3.4: The neutralization potency of 747(4)B7

Means and standard errors of test results were calculated from three or more independent ViBNA measurements per serotype. Data for HIG are in black while monoclonal antibodies are in grey. The numbers next to the bar denote the numbers of blood meals. The y-axis value of 100 means the mAb blocked DENV infection of mosquitoes completely, relative to the PBS cohort.

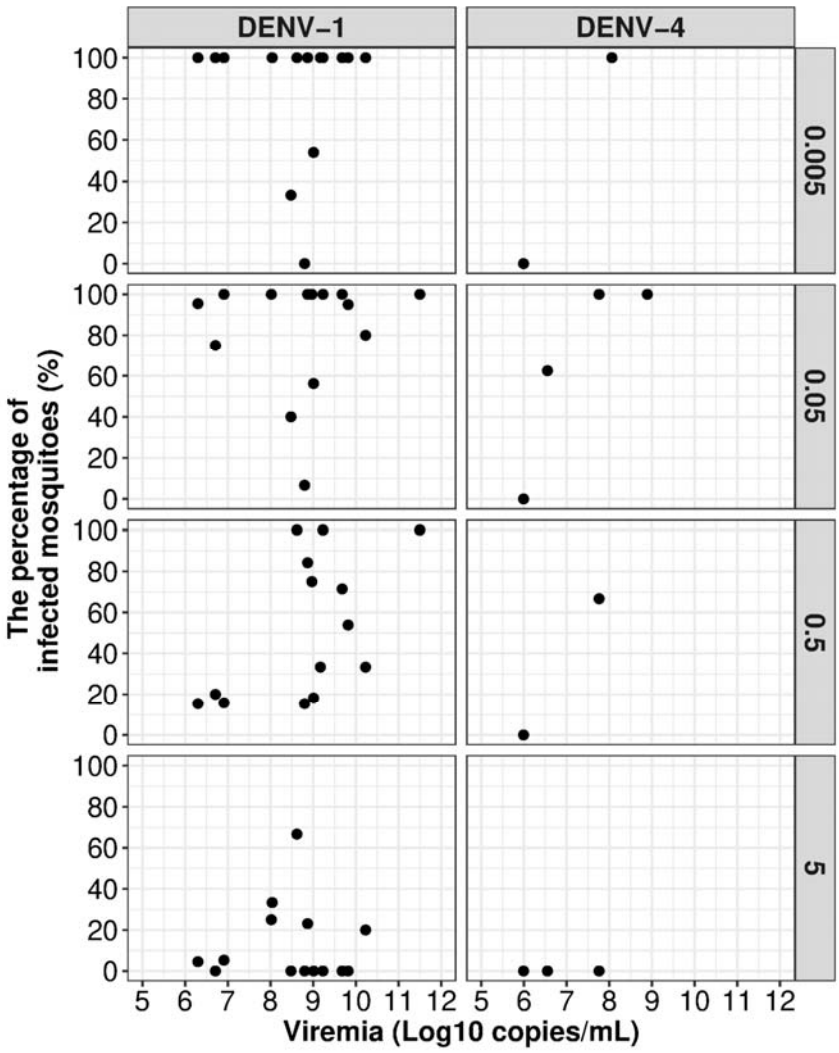


Figure 3.5: Dose response scatterplot of the percentage of infected mosquitoes for 753(3)C10 by plasma viremia

The mAb was diluted to obtain four concentrations (in µg/mL), indicated by rows. Each dot represents a blood meal. The serotype of DENVs in the blood is shown by the column header.

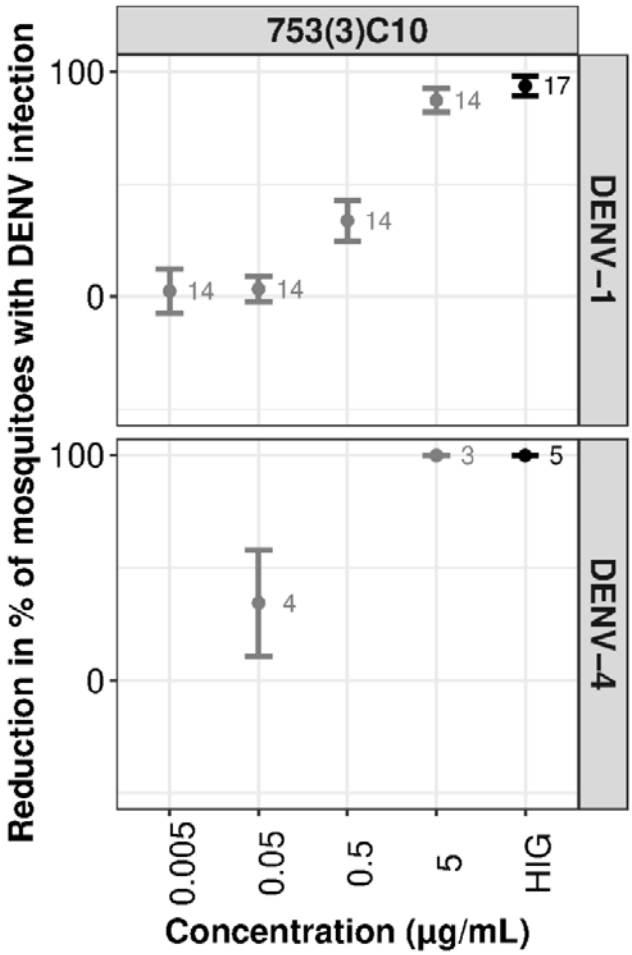


Figure 3.6: The neutralization potency of 753(3)C10

Means and standard errors of test results were calculated from three or more independent ViBNA measurements per serotype. Data for HIG are in black while monoclonal antibodies are in grey. The numbers next to the bar denote the numbers of blood meals. The y-axis value of 100 means that the mAb blocked DENV infection of mosquitoes completely, relative to the PBS cohort.

3.3.2 The purification of wildtype 14c10, 14c10-LALA

Before investigating EC_{50} of the remaining neutralizing mAb (14c10), we confirmed its purification using gel electrophoresis. We also performed gel electrophoresis for its LALA mutant (14c10-LALA). Results showed that each mAb variant had two distinct bands, corresponding to the Fab and Fc, suggesting that they were highly purified (Figure 3.7). The thickness of bands was also visually similar, implying that their stock concentrations were consistently at 10 µg/mL, as previously defined by the supplier (GSK).

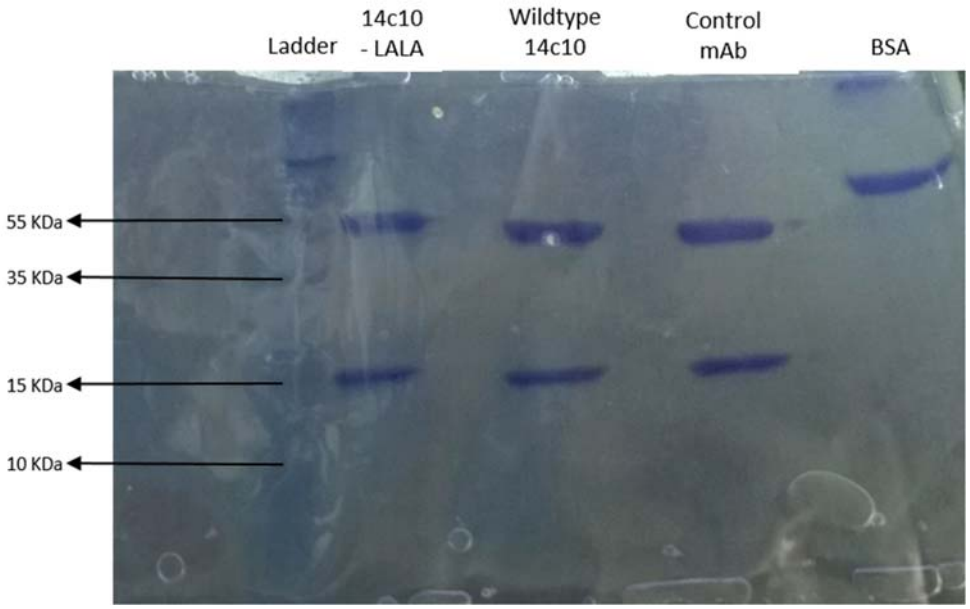


Figure 3.7: The purification of wildtype 14c10, 14c10-LALA, and the control mAb was high.

After receiving the three mAbs from the supplier (GSK), we tested them with protein electrophoresis for their purification. The clone names of mAbs are shown at the top of each panel. Each mAb was added into a corresponding well at the amount of 5

µg/well. Bovine serum albumin (BSA), also added at 5 µg/well, was served as positive controls. The control mAb binds to RSV. The molecular weight marker is shown.

3.3.3 The potency of wildtype 14c10, 14c10-LALA

To investigate the engagement of Fc, we assessed the potency of wildtype 14c10 and 14c10-LALA. The two mAb variants were tested in parallel using the ViBNA. Scatter plots were drawn to show the percentage of infected mosquitoes by viremia (

Figure 3.8). Both of the 14c10 variants reduced DENV-1 infection of mosquitoes from the concentration of 10 µg/mL to 0.1 µg/mL. When wildtype 14c10 and 14c10-LALA was diluted to the concentration of 0.01 µg/mL, DENV-1 infection of mosquitoes was not neutralized, and the scatter plots followed dose-response patterns. When the reduction in percentage of mosquitoes with DENV infection was plotted against concentrations of mAb variants, the EC_{50} were identified at 0.013 µg/mL (95% CI = [0.005, 0.020]) for wildtype 14c10 and 0.026 µg/mL (95%CI = [0.006, 0.045)] for 14c10-LALA (Figure 3.9). In summary, both wildtype 14c10 and 14c10-LALA neutralized DENV-1 infectivity for mosquitoes effectively.

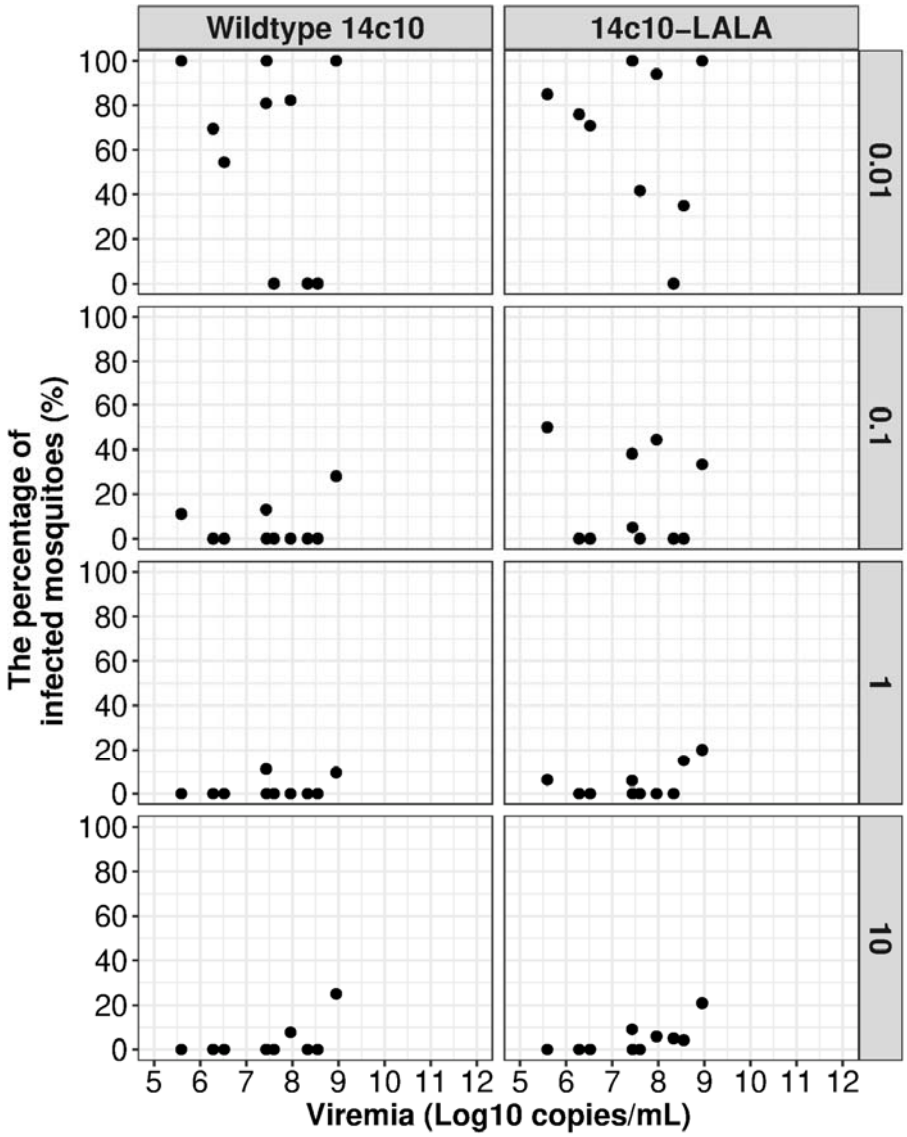


Figure 3.8: Dose response scatterplot of the percentage of infected mosquitoes by plasma viremia

Clone names of mAb variants are shown by columns. Both wildtype 14c10 and 14c10-LALA were diluted to obtain four concentrations, indicated by rows. The unit of concentration is µg/mL.

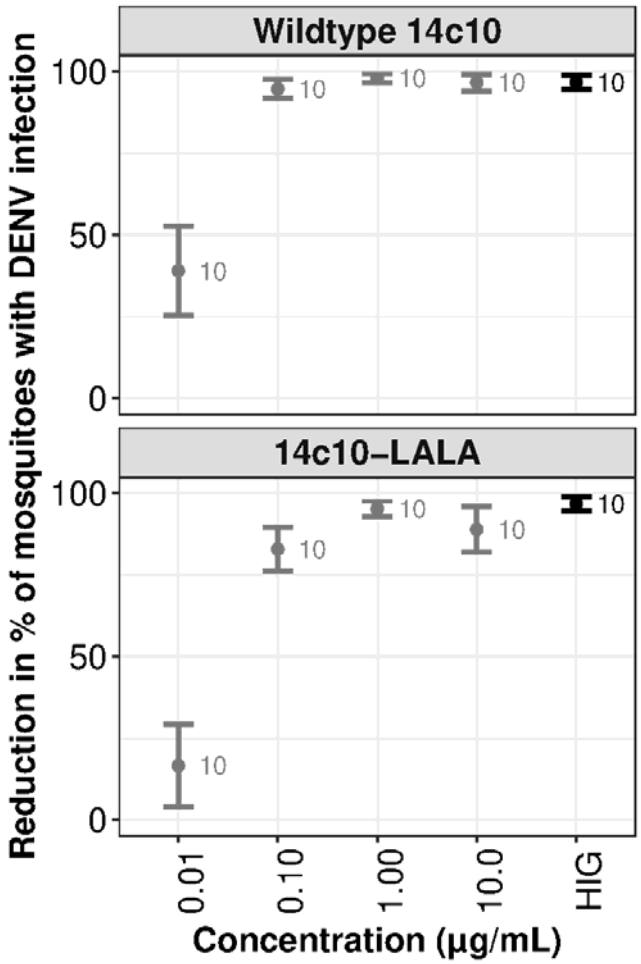


Figure 3.9: The potency of 14c10 remained unaffected without Fc engagement. DENV-1 viremic blood was spiked with concentrations of either 14c10 or 14c10-LALA. Means and standard errors of ten blood meals are shown, indicated by the number next to the bars. Data for HIG are in black while monoclonal antibodies are in grey. The numbers next to the bar denote the numbers of blood meals. The y-axis value of 100 means the mAb blocks DENV infection of mosquitoes completely, relative to the PBS cohort.

3.3.4 The residual infectivity of DENV-1 after incubating with 14c10

DENV-1 is believed to be neutralized while we incubate viremic blood with 14c10 variants at 37°C for 30 minutes. To examine whether there was residual infectivity of DENV-1 in viremic blood, I incubated 14c10 variants with DENV-1 in viremic blood, then inoculated plasma onto C6/36 cells for two passages, and detected DENV-1 via RT-PCR on the supernatant of C6/36 cells after passage two. The tested treatment groups were HIG (positive controls), the RSV-specific mAb (negative controls), wildtype 14c10, and 14c10-LALA.

Due to the unavailability of C6/36 cells, I performed this experiment with eight out of ten DENV-1 plasma samples, derived from blood used in the EC_{50} experiments for 14c10. Results were excluded from analysis for one plasma sample because of a technical error (lack of the RSV-specific treatment group). Results were also excluded from analysis for three plasma samples because of the death of C6/36 cells (unknown reasons). As such, the residual infectivity was investigated with four biological replicates (plasma samples). After two passages in C6/36 cells, infectious DENV-1 was present in viremic blood after being treated with all groups including HIG, RSV-specific mAb, wildtype 14c10 (10 μg/mL), and 14c10-LALA (10 μg/mL) (Table 3.3). Interestingly, HIG failed to completely neutralize DENV-1, with three of four C6/36 supernatants positive with DENV-1 RNAs

.Table 3.3: Residual infectivity of DENV-1 were detected irrespective of added antibodies.

Viremic blood samples were spiked with antibodies, then incubated at 37°C for 30 minutes. Plasma was then isolated and inoculated onto C6/36 cells.

Group	Number of plasma samples	Number negative[a]
Hyper-immune globulin (HIG)	4	1
RSV-specific mAbs	4	0
Wildtype 14c10	4	0
14c10-LALA	4	0

[a]The assessment of number negative is based on the presence of DENV-1 RNAs in the passage-two supernatant of C6/36 cells.

3.3.5 The 14c10-mediated neutralization of DENV-1 in the saliva of mosquitoes

To examine the capacity of 14c10 to neutralize DENV-1 in the saliva of mosquitoes, I incubated 14c10 with pools of saliva from DENV-1 infected mosquitoes. The mix was then injected into the thoraxes of secondary mosquitoes, and the presence of DENV-1 in pools of secondary mosquitoes was detected seven days after the injection.

Results shows that HIG neutralized DENV-1 in the saliva of index mosquitoes. This was in contrast to PBS where we detected DENV infection of secondary mosquitoes. Interestingly, we found breakthrough DENV infection in mosquitoes into which mix of saliva-14c10 was inoculated, demonstrating incomplete 14c10-mediated neutralization of DENV-1. In particular, 20% of the pools derived from 14c10 at 10 µg/mL were positive with DENV-1, and the frequency of infection was higher when the concentration of 14c10 was reduced to 1 µg/mL (Table 3.4). In summary, 14c10 (1 µg/mL) failed to neutralize DENV-1 in the saliva of mosquitoes, compared with HIG (Chi square test, X-squared = 7.97, df = 1, p = 0.005)

Table 3.4: 14c10 failed to neutralize DENV-1 in the saliva of infected mosquitoes. HIG and PBS stand for hyper-immune globulin and phosphate buffer saline, respectively.

	PBS	14c10 (1 µg/mL)	14c10 (10 µg/mL)	HIG
Experiment 1 - % (n)[a]	100 (5)	33 (3)	20 (5)	0 (4)
Experiment 2 - % (n)	100 (5)	80 (5)	20 (5)	0 (5)

[a]Numbers in the table show the percentage of positive pools, and the numbers of pools of mosquitoes.

3.4 Discussion

The mAbs characterized as neutralizing antibodies via the ViBNA could plausibly be employed as therapeutic antivirals in dengue. To support the development of this application, I examined and found EC_{50} values ranging from 0.013 μg/mL to 1.289 μg/mL for 747(4)B7, 753(3)C10, and 14c10. My results indicate the amount of mAbs that neutralize DENVs in viremic blood, which may have implications for the dosing strategy of therapeutic (or prophylactic) mAbs against dengue.

I found that while the EC_{50} value of 747(4)B7 against DENV-1 was 0.056 μg/mL; that of 753(3)C10 against the same serotype was ten-fold higher at 0.643 μg/mL. Previous studies of these two mAbs also revealed different EC_{50} values against DENV-1 [82]. Because both 747(4)B7 and 753(3)C10 required 180 molecules to fully occupy a DENV-1 virion [82, 178], the difference in EC_{50} values between the two mAbs cannot be readily explained by a difference in occupancy. Instead, variations in avidities of mAbs may explain their variance in potency against DENVs.

The 14c10-mediated neutralization of DENV-1 occurred independently of Fc engagement, indicated by comparable levels of neutralization by a LALA variant – when tested in parallel. The rationale and hypothesis for testing a LALA variant of 14c10 was that the Fc engagement to its receptors may form immune complexes, which could be pathogenic in dengue. Therefore, mAb therapeutic antivirals for dengue is likely to be engineered to be devoid of Fc receptor binding [174-176], and the potency of wildtype mAbs would be

reduced by this mutation. My results suggest that DENV-reactive mAbs remains effective without the engagement of Fc component.

The EC_{50} value of 14c10 to neutralize DENV-1 in the ViBNA was lower than in the PRNT (ViBNA: 0.013 μg/mL, cell-culture systems: 1.5 μg/mL [142]). The PRNT relied on the number of plaques, generated by cultured-DENV infection of BHK-21 cells. The difference in EC_{50} values could be explained fundamentally by different assays, performed at different times in different laboratories. Additionally, another explanation for the lower EC_{50} value in the ViBNA could be based on recent studies on the structural biology of DENVs and 14c10. In particular, circulating DENVs, derived from patients, seem to be more mature than cultured DENVs, derived from C6/36 or Vero cells [82, 134]. Because 14c10 binds to quaternary epitopes on mature DENV-1 [142], it would be expected that 14c10 would neutralize circulating patient-derived DENVs more effectively than cultured DENVs, leading to the lower EC_{50} values. However, contrary to this explanation, 14c10 neutralized DENV virions derived from patients less effectively than those derived from C6/36 and Vero cells [134]. These contrasting results suggest additional, well-controlled experiments are needed to determine the potency of 14c10 against circulating and cultured DENVs.

Despite the contrast, my results, along with those previously-published, demonstrate that 747(4)B7, 753(3)C10, and 14c10 may neutralize DENVs derived from both patients and cultured cells. Results generated in my study provide confidence that these three mAbs

could act not only as a blocker of transmission to mosquitoes but also as therapeutic antibodies.

The therapeutic use of antibodies was indicated in two case reports of severe dengue, being managed by human blood products enriched for immunoglobulin (Ig) antibodies. In the first report, the administration of an Ig-enriched blood product was correlated with a rise in the platelet count, and the recovery of the patient [179]. In the second report, the patient also recovered after the administration of another Ig-enriched blood product [180]. However, the clinical benefit of these blood product remain controversial because without this Ig therapy, most patients with severe dengue could still recover with adequate supportive care, such as fluid management. Moreover, because the enriched-Ig was administered late (after the patients developed shock syndromes), how Ig functions in patients with severe dengue remains unknown.

The antibody therapeutic antiviral, administered during the febrile phase of dengue (before patients develop severe diseases) would be more useful. The mAbs, which were effective in neutralizing DENV infectivity for mosquitoes in my study, for cultured cells and animals in other studies, may become therapeutic antiviral candidates in dengue. To this end, cross-reactive mAbs would be more useful than type-specific mAbs because cross-reactive mAb may not require laboratory tests to match the specificity with the infecting serotype. A concept for the use of therapeutic mAbs is that they could be administered into patients with dengue when viremia is detectable and high [27, 181]. In this situation, administered

mAbs are expected to accelerate the clearance rate of viremia, reduce the symptomology, and mitigate the risk of developing severe complications. The therapeutic use of mAbs was indicated in Ebola when a cocktail of mAbs (named ZMapp) reduced the fatality of the disease, but the reduction did not meet the pre-specified statistical threshold for efficacy [174, 182].

For mAb therapeutic approach in dengue, cost-effectiveness would be a challenge. Because there are no licensed therapeutic mAbs in dengue, the exact cost of this approach is unknown. In a study evaluating economic aspects of licensed mAbs for rare diseases, the incremental cost per effectiveness gained for monoclonal antibody therapy was estimated from US$4,669 [183]. Although dengue is not a rare disease in Vietnam, the estimated cost is an impediment to using mAb therapy because the total cost to manage a dengue case in Vietnam was estimated at less than US$300 [184].

One option to improve the cost-effectiveness is that the mAb could be used in patients who have non-severe symptoms, but have a high risk of developing complications of dengue. Predictions of developing severe symptoms can be performed with the use of rapid point-of-care tests in combination with useful and validated algorithms [173, 185-188], which are currently under research and development. In this situation, potential clinical benefits of mAb therapy would be a decreased risk of complications such as shock, severe plasma leakage, severe hemorrhage, and severe organ impairments.

If the cost of using mAbs is affordable to manage non-severe but high-risk dengue patients, the mAb therapeutic antivirals could be used routinely. In this scenario, clinical benefits would be an acceleration of viremia clearance and positive changes in clinically important parameters such as platelet counts, hematocrits, ALT, AST. As a result, complications such as shock, severe hemorrhage, and severe organ impairments could be reduced. However, it is important to note that antibody therapy for dengue needs to be very safe because the proportion of dengue patients, who will develop severe diseases, is very small and most patients will not have any complications if standard of supportive care is provided only.

The next step in progressing toward the use of mAbs as a therapy would involve to assess essential values such as dosing, volume of administration, infusion rate and duration, the maximum concentration of administered antibodies in plasma, time to that maximum concentration, elimination half-life, i.e. time that the maximum antibody concentration reduced by a half of its original value. These parameters would be measured in clinical trials to optimize safe and efficacious therapeutic mAbs.

The antiviral potency for neutralizing mAbs against DENV-2 and DENV-3 was not defined due to the serotype prevalence in Ho Chi Minh City in the study period I performed my study. Thus, I could not identify the EC_{50} of 747(4)B7 and 753(3)C10 required to neutralize DENV-2 and DENV-3. I also could not identify the EC_{50} for 2D22 and 1L12 against DENV-2. As such, the employment of the ViBNA to test these mAbs should be

continued to measure their potency against all four DENV serotypes, to enable the development of effective mAbs, irrespective of DENV serotypes, as antivirals in dengue.

There was a discrepancy in neutralization capacity of 747(4)B7 against DENV-4 between Chapter 2 and Chapter 3 of my thesis. 747(4)B7 was characterized in Chapter 2 as being able to neutralize DENV-4 at the highest concentration of 3.7 μg/ml, while in the present set of experiments, it failed to neutralize DENV-4. The number of DENV-4 blood meals spiked with 747(4)B7 was low in Chapter 3 (three blood meals with 10 μg/mL of 747(4)B7), so sample size could be one possible explanation. Another one is that the antiviral potency of 747(4)B7 may differ among the 5 genotypes of DENV-4 [189]. In other words, if there was a change in the genotypes of DENV-4 from the time we performed the ViBNA in Chapter 2 to that of Chapter 3, this might lead to an escape from being neutralized by 747(4)B7. A virus sequencing experiment to examine DENV-4 genotypes could demonstrate this possibility.

Infectious DENV-1 was still present in the viremic blood after incubating with 10 μg/mL of 14c10. A reason for this observation is that although a majority of DENVs were neutralized by 14c10, some of them might escape from being neutralized and infect C6/36 cells. This set of experiments may highlight a drawback of the ViBNA when the assay did not detect the residual infectivity of DENVs. Instead, it just measures the ability of mAb to reduce the infectious titer to below the concentration needed to infect blood feeding mosquitoes.

While 14c10 neutralized DENV-1 in viremic blood from patients with dengue, the mAb did not block DENV-1 in the saliva of infected mosquitoes. The reason for this observation may relate to the continuum of maturation states of DENVs [190]. DENVs are completely mature when all 180 E proteins are arranged as 90 E dimers. In contrast, DENVs are wholly immature when 180 E proteins are arranged as 60 trimeric spikes. However, most DENVs are in-between the two maturation states. Because 14c10 binds to two adjacent E dimers, present only on mature DENVs [142], a hypothesis is that DENVs produced in human tissues of patients with dengue may be of higher maturation states than those produced from mosquitoes. This is supported by previous studies, demonstrating that virus from mosquito cells are in a less mature state than viruses that have replicated in mammalian cells [82, 134]. Further studies into the structural heterogeneity of DENVs produced in humans and mosquitoes will provide insights into their antigenic states and support rational vaccine and therapeutic development.

3.5 Conclusion

Four main results of this Chapter are:

1. 747(4)B7, 753(3)C10, and 14c10 neutralized DENVs with EC_{50} values ranging from 0.013 μg/mL to 1.289 μg/mL, indicating the amount of mAbs that are required to neutralize DENVs in viremic blood.
2. Both wildtype 14c10 and 14c10 neutralized DENV-1, suggesting 14c10-mediated neutralization of DENV-1 was independent of Fc engagement in the ViBNA.
3. DENV-1 remained infectious after incubating with 10 μg/mL of 14c10, highlighting a drawback of the ViBNA.
4. 14c10 failed to neutralize DENV-1 in the saliva of mosquitoes, which may be explained by variations in the maturation states between DENVs produced by patients and those produced by mosquitoes.

These results may improve the understanding of the functional antiviral potency of these neutralizing mAbs, in particular their transmission blocking features.

Chapter 4

Immunological and virological factors in pregnant women with dengue.

Abstract:

Dengue during pregnancy may be harmful to both the mother and their fetus. The overarching aim of Chapter 4 is to compare immunological and virological factors between pregnant and non-pregnant women with dengue. In particular, I assessed lymphocyte subsets, cytokines, DENV viremia, and the percentage of cases that were NS1-positive at enrolment. While viremia and the percentage of NS1-positive cases were comparable between the two arms, the percentage of CD8+ CD27- CD45RA- T cells, concentrations of three chemokines including eotaxin, MCP-1, and MDC were lower in pregnant patients. Furthermore, I detected DENV-reactive IgM in two cord blood samples of babies, born to pregnant participants, which may suggest a fetal response to *in utero* DENV infection. Results of my study contribute to the understanding of dengue pathogenesis in pregnant patients, and support further studies on immunological and virological aspects of dengue during pregnancy.

4.1 Introduction

The classical view holds that pregnancy is associated with constant suppression of the maternal immune response across the three trimesters, and that complications during pregnancy such as preterm birth or stillbirth result from failure of the body to maintain this suppression [149].

However, maternal immunity is both suppressed and stimulated to varying degrees throughout the pregnancy [147, 191] to maintain a balance between protecting against microbial infections and tolerating the fetus. In general, maternal immunity appears to be driven by gestational stages [147]. Specifically, the maternal immune response is pro-inflammatory in the first gestational stage, followed by an anti-inflammatory response during the mid-phase, returning later to a pro-inflammation profile at the last gestational stage. While the pro-inflammation response may be necessary to protect the mothers from being infected with microbes, the anti-inflammatory stage, which is thought to last the longest, may predispose the pregnant women to increased severity of infections.

In addition to bacterial and fungal infections, those caused by viruses such as cytomegalovirus, influenza, hepatitis E, and herpes simplex virus are detected during pregnancy, and may result in severe maternal and fetal complications [158]. While maternal immune responses against those viruses have been studied extensively, what we know about immunity against flaviviruses is still just emerging. Flaviviral infections of pregnant women were relatively neglected until 2016 when ZIKV re-emerged in the

Americas, with the infection being associated with microcephaly, a severe complication occurring to some neonates born to pregnant women with ZIKV. While ZIKV infection of pregnant women was associated with immunosuppression profiles [159, 160], the immune response of pregnant women with dengue are generally poorly understood.

Dengue during pregnancy can lead to both maternal and fetal consequences [3, 192], as revealed by the results of two prospective studies. In the first study of 24 pregnant women who had dengue just before delivery (~1 week before, on average), 13 patients developed plasma leakage, and two died (both the mothers and the fetuses) [193]. The other study of 73 pregnant patients found dengue with warning signs in mothers was a risk factor for peripartum hemorrhage [194]. Retrospective epidemiological studies have highlighted preterm birth and low birthweights as outcomes associated with dengue during pregnancy [38, 39, 195]. These studies examined clinical and obstetric outcomes. Immunological and virological factors, as well as the potential fetal response to *in utero* DENV infection were not investigated.

Both innate and adaptive immune components, including but not limited to CD8+ T cells, B cells, NK cells, and cytokines are involved in the response to DENV infections.

- CD8+ memory T cells, which function in the cytotoxic response towards DENV-infected cells, are activated and mature through a process of cellular differentiation. CD8+ naïve, CD8+ CM, CD8+ EM, or EMRA T cells are four major memory subsets. Among them, CD8+ EM and EMRA T cells

are endowed with effector functions. The specificity of CD8+ memory T cells to DENVs can be detected using tetramers, which comprise DENV peptides complexed with HLA class I. The detection of DENV-specific CD8+ memory T cells during the febrile phase of dengue indicates a secondary infection.

- B cells, which play a major role in the humoral adaptive immune response, are classified into memory B cells (CD27+ CD38-) and plasmablasts (CD27+ CD38+). While the proportion of memory B cells is reduced, that of plasmablasts is increased in the third trimester of healthy pregnancy [152]. Plasmablasts also represented the large percentage of peripheral B cell population in patients with secondary DHF [120], suggesting a pathogenic role for this B cell subset in dengue.
- NK cells are well-known for their cytokine-secreting and cytotoxic activities against DENV-infected cells. NK cells can be classified into cytokine-secreting NK cells (CD16- CD56high) or cytotoxic NK cells (CD16+ CD56+) [196]. The effector functions (phenotypes) such as cytokine secretion or cytotoxicity may be enhanced by the expression of activating markers such as CD57, or may be suppressed by the expression of inhibitory markers such as NKG2A.
- Cytokines comprise a broad category of small proteins that play a role in communicating between cells and in orchestrating innate and adaptive immune responses. At least 20 cytokines, both pro- and anti-inflammatory

(Table 1.1), are associated with immune responses to DENV infection and to the severity of dengue.

Our understanding of the immune response to DENV infections has been almost exclusively acquired from male and non-pregnant female patients. How CD8+ memory T cells, B cell, NK cells, and cytokines are altered in the context of dengue during pregnancy remains unknown. Therefore, using samples from a matched cohort study that enrolled pregnant and non-pregnant women with suspected dengue hospitalized at a referral hospital in Ho Chi Minh city, I investigated a range of immunological (CD8+ memory T cells, B cells, NK cells, and cytokines) and virological (viremia, NS1) factors. Moreover, I examined the presence of immunological (DENV-reactive IgM, IgG) and virological (viremia, NS1) factors in the cord blood of the babies born to the pregnant participants. The aim of this work is to compare immunological and virological profiles between pregnant and non-pregnant women with dengue and determine whether the fetus respond to DENV infection *in utero*.

Because the anti-inflammatory stage lasts the longest during pregnancy, my overarching hypothesis for this research is that pregnancy may be associated with a less pronounced immune response to DENVs. This may manifest as higher virological parameters (e.g. viremia, NS1 antigenemia) in pregnant patients.

4.2 Methods

4.2.1 Study design

Pregnant and non-pregnant women, who were suspected to have dengue and were hospitalized at HTD, were enrolled into a cohort. The study was designed to recruit two non-pregnant patients matched by day of illness (the number of days with symptoms at study enrollment) and age range (approximately 5 year age brackets: 18-24, 25-29, 30-34, 35-39, 40-45) for each pregnant patient enrolled. Non-pregnant status was confirmed before the enrolment using a standard pregnancy test. Inclusion criteria were: (1) clinically suspected to have dengue, (2) between 18 and 45 years of age, (3) written informed consent provided. Exclusion criteria were: (1) clinically suspected with other diseases rather than dengue, (2) managed in the outpatient ward of HTD (not hospitalized) (3) home address far from the hospital making follow up likely to be difficult due to long travelling distance. For all participants, clinical signs and symptoms and standard laboratory results were obtained and recorded according to a standard schedule. The study was approved by OxTREC (OxTREC 37-16) and the Ethical Committee of HTD (CS/ND/16/29). The enrollment of patients were conducted by research doctors.

I grouped enrolled patients into three populations for measuring immunological and virological factors (Figure 4.1). The three populations were not distinct, i.e. a patient might be included in more than one population. The three populations are:

1. FACS population: A subset of suspected-dengue patients recruited when FACS reagents were available, irrespective of pregnancy status or dengue diagnosis. Samples from the FACS population were used to assess the lymphocyte phenotypes. All assays were performed blind to pregnancy status and dengue diagnosis.
2. VCD population: Dengue cases were confirmed by RT-PCR (previously described in Section 2.2.2.4) or NS1 ELISA (described in Section 4.2.5 below). Here, I examined viremia and NS1 antigenemia at enrolment.
3. Cytokine population: Pregnant patients, and their two controls matched by day of illness and age range. All patients in the cytokine population were also virologically confirmed with dengue. Cytokine assays (described in Section 4.2.3 below) are expensive so they were only examined in this population to maximize cost effectiveness.

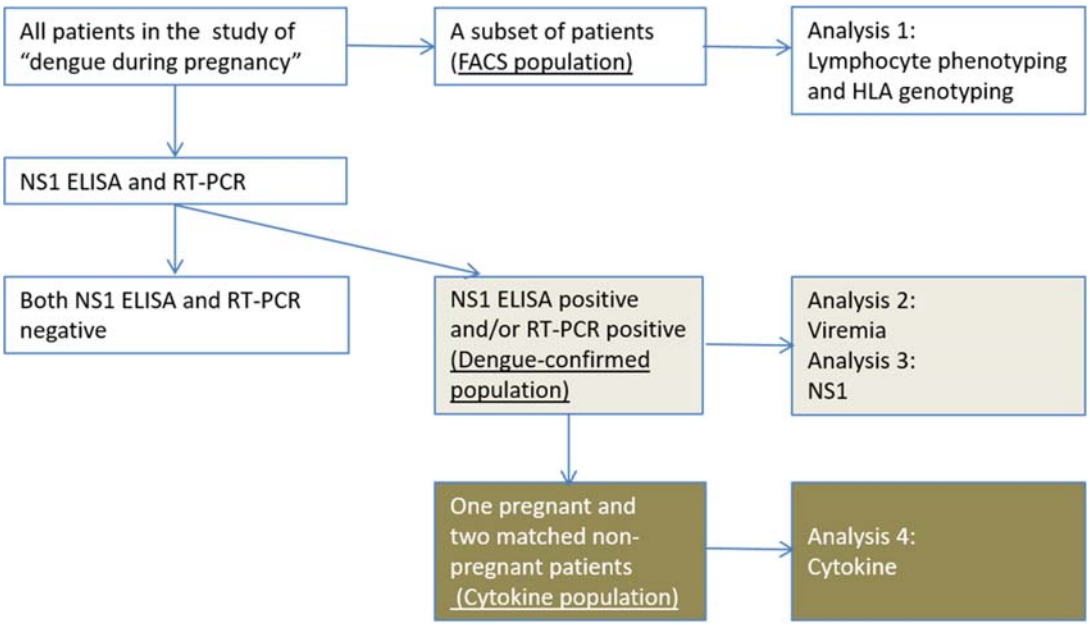


Figure 4.1: Flow chart of progression of samples

Pregnant and non-pregnant patients with suspected dengue were enrolled. A subset of enrolled patients, allocated into the FACS population, were examined for the phenotype of lymphocytes and the genotype of HLA. Enrolled patients who were virologically confirmed with dengue were allocated into the VCD population. DENV viremia and NS1 antigenemia were examined with patients in the VCD population. Cytokine were measured in a subgroup of VCD patients, including one pregnant women and their two matched non-pregnant women.

Research nurse collected two research blood samples from participants during their hospitalization, one at enrolment (5 mL) and one at discharge (2 mL). All participants were also asked to return to HTD once between days 10 to 14 post illness onset for a clinical assessment and to collect another blood sample (2mL). Using blood samples collected at these three time points (enrolment, discharge, and day 10-14 follow-up), I assessed the phenotype of lymphocytes. In contrast, the viremia and NS1 assays were conducted using only the blood samples at enrolment because these parameters are likely to be undetectable at discharge and follow-up (Figure 4.2).

Pregnant participants, who had either virologically confirmed dengue or OFI, were followed until delivery by members of the Dengue group. Among those who delivered at three of the major obstetric hospitals in Ho Chi Minh City (Tu Du, Hung Vuong, and Mekong hospitals), nurses in those hospitals were able to collect cord blood samples within 24-48 hours after delivery. Using these samples we investigated the presence of viremia (previously described in Section 2.2.2.4), NS1 (described in Section 4.2.5 below), and DENV-reactive IgM and IgG (described in Section 4.2.6.1 above and Section 2.2.9 below, respectively).

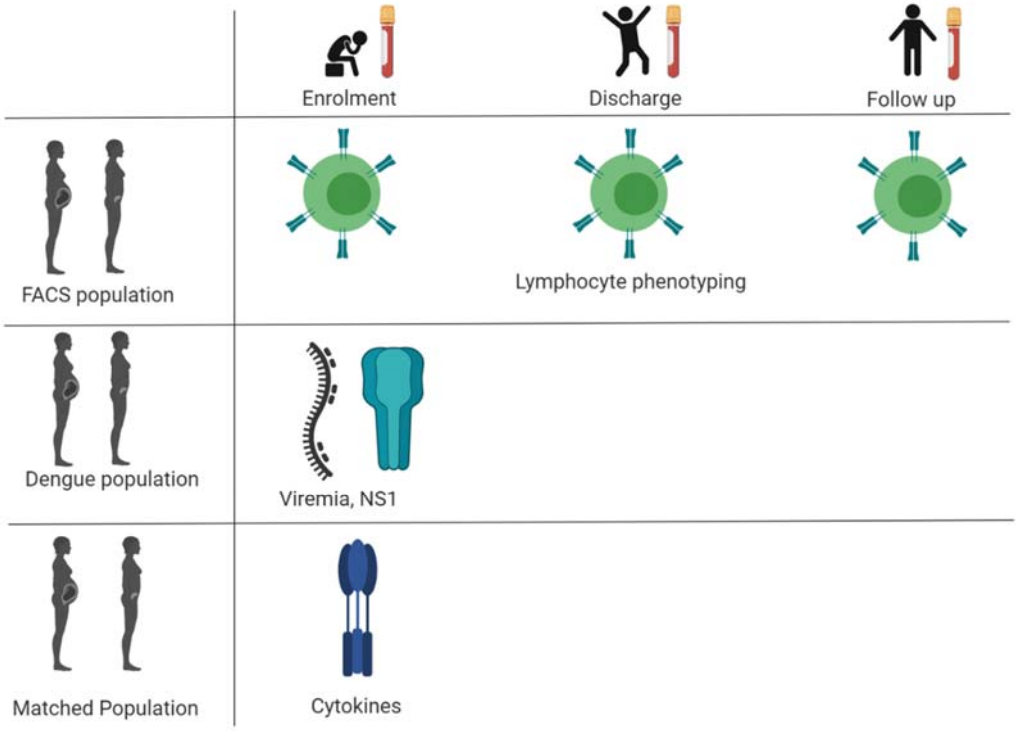


Figure 4.2: Schematic overview of sample collection and assays performed on respective samples

Lymphocyte phenotyping was performed for a subset of patients, using blood samples collected at enrolment, discharge and follow-up. In contrast, viremia, NS1, and cytokines were examined using enrolment samples only.

4.2.2 Lymphocyte phenotyping

I performed procedures to phenotype lymphocytes in serial samples from patients in the FACS population. Lymphocytes were of particular cell interest due to their immunological relevance to DENV infection. I examined four subsets of CD8+ memory T cells including CD8+ CD27+ CD45RA+ T cells, CD8+ CD27+ CD45RA- T cells, CD8+ CD27- CD45RA- T cells, and CD8+ CD27- CD45RA+ T cells. Two subsets of B cells, consisting of plasma cells and plasmablasts, were also investigated. Finally, I examined four subsets of NK cells including CD16- CD56high CD57+ NKG2A- NK cells, CD16- CD56high CD57- NKG2A+ NK cells, CD16+ CD56+ CD57+ NKG2A- NK cells, and CD16+ CD56+ CD57- NKG2A+ NK cells.

I supplemented cell pellets, collected after centrifugation, with growth medium (RPMI 10% inactivated fetal calf sera) to compensate for the volume of plasma removed. The suspension of cell pellet and growth medium was then divided equally into three aliquots, and centrifuged to collect the cell pellet. Cell pellets from each aliquot were stained with individual panels of antibody markers (Table 4.1), and then the red blood cells were lysed and removed. Stained white blood cells were analyzed using a FACS Canto.

In the CD8+ T cells antibody panel (Table 4.1), I used tetramers to assess the specificity to DENVs. Tetramers consist of 11-mer epitopes, located between position 133 and 143 of the NS3 protein, and restricted by HLA-A*11:01. Two separate variants of epitopes were used: GTSGSPIINRK from DENV-3 and DENV-4 [116], GTSGSPIIDKK from DENV-2

[118]. I also prepared one more FACS tube for CD8+ memory T cell panel which is the fluorescence minus one (FMO). Here, I added all antibody markers of the CD8+ memory T cell panel except for the tetramers. This was applied to a group of samples in the beginning to define the threshold for separating tetramer-positive from tetramer-negative fluorescent signals.

I designed the gating strategy for subsets of CD8+ memory T cells (Figure 4.3), and NK cells (Figure 4.4). Several markers did not work during these experiments of lymphocyte phenotyping. They include the tetramers for the CD8+ memory T cell panel; CD38, IgG, CD27 of the B cell panels; NKp30, CD69 of the NK cell panel. As a result, gating strategies for subsets of B cells could not be performed. These markers were also not included into the gating strategy for subsets of CD8+ memory T cells and NK cells.

In the gating strategy for subsets of CD8+ memory T cells, because the fluorescent intensity of several markers (CD3, CD8, CD27, CD45RA) varied substantially between patients, I performed individual adjustment of the gating for those patients.

From the gating strategy I used, I did not detect CD16- CD56high CD57+ NKG2A- NK cells (Figure 4.4). Therefore, I calculated the percentage of the parental cells for seven subsets of lymphocytes. These seven subsets of lymphocytes were:

1. CD8+ CD27+ CD45RA+ T cells
2. CD8+ CD27- CD45RA- T cells
3. CD8+ CD27+ CD45RA- T cells
4. CD8+ CD27- CD45RA+ T cells
5. CD16- CD56high CD57- NKG2A+ NK cells
6. CD16+ CD56+ CD57- NKG2A+ NK cells
7. CD16+ CD56+ CD57+ NKG2A- NK cells

Table 4.1: Lists of antibody markers used for lymphocyte phenotyping.

Markers are grouped on the basis of target cells, FACS parameters, antibody markers, suppliers, catalogue numbers, and the role for antibody markers are indicated.

CD8+ T cells panel			
Fluorochrome	**Antibody marker**	**Catalogue #**	**Used for**
FITC	CD45RA	555488	Gating memory cells
PerCP	CD8	565310	Gating CD8+ T cells
PE-Cy7	CD3	563423	Gating CD8+ T cells
APC-Cy7	CD14	560180	Excluding monocytes
APC-Cy7	CD19	560177	Excluding B cells
Pacific blue/BV421	CD27	562513	Gating memory cells
BV510	Live/Dead aqua[b]	L34957	Excluding dead cells
PE	Tetramers[a]	F2B-G	Gating cells specific to DENVs
B Cells panel			
Fluorochrome	**Marker**	**Catalogue #**	**Used for**
FITC	CD19	340409	Gating B cells
PerCP	CD20	560736	Gating B cells
PE-Cy7	CD38	560677	Gating plasmablasts and plasma cells
APC	CD21	559867	Gating activated B cells
APC-Cy7	CD27	560222	Gating plasmablasts and plasma cells
Pacific blue/BV421	IgG	562581	Gating activated B cells
BV510	Live/Dead aqua[b]	L34957	Excluding dead cells
BV510	CD14	563079	Excluding monocytes
BV510	CD3	740187	Excluding T cells
BV510	CD16	563830	Excluding NK cells

NK cells panel			
Fluorochrome	**Marker**	**Catalogue #**	**Used for**
FITC	CD16	555406	Gating NK cells
PE	NKG2A/CD159a[c]	IM3291U	Gating inhibitory NK cells
PerCP	CD3	347344	Excluding T cells
PE-Cy7	CD56	335791	Gating NK cells
APC	NKp30 AF647	558408	Gating activated NK cells
APC-Cy7	CD69	560737	Gating activated NK cells
Pacific blue/BV421	CD57	563896	Gating activated NK cells
BV510	Live/Dead aqua[b]	L34957	Excluding dead cells

All markers were supplied by BD, except for [a]ProImmune, [b]Life Technologies, [c]Beckman Coulter

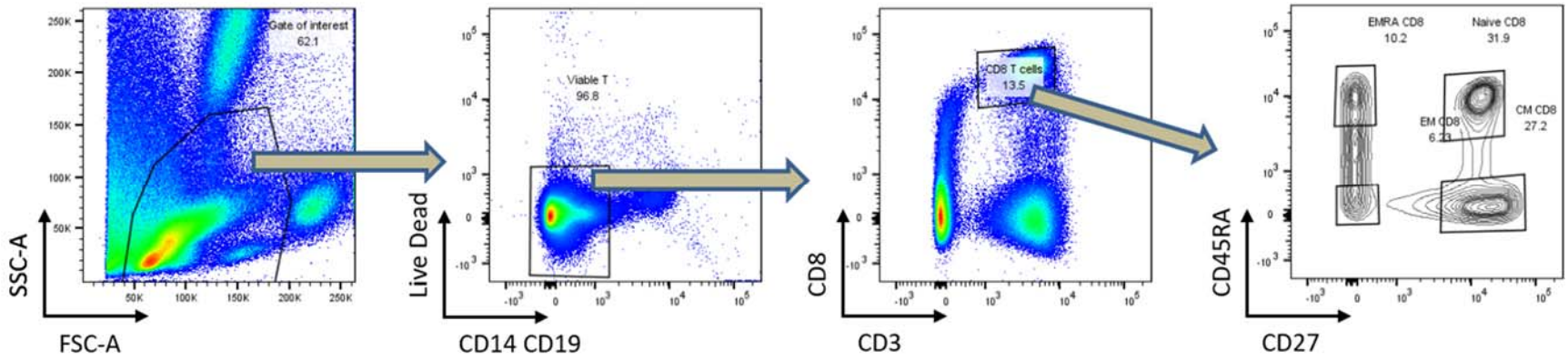


Figure 4.3: Example of the gating strategy employed for subsets of CD8+ memory T cells

From the entire cell population cells of interests were selected. I then excluded monocytes, B cells, and dead cells by gating for CD14- CD19- and Live_Dead-. In the next step, I selected CD8+ T cells characterized by CD3+ CD8+. Subsets of CD8+ memory T cells were then identified based on CD27 and CD45RA.

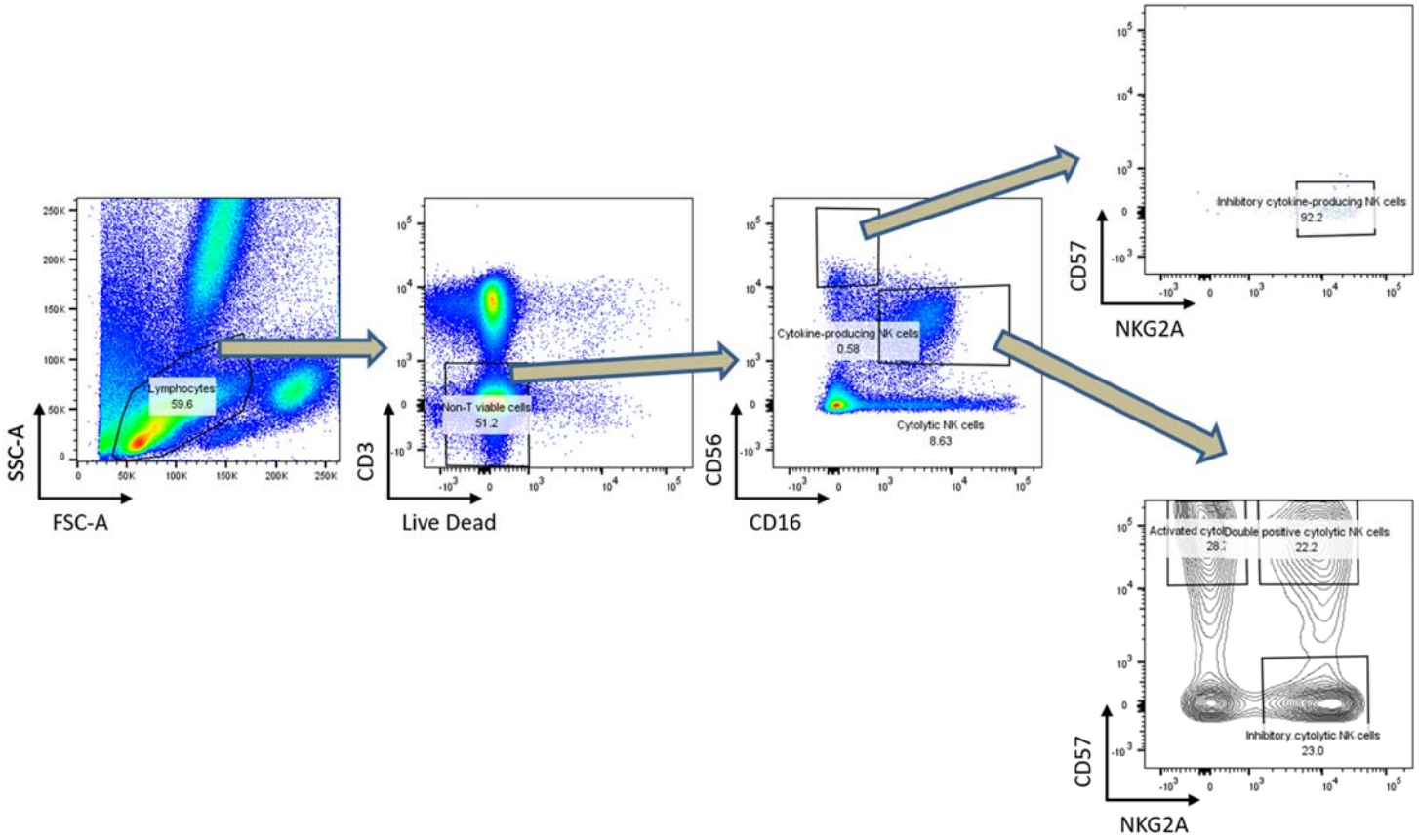


Figure 4.4: Example of the gating strategy employed for subsets of NK cells

From the entire cell population cells of interests were selected. I then excluded cells that expressed CD3, or dead cells. Subsets of NK cells are then shown, including CD16- CD56high NK cells, or CD16+ CD56+ NK cells. For both of the NK cell subsets, I gated for activating marker (CD57+) and inhibitory marker (NKG2A+).

HLA genotyping

To assess the specificity of subsets of CD8+ memory T cells for DENVs, I used tetramers, consisting of 11-mer epitopes, located between position 133 and 143 of the NS3 protein, and restricted by four HLA-A*11:01 molecules (Table 4.1). The selection of tetramers was based on previous studies [116, 118]. However, I failed to detect CD8+ memory T cells that bound specifically to HLA-A*11:01 tetramers (Section 4.3.2.4). Therefore, I genotyped HLA-A loci, using residual pellet samples collected at enrolment, to investigate whether the tested patients had other HLA-A types rather than HLA-A*11:01.

I carried out three main procedures to genotype HLA-A. First, I extracted nucleic acid of the patients, then amplified the HLA-A loci, and then visualized the products on gel. For DNA purification, I used the "Illustra Nucleon Genomic DNA Extraction kit". The principle of this assay is that after the cells are lyzed, proteins are removed using sodium perchlorate, chloroform, and nucleon resin. Nucleic acid was then precipitated in ethanol and eluted in water. Detailed procedure is followed:

1. Cell lysis - add 4 times the volume of reagent A to the blood sample. Rotary mix for 4 minutes at room temperature, centrifuge at 1300 g for 5 minutes, discard the supernatant
2. To the pellet add 350 µL of reagent B, vortex briefly to resuspend the pellet, transfer the suspension to a 1.5 mL microcentrifuge tube
3. Deproteinization – add 100 µL of sodium perchlorate solution, invert the capped tube at least 7 times
4. DNA extraction – add 600 µL of chloroform, invert the capped tube at least 7 times
5. Add 150 µL nucleon resin, centrifuge at 350 g for 1 minute

6. Without disturbing the nucleon resin layer (brown in color), transfer the upper phase to a clean 1.5 mL microcentrifuge tube
7. Add 900 μL of cold absolute ethanol, mix by inversion until the precipitate appears
8. DNA washing - centrifuge at 4000 g for 5 minutes to pellet the DNA, discard the supernatant
9. Add 1 mL cold 70% ethanol, mix several times by inversion, re-centrifuge and discard the supernatant.
10. Air dry the pellet for 10 minutes, ensuring that all the ethanol has been removed. Re-dissolve the DNA in 50 μL of water

The concentration and purity of nucleic acid samples were assessed using a nanodrop system. For subsequent procedures which target DNAs the concentration of nucleic acids should be from 25 to 200 ng/μL and A260/A280 ratio should be from 1.55 to 1.90. Any values lower than 1.55 or higher than 1.90 indicate high rates of protein and RNA contamination, respectively.

HLA-A loci were amplified using "Micro SSP™ HLA DNA Typing Trays", produced by One Lambda. Here, the PCR with sequence-specific primer (PCR-SSP) technology uses primer pairs designed to have perfect matches with a single allele. Those primer pairs are already put in the typing trays by One Lambda. Under PCR conditions, primer pairs result in the amplification of target sequences (i.e. positive result) while mismatched primer pairs do not result in amplification (i.e. negative result). An internal-control primer pair is included in every PCR reaction. The control primer pair amplifies a conserved region of a human β-globin gene, which is present in all human DNA samples. The positive reaction for a specific HLA allele is visualized on the gel as an

amplified DNA fragment between the internal-control product band and the incorporated primer band. Detailed procedure is followed:

1. Add 1 µL of DNA diluent to the negative control reaction tube on the typing tray
2. Add 3 µL of Taq polymerase (from One Lambda, 5 units/µL) to the D-mix tube
3. Transfer 9 µL of the Taq-D-mix to the negative control reaction tube
4. Add 59 µL of DNA sample to the Taq-D-mix
5. Aliquote 10 µL of the sample-Taq-D-mix into each reaction tube, except for the negative control
6. Cover the reaction tubes, and place the tray in the thermal cycler
7. Start the PCR program (below), set the ramp speed at 1˚C/s
8. Transfer each PCR reaction (10 µL) in s sequence to a 1.5% agarose gel with Nancy
9. Electrophorese the samples at 140 – 150 volts for 45 minutes
10. Photograph the gel on a UV transilluminator
11. Interpret the typing results using the worksheet provided by One Lambda

Number of cycles	Step	Temp. (˚C)	Time (sec.)
1	1	96	130
	2	63	60
9	1	96	10
	2	63	60
20	1	96	10
	2	59	50
	3	72	30
End	1	4	

4.2.3 Cytokine measurements

Concentrations of cytokines were measured using enrolment plasma samples from the cytokine population. A collaborator in Singapore measured 41 cytokines using the Luminex multiplex assay while I analyzed the clean data.

To this end, we used a Milliplex MAP kit, cat# HCYTOMAG-60K-PX41. This kit is based on Luminex technology which uses color-coded microspheres with two fluorescent dyes. Using precise concentrations of these dyes, groups of distinct beads can be generated. Each of them is coated with an antibody specific to a cytokine. After the cytokine is captured by the beads a biotinylated detection antibody is added. The reaction mixture is then incubated with streptavidin-PE conjugate to allow detection of the cytokine. Concentrations of cytokines are then defined using standard curves.

When comparing observed concentrations across plates, normalization was implemented to remove plate effects. The median fluorescent intensity of each full plate was used to normalize. This normalization is possible as we designed the plate layout such that equal ratios of non-pregnant and pregnant patients were allocated to each plate. There were also two internal controls which were used to examine the accuracy of the normalization.

Among the 41 measured cytokines, the concentrations of 15 cytokines were considered for primary statistical comparisons to avoid the issue of multiple testing (Table 4.2). The 15 selected cytokines were of interest because of their immunological relevance to dengue and severe dengue (Section 1.4.1). The analysis for the other 26 cytokines was exploratory. The procedure for cytokine measurements is described in Appendix, entitled "SOP for – Milliplex MAP kit, human cytokine/chemokine Panel 1, cat# HCYTOMAG-60K-PX41".

Table 4.2: Summary of 15 cytokines of interest

Cytokines	Relevance[a]	Reference
IL-1α	Elevation with dengue	[86]
IL-1ra	Elevation with dengue, and severe dengue	[86]
IL-6	Elevation with dengue	[86]
IL-8	Elevation with dengue, and DHF	[86, 87] [92]
IL-10	Elevation with dengue, and DHF	[92]
IL-12p70	Elevation with DSS	[90]
Ootaxon	Elevation with dengue	[86]
FGF-2	Elevation with dengue	[86]
Fin	Elevation with dengue	[86, 87]
IP-10 (CXCL10)	Elevation with dengue, DHF, DSS and severe dengue	[86-88] [90]
MCP-1 (CCL2)	Elevation with dengue	[86, 97]
MDC	Elevation with dengue	[86]
sCD40L	Elevation with dengue	[86]
TNFα	Elevation with dengue, and DSS	[86, 87] [90]
VEGF	Elevation with DHF	[91]

[a]The classification of dengue is based on the references.

4.2.4 Quantitative assessment of viremia

Viremia was assessed in the VCD population and in the cord blood of babies born at one of the three hospitals at which cord sampling was possible. Members of the Dengue group conducted qtrs.-PCR (previously described in Section 2.2.2.3). The unit of measurement was RNA copies/mL. The detection limits for DENV-1, DENV-2, DENV-3, and DENV-4 were 5, 2, 5, and 10 RNA copies/mL, respectively [23]. For the statistical analysis I replaced values below the detection limit (i.e. negative) by numbers corresponding to half of the appropriate detection limits.

4.2.5 Qualitative assessment of NS1 status

Members of the Dengue group conducted NS1 assays, using plasma samples obtained from the blood of the VCD population at enrolment, and also from the cord blood of babies born to pregnant women. Detection of NS1 was performed using the Platelia^{TM} Dengue NS1 Ag Kit from Bio-Rad. The kit is based on a one-step sandwich format of an ELISA for qualitative detection of NS1 in human plasma. The kit uses NS1-specific monoclonal antibodies for capture while peroxidase-conjugated antibodies are used for color development. The optical density reading is then obtained using a spectrometer at 450/620 nm. The kit provides positive and negative controls to validate the experiment, and also two calibrators. The mean of the two optical density values of the calibrators is the cut-off value. The cut-off value should be higher than 0.2 for a valid experiment.

A sample ratio is calculated for each specimen, as a ratio of the optical density value of the sample and the cut-off value. The experiment is valid if the sample ratio for negative controls is less than 0.4 while that for positive controls is higher than 1.5. Tested samples with the ratio of < 0.5 or of ≥ 1 are negative or positive for NS1, respectively. Samples with values that fall within the

intermediate zone are considered equivocal for NS1. Equivocal results were excluded from statistical analysis without repeating the experiment. The standard operation procedure for NS1 Ag kit is described in Appendix, entitled “NS1 qualitative ELISA assay”.

4.2.6 Dengue Serology

Dengue serology refers to the methods used to detect DENV-reactive IgM for the purpose of diagnosing dengue, and DENV-reactive IgG in order to classify the DENV confirmed patients with primary or secondary dengue (i.e. to assess their immune status). I also examined the presence of these two antibody classes in cord blood of babies born to pregnant participants. While IgM was measured using a capture ELISA only, IgG was assessed using either a capture or an indirect ELISA. The capture ELISA (described below) is less sensitive than the indirect ELISA and is typically used to measure high titers of IgG such as are seen during covalescence from an acute infection. On the other hand, the indirect ELISA, previously described in Section 2.2.9, can measure very low titers of IgG, such as are present after previous exposure to a DENV. Members of the Dengue group conducted all dengue serology assays.

4.2.6.1 Panbio Dengue IgM/IgG capture ELISA

The Panbio Dengue IgM/IgG Capture ELISA with 96-well-plate format was used to detect the presence of DENV-reactive IgM/IgG. In the micro-plate each well contains anti-human-IgM/IgG antibodies coated on the bottom. Plasma samples are added into the wells; if IgM/IgG antibodies are present in the plasma, these should bind to the anti-human-IgM/IgG antibodies. Antigens from DENV-1 to DENV-4 are then added, followed by the addition of DENV-reactive mAbs conjugated with horseradish peroxidase. The colorless TMB (the chromogen) is used and hydrolyzed by

horseradish peroxidase to become blue. Acid is then added to stop the reaction and turn the solution to yellow. The color development is indicative of the presence of DENV-reactive IgM/IgG in the test sample. The color absorbance of each well is then read at a wavelength of 450 nm with a reference filter of 600-650 nm. In the 96-well-plate format, three wells function as calibrators to calculate the average absorbance and the Panbio unit, following the equation below:

$$\text{Panbio unit} = \frac{\text{Sample absorbance} \ * \ 10}{\text{Average absorbance of calibrators} \ * \ \text{calibration factor (0.62)}}$$

For IgM, a calculated Panbio unit of less than 9 indicates the corresponding samples are negative for DENV IgM. Samples are considered positive for DENV IgM if the Panbio unit is more than 11. Any samples with intermediate Panbio units are considered equivocal for DENV IgM.

For IgG, calculated Panbio units less than 18 indicate that the corresponding sample is negative. Samples are considered positive if the Pabio unit is more than 22, and samples with Panbio units between these two cutoffs are considered equivocal. The standard operation procedure for DENV-specific IgM/IgG capture ELISA is described in Appendix, entitled “DENV IgM/IgG ELISA”.

4.2.6.2 Classifying primary/secondary immune status in study participants

In this context immune status refers to whether VCD patients have previous exposure to a heterologous DENV serotype or not. If the individual is infected with a DENV for the first time during the current illness, the disease is characterized as primary dengue. Otherwise, it is considered to be post-primary dengue – i.e. all infections after the first exposure. For simplicity,

this is referred to from here on as ‘secondary dengue’. I developed criteria to distinguish primary from secondary dengue cases. I also classified primary/secondary immune status in study participants.

For the work described in this Chapter, the characterization of the immune status of patients with dengue was based solely on detection of DENV-reactive IgG in plasma collected at enrolment and discharge. The capture ELISA (previously described in Section 4.2.6.1) was used to measure IgG in each discharge sample – i.e. in samples obtained when IgG levels are expected to be high. On the other hand, indirect IgG ELISA (previously described in Section 2.2.9) was used with enrolment samples obtained before 5 days of illness, when DENV-specific IgG levels are expected to be low - the purpose of using indirect IgG ELISA at this time was to detect an IgG response to past exposure with DENVs. Enrolment samples drawn from patients after 5 days of illness were not used to assess indirect IgG because this antibody class may be detectable at this time irrespective of primary or secondary dengue.

Patients in the VCD population were classified as having primary dengue if all three criteria below were met:

1. Two consecutive samples, collected at enrolment and discharge, were both IgG-negative. Enrolment samples were negative for indirect IgG, and discharge samples were negative for capture IgG.
2. The discharge sample was collected at day of illness ≥ 6
3. The day of collection of the discharge sample was > 2 days apart from the enrolment sample.

Patients in the VCD population were classified as having secondary dengue if either or both the enrolment and the discharge sample (provided this was taken at day of illness < 10), were IgG positive. If discharge samples were obtained at day of illness ≥ 10, DENV-reactive IgG may be detectable, irrespective of the immune status.

Patients who fell into neither the primary nor the secondary dengue categories using these rules were classified as inconclusive immune status.

4.2.6.3 Detection of DENV-reactive antibodies in cord blood

Cord blood samples were only collected when we were able to receive them within the first 48 hours post-delivery. To evaluate the infant's response to the maternal infection we assessed DENV-reactive IgM using the capture ELISA (previously described in Section 4.2.6.1) and IgG using the indirect ELISA (previously described in Section 2.2.9). Members of the Dengue group conducted the capture IgM and indirect IgG ELISA.

4.2.7 Statistical analysis

I developed the statistical analysis plan and performed all statistical analyses for this Chapter. A statistician helped me decide what tests to use.

4.2.7.1 Descriptive analysis

Boxplots were used to visualize the median and IQR for continuous data while dot plots were used to visualize categorical data. To observe changes over time, all data were plotted by day of illness,

or grouped by week of illness for plots showing results of serial samples from the same individuals. Plots used to illustrate the variation between the pregnant and non-pregnant arms are as follows:

- Median (IQR) percentage of subsets of lymphocytes
- Median (IQR) concentration of each cytokine
- Median (IQR) log10 viremia
- The percentage of NS1 positive samples

4.2.7.2 Comparative analysis

I summarized and compared the following between the pregnant and non-pregnant patients with dengue (reference group):

- Percentages of subsets of CD8+ T cells, and of NK cells
- Concentrations of cytokines
- Log10 viremia, measured in RNA copies/mL
- NS1 status

For the percentages of lymphocyte subsets, the analysis was only conducted for virologically-confirmed dengue patients because there were few patients with OFI. Therefore, the lymphocyte phenotyping data of patients with OFI were excluded.

Comparisons between pregnant and non-pregnant patients with dengue were made based on generalized estimating equations. Linear regression models were used for continuous data (percentages of immune cell subsets, cytokines, $\log_{10}$ viremia) while a logistic regression model was used for the categorical data (NS1 status). Clusters – i.e. patients matched by day of illness

and age group, or with sequential samples collected from one patient - were taken into account by using robust standard errors. Because the generalized estimating equation only allows one factor to be included in a cluster, the selection of the cluster depended on the specific analyses. In particular, matching of patients by day of illness and age group was used for clusters involving cytokines, $\log_{10}$ viremia, and NS1 status, while the fact that sequential samples were collected from one patient was considered as the cluster for evaluating percentages of immune cell subsets.

For all analyses, I adjusted for baseline confounding factors, including:

- Serotype
- Patient age (measured in years)
- Immune status (see above, Section 4.2.6.2)
- Day of illness
- The presence of any of warning signs. Patients with dengue are often hospitalized if they have any of a range of warning signs when seeking medical care. Although not formally included in the list of warning signs, pregnancy is clinically considered as a risk factor for obstetric consequences, so many pregnant women were hospitalized (and thus could be enrolled into this study) without the presence of warning signs. Therefore, in this situation, the presence of warning signs at baseline may be associated with both pregnancy status and with the analyses we performed. The warning signs we looked at were as indicated by WHO in their 2009 publication [18]. Persistent vomiting was defined as vomiting more than three times within 24 hours.

Other adjustments were made in addition to those listed above, including:

- Hematocrit, for viremia and cytokines. The rationale is that hematocrit values are influenced by pregnancy because the intravascular fluid volume of pregnant women is higher than the normal population. The difference in hematocrits between pregnant and non-pregnant states is an indicator of volume status and may influence the results of cytokine concentrations and viremia levels.

Although day of illness and age group were matched between the two arms, these variables were still included in the models as confounding factors. This is to improve the power of the analyses.

4.2.7.3 Multiple testing adjustment

A total of 24 comparisons were conducted: seven for the phenotype of lymphocytes, 15 for cytokines, one for viremia, and one for NS1 detection. Therefore I adjusted for multiplicity (to protect type I error) using the Bonferroni method. In particular, a p-value of ≤ 0.002 (0.05 / 24) was considered as the level for statistical significance.

4.3 Results

4.3.1 Patient enrolment and follow-up

A total of 663 women were recruited between October 2016 and February 2018 (Table 4.3). The FACS population comprised 78 consecutive participants enrolled between September and November 2017. The VCD population included all 541 virologically-confirmed dengue cases enrolled in the study, while the cytokine population consisted of 273 virologically-confirmed patients, grouped into 81 pregnant and 192 non-pregnant patients.

Baseline information for patients in the three populations is indicated in Table 4.3. Median (IQR) day of illness at enrolment were 4 (3, 5) days for VCD pregnant patients, and 4 (4, 5) days for VCD non-pregnant individuals. Among the VCD pregnant participants, median (IQR) length of gestation at enrolment was 20 (12, 28) weeks. The percentage of pregnant patients with dengue who had at least one warning sign at enrolment was lower than among the non-pregnant patients, indicating that pregnant patients were generally less severe in this study. The majority of the dengue patients had secondary serological profiles. DENV-1 was the most prevalent serotype, followed by DENV-2 and DENV-4.

Table 4.3: Baseline information of FACS, VCD, and cytokine populations

	FACS population		VCD population		Cytokine population	
	Pregnant	**Non-Pregnant**	**Pregnant**	**Non-pregnant**	**Pregnant**	**Non-pregnant**
n	26	52	212	329	91	182
Age in years						
Median (IQR)	30 (26,32)	30 (25-32)	27 (24,30)	27 (23,30)	27 (24,30)	27 (23,31)
Day of illness at enrolment						
Median (IQR)	5 (4,6)	4 (4,5)	4 (3,5)	4 (4,5)	4 (4,5)	4 (4,5)
Gestational week at enrolment						
Median (IQR)	22 (15-33)	NA	20 (12,28)	NA	19 (12,27)	NA
Any warning signs at enrolment[a]						
n (%) yes	19 (73)	40 (77)	120 (57)	242 (74)	58 (64)	139 (76)
Immune status[b]						
n (%) primary	2 (8)	1 (2)	9 (4)	13 (4)	3 (3)	7 (4)
n (%) secondary	15 (58)	42 (81)	186 (88)	288 (87)	82 (90)	160 (88)
n (%) Inconclusive	0	1 (2)	17 (8)	28 (9)	6 (7)	15 (8)
OFI	9 (34)	8 (15)	NA	NA	NA	NA
DENV serotypes[b]						
n (%) DENV-1	10 (38)	23 (44)	125 (59)	194 (59)	49 (54)	109 (60)
n (%) DENV-2	6 (23)	11 (21)	40 (19)	68 (21)	21 (23)	35 (19)
n (%) DENV-3	1 (4)	0	1 (0.5)	0 (0)	1 (1)	0 (0)
n (%) DENV-4	0	5 (10)	32 (15)	46 (14)	14 (15)	26 (14)
n (%) inconclusive	0	5 (10)	14 (6.5)	21 (6)	6 (7)	12 (7)
OFI	9 (35)	8 (15)	NA	NA	NA	NA
Severe dengue[a,b,c]						
n (%) yes	2 (12)	4 (9)	11 (5)	23 (7)	3 (3)	10 (5)

[a]The presence of warning signs at enrolment and the final classification of severe dengue were based on 2009 WHO guidelines [18].
[b]A number of 17 patients (9 pregnant, 8 non-pregnant) in the FACS population were not virologically confirmed with dengue, so they are classified as having OFI.
[c] Three patients in the VCD population were not classified for the severity of dengue due to the lack of clinical data.
NA = Not applicable.
IQR = Interquartile range

4.3.2 Lymphocyte phenotyping

In the FACS population, a total number of 203 blood samples was stained with antibody markers to assess subsets of CD8+ T cells and NK cells (Figure 4.5). Reasons for several discharge and follow-up samples not being tested are indicated in Figure 4.5.

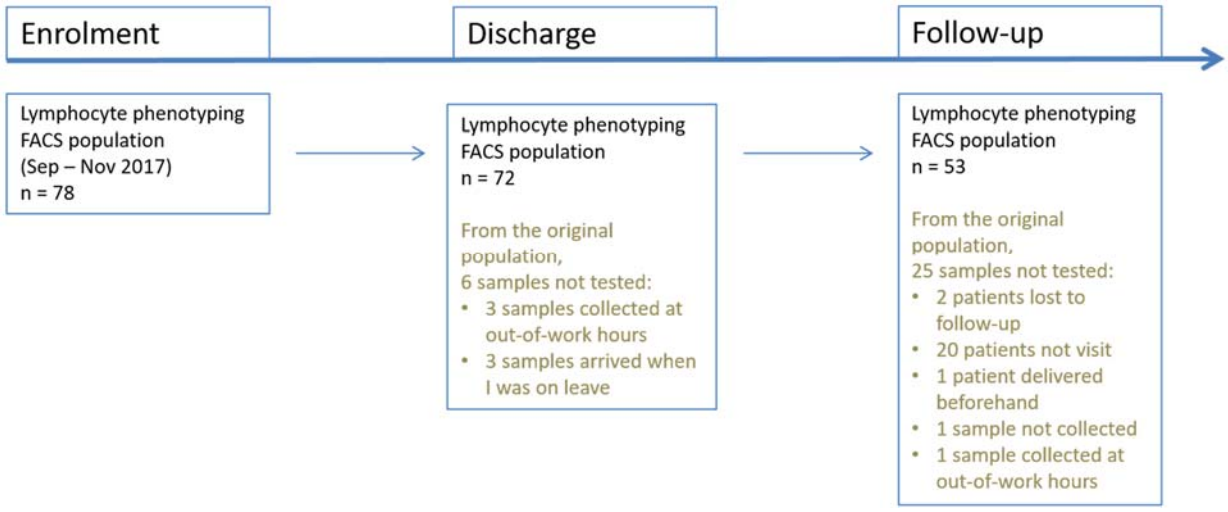


Figure 4.5: The progression of samples used in immune cell phenotyping

4.3.2.1 Dynamics of NK cell subsets

While analyzing the data for NK cells I excluded 60 of the 203 samples. Reasons for the exclusion are indicated in Figure 4.6. The remaining 143 samples were analyzed for subsets of NK cells including CD16- CD56high CD57- NKG2A+ NK cells, CD16+ CD56+ CD57- NKG2A+ NK cells, and CD16+ CD56+ CD57+ NKG2A- NK cells. There is no pattern in the percentage of these three NK cell subsets across the range of day of illness (Figure 4.7). The statistical analyses shows that none of the three subsets of NK cells differed between pregnant and non-pregnant women with dengue (Table 4.4).

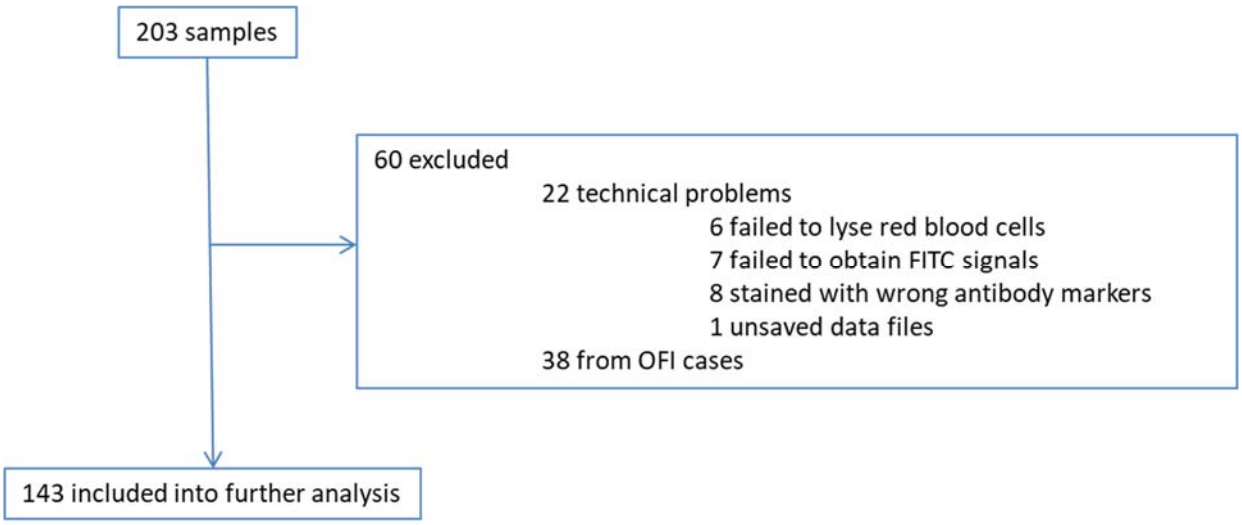


Figure 4.6: Flow chart for numbers of samples included into the descriptive analysis of NK cells

Table 4.4: Statistical analyses for subsets of NK cells

The percentage of CD16- CD56high CD57- NKG2A+, CD16+ CD56+ CD57- NKG2A+, and CD16+ CD56+ CD57+ NKG2A- NK cells were compared between pregnant and non-pregnant women with dengue, using the generalized-estimating-equation method. Non-pregnant patients were the reference.

	Mean difference in percentage (95% CI)	p -value
CD16- CD56high CD57- NKG2A+ NK cells	5.10 (-8.55, 18.76)	0.46
CD16+ CD56+ CD57- NKG2A+ NK cells	-0.03 (-0.08, 0.03)	0.34
CD16+ CD56+ CD57+ NKG2A- NK cells	-0.02 (-0.09, 0.05)	0.54

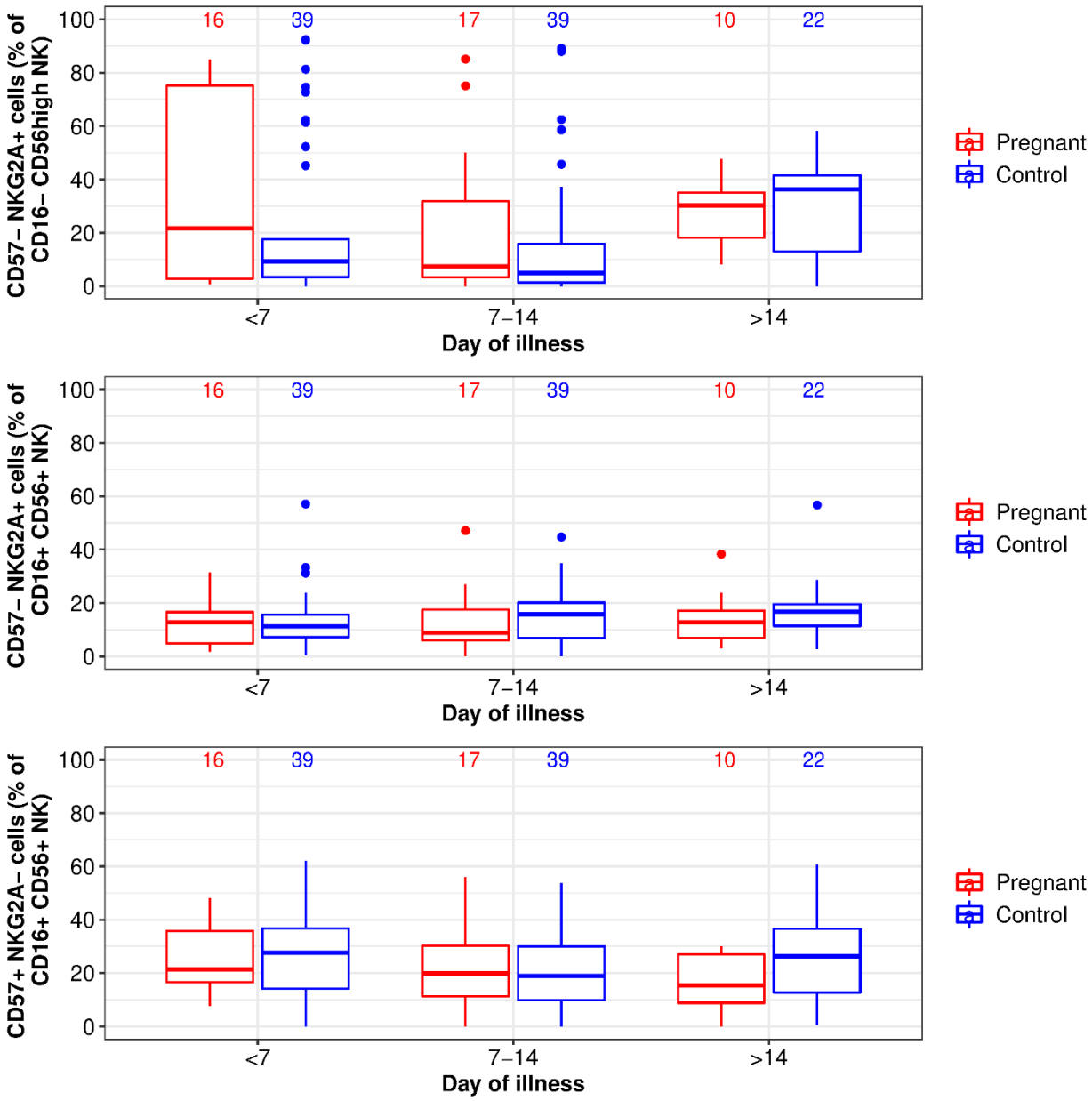


Figure 4.7: Dynamics of subsets of NK cells

The percentages of CD16- CD56high CD57- NKG2A+ NK cells (top), CD16+ CD56+ CD57- NKG2A+ NK cells (middle), and CD16+ CD56+ CD57+ NKG2A- NK cells (bottom) were shown across three weeks of illness. The number of tested blood samples is indicated within each panel.

4.3.2.2 Dynamics of CD8+ T cell subsets

While analyzing data of CD8+ cells, I excluded 57 of the 203 samples. Reasons for the exclusion are indicated (Figure 4.8). I proceeded with the remaining 146 samples for subsets of CD8+ T cells including CD27+ CD45RA-, CD27+ CD45RA+, CD27- CD45RA-, CD27- CD45RA+ cells. Figure 4.9 shows that the percentages of CD27+ CD45RA+ and CD27+ CD45RA- CD8+ T cells were higher than those of CD27- CD45RA- and CD27- CD45RA+ CD8+T cells. Moreover, while the percentage of CD8+ CD27+ CD45RA- T cells peaked at 7-14 day of illness, those of other CD8+ T cell subsets did not show any patterns (Figure 4.9). Notably, pregnancy was significantly associated with a reduction in the percentage of CD8+ CD27- CD45RA- cells (Mean difference [95% CI] = -7.79 [-12.38, -3.20], $p < 0.001$) (Figure 4.9, Table 4.5). In contrast, the percentage of CD27+ CD45RA+, CD27+ CD45RA-, and CD27- CD45RA+ CD8+ T cells were not significantly different between the two groups.

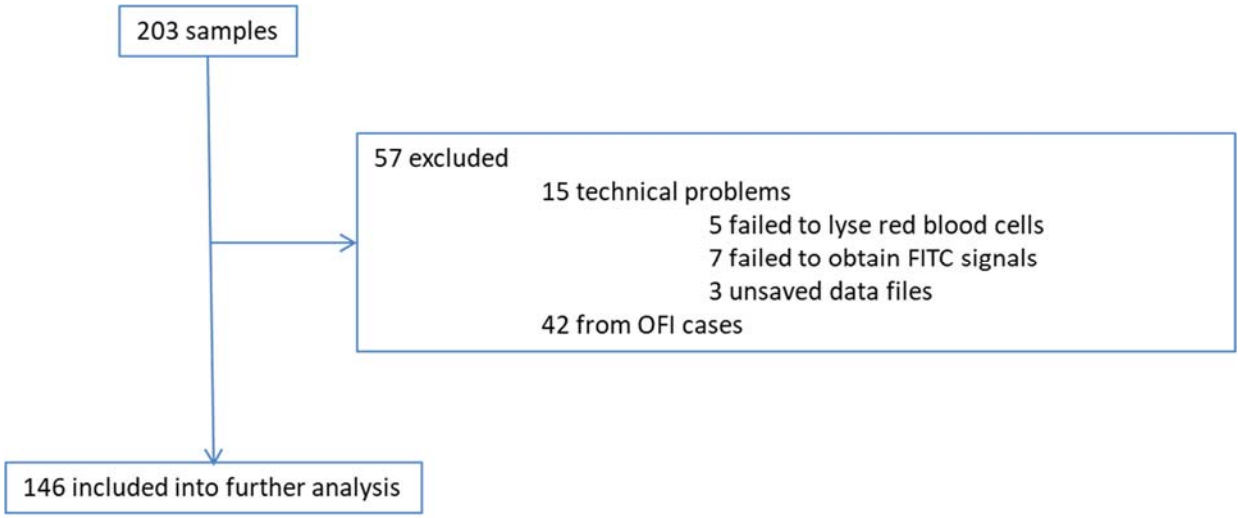


Figure 4.8: Flow chart for numbers of samples included into the analysis of CD8+ memory T cells

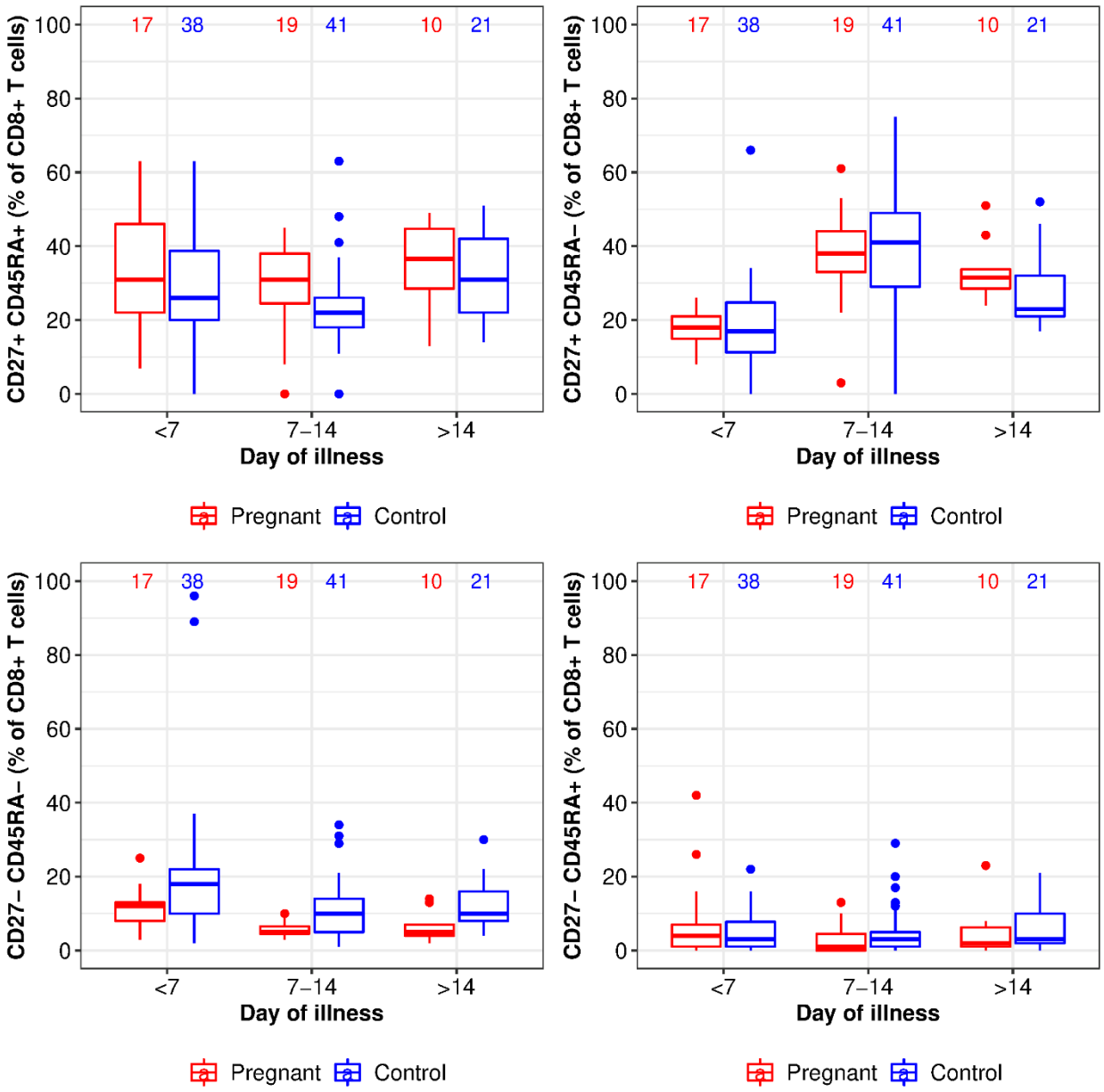


Figure 4.9: Dynamics of subsets of CD8+ T cells

The percentages of CD8+ CD27+ CD45RA+ T cells (top left), CD8+ CD27+ CD45RA- T cells (top right), CD8+ CD27- CD45RA- T cells (bottom left), and CD8+ CD27- CD45RA+ T cells (bottom right) are shown across three weeks of illness. The number of tested blood samples is indicated within each panel.

Table 4.5: Statistical analyses for subsets of CD8+ T cells

The percentages of CD8+ CD27+ CD45RA+, CD8+ CD27+ CD45RA-, CD8+ CD27- CD45RA-, CD8+ CD27- CD45RA+ T cells were compared between pregnant and non-pregnant women with dengue. Non-pregnant patients were the reference.

	Mean difference in percentage (95% CI)	p -value
CD8+ CD27+ CD45RA+ T cells	4.73 (-1.23, 55.67)	0.12
CD8+ CD27+ CD45RA- T cells	-0.25 (-4.72, 5.22)	0.92
CD8+ CD27- CD45RA- T cells	-8.21 (-12.56, -0.04)	**0.002**
CD8+ CD27- CD45RA+ T cells	0.02 (-3.57, 3.61)	0.99

4.3.2.3 Failed antibody markers

In the lymphocyte phenotyping, several markers did not work. They included CD38, IgG, and CD27 in the B cell panel, so I could not identify subsets of B cells, including memory B cells (CD38+ CD27-) and plasmablasts (CD38+ CD27+). Additionally, NKp30 and CD69 markers in the NK cell panel did not work. An explanation for the failure of these antibody markers is that I stained whole blood with them. If those antibody markers were stained after red blood cells were lyzed, they may bind to their cognate receptors on immune cells.

I also examined the specificity of subsets of detected CD8+ memory T cells to DENVs. Due to small volume of the reagent (tetramers), among 78 patients in the FACS population, 36 patients

were examined for subsets of DENV-specific CD8+ memory T cells. Here, tetramers consisting of HLA-A*11:01-restricted DENV peptides were used to bind to T cell receptors.

While performing FACS, I noticed that CD8+ memory T cells that bound specifically to tetramers were not detected (Figure 4.10). There are two possible hypotheses for this observation. First, tetramers may not be folded properly, so they did not bind to CD8+ T cell receptors. Secondly, the patients tested might have other HLA-A types rather than HLA-A*11:01 and therefore they tetramers did not bind.

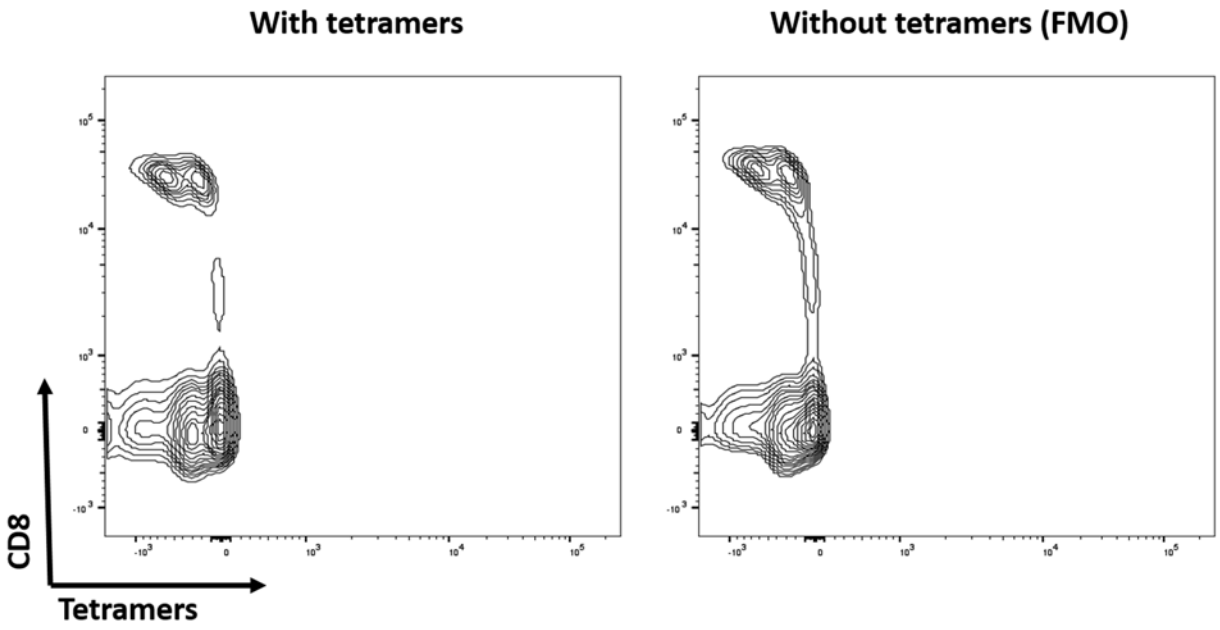


Figure 4.10: DENV-reactive CD8+ T cells were not identified using tetramers.

Contour plot shows lymphocytes of a samples stained with tetramers (left) and without tetramers (right).

4.3.2.4 HLA genotyping

To determine whether the tested patients had other HLA-A genotypes rather than HLA-A*11:01, the HLA-A experiment was carried out with enrolment samples from the 36 patients whose data on DENV-specific CD8+ memory T cells were available. Of the 36 samples, the DNAs from eight samples was not suitable for the subsequent PCR and electrophoresis procedure. In particular, samples had degraded DNAs while in the other six samples the DNAs were contaminated with RNAs and proteins, as indicated by the A260/A280 ratio being outside the range of 1.55 – 1.90. According to the manufacturer of the HLA genotyping kit, the RNA and protein contamination may result in no bands or faint bands after electrophoresis.

I carried out PCR and electrophoresis with 28 samples, of which ten samples failed to show any positive bands after gel electrophoresis despite the presence of internal-control bands. As such, the results of HLA-A genotyping were inconclusive for these ten samples. HLA-A genotypes were characterized for the remaining 18 samples, of which 12 samples (67%) were HLA-A*11:01 (Figure 4.11). Because the hypothesis was falsified with the detection of HLA-A*11:01 allele in 12 patients, DENV-specific CD8 T cell responses should have been detectable in the pellet samples from these patients. Therefore, an explanation for the failure of tetramers is that these commercial products were not properly folded, so they did not bind to CD8+ T cell receptors.

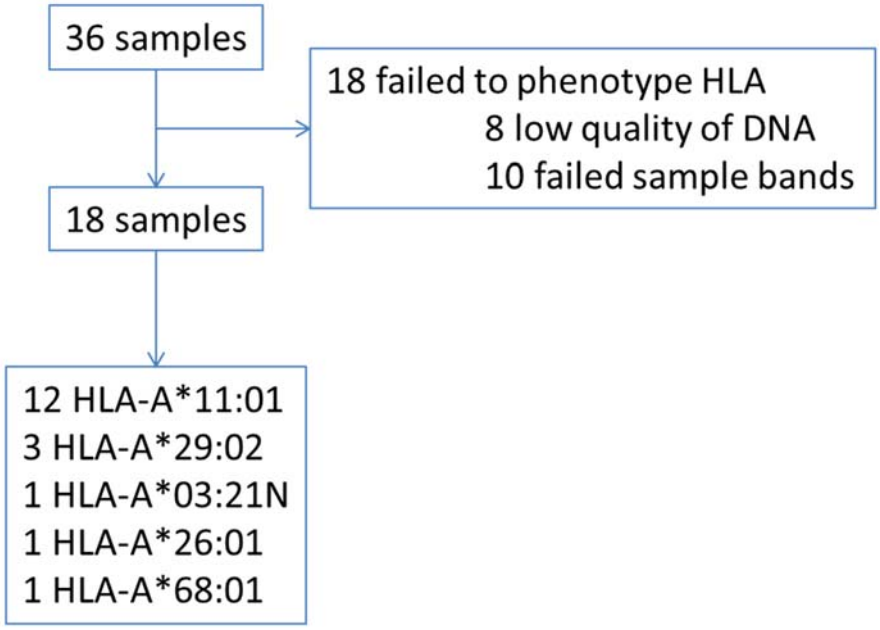


Figure 4.11: Summary of the HLA-A genotyping experiment

4.3.3 Cytokine dysregulation

I hypothesized that concentrations of pro-inflammatory cytokines in pregnant women with dengue may be lower while those of anti-inflammatory cytokines may be higher. To address this hypothesis, I explored cytokines in plasma specimens collected at the time when the 273 patients in the cytokine population were enrolled into the study. The number of pregnant patients who had cytokines measured on day 2, 3, 4, 5, and 6 of illness were 1, 10, 37, 24, and 19, respectively. The number of non-pregnant women with cytokine data was double that of pregnant women.

Among the 41 tested cytokines, concentrations of 8 cytokines were below the lower limit of detection for more than 50% of patients. They include Flt-3L, IL-6, IL-12p70, IL-13, IL-9, MCP-3, TGF-α, TNFβ. The other 33 cytokines had values that fell within the assay detection limits for more than 50% of patients.

Due to their previously reported relevance to dengue and severe dengue, the primary analysis included 15 out of 41 cytokines (Figure 4.12). Among these 15 cytokines, concentrations of three cytokines were significantly lower in pregnant patients: eotaxin (mean difference [95%CI] = -11.86 [-18.63, -5.09], $p < 0.001$); MCP-1 (mean difference [95%CI] = -168.87 [-265.93, -71.82], $p < 0.001$); and MDC (mean difference [95%CI] = -73.40 [-118.36, -28.44], $p < 0.001$) (Table 4.6). Among cytokines with p values ≤ 0.05 but not below the threshold for significance ($p < 0.002$) chosen for this research, concentrations of 6 cytokines (GM-CSF, IFNα2, IFNγ, IL-1a, IL-8, IP-10) were also lower in pregnant than non-pregnant patients. In contrast, the concentrations of 2 cytokines (PDGF-AA, PDGF-AB/BB) were higher in the pregnant participants.

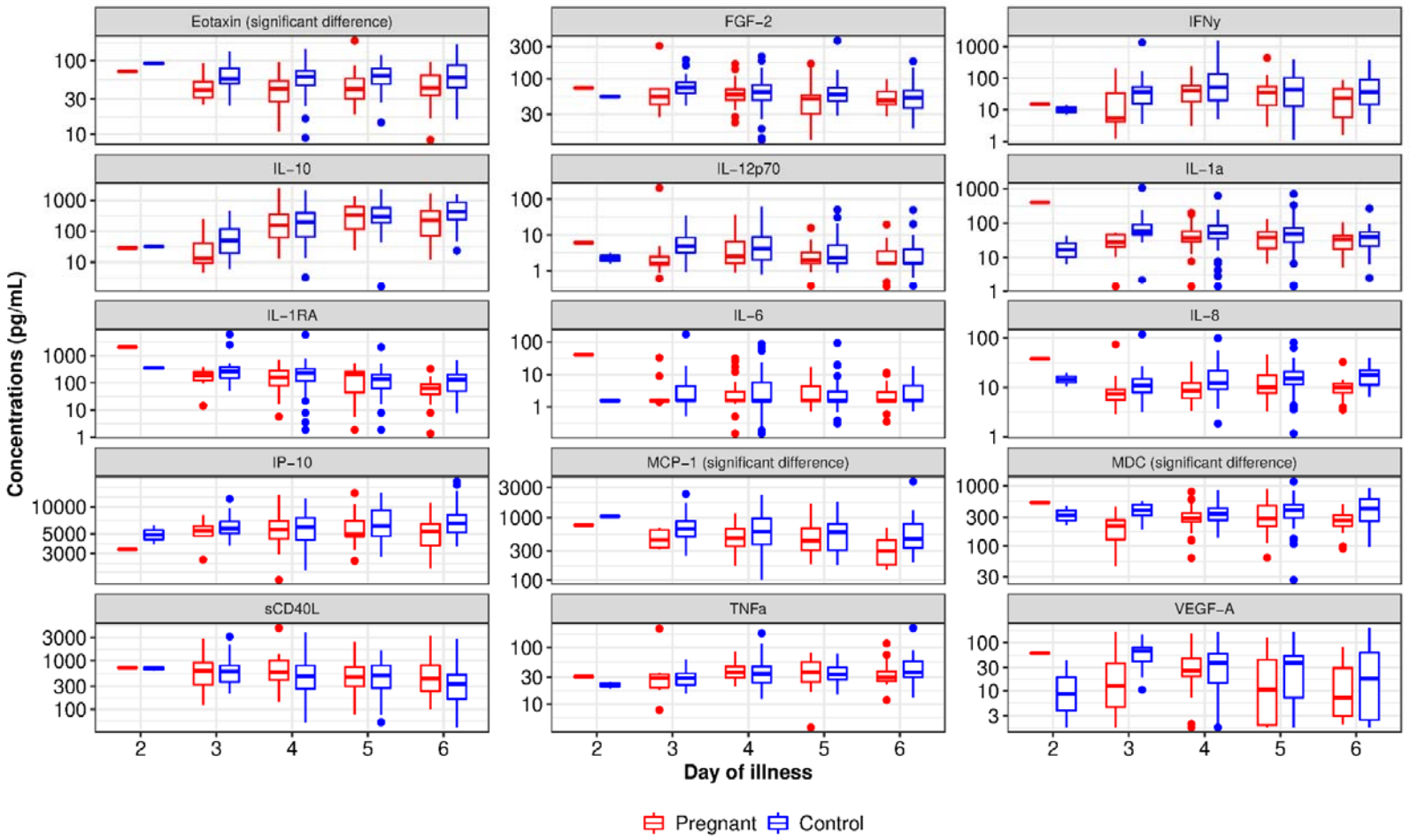


Figure 4.12: Primary-analysis cytokines in 91 pregnant and 182 non-pregnant women with dengue

Names of cytokines are indicated at the top of each panel, and listed alphabetically. Eotaxin, MDC, and MCP-1 differ statistically between the two arms. Values under the lower limit of detection were replaced by half of the limit of detection.

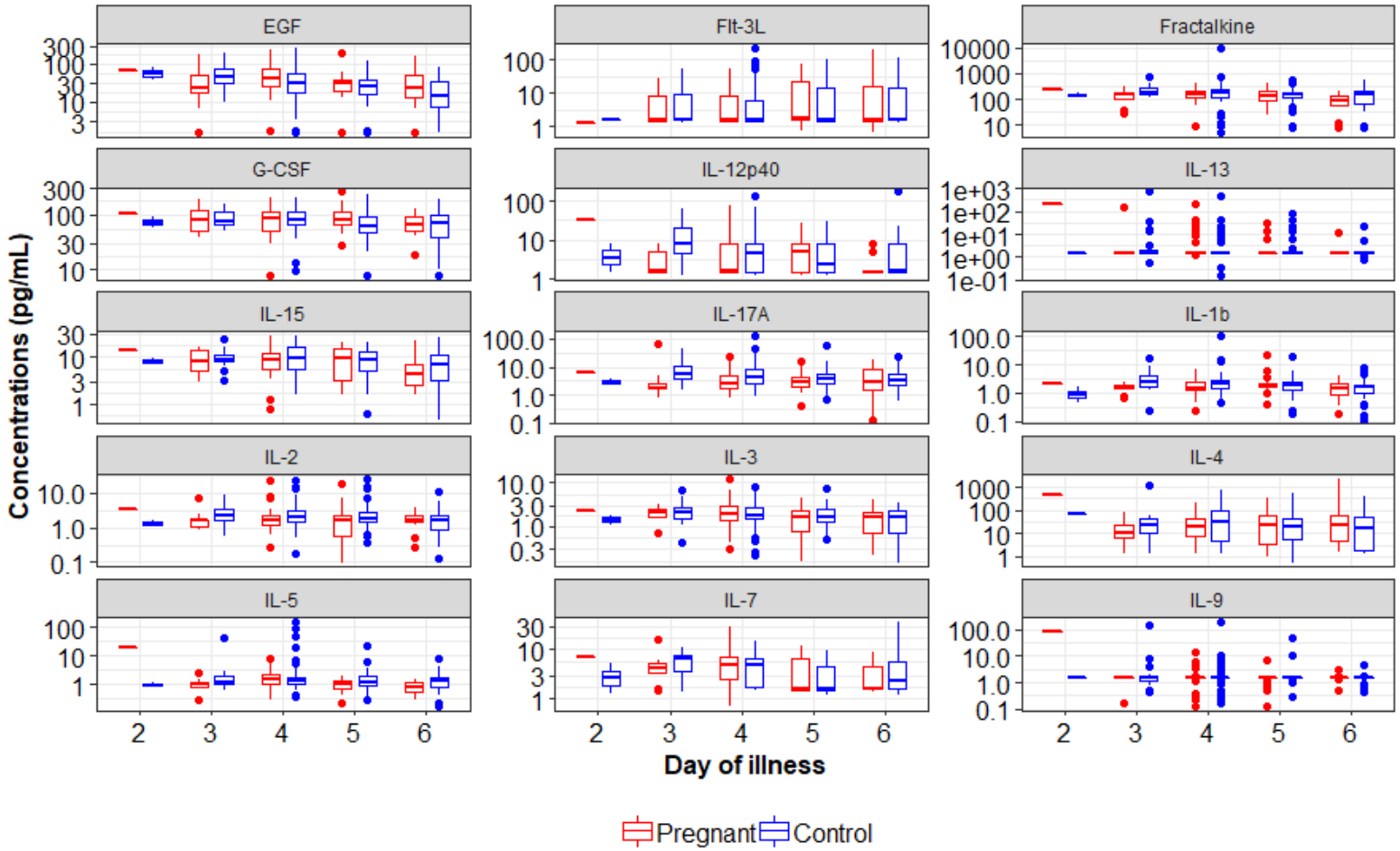


Figure 4.13: First set of exploratory cytokines in 91 pregnant and 182 non-pregnant women with dengue

Names of cytokines are indicated at the top of each panel and are listed alphabetically. Values under the lower limit of detection were replaced by half of the limit of detection.

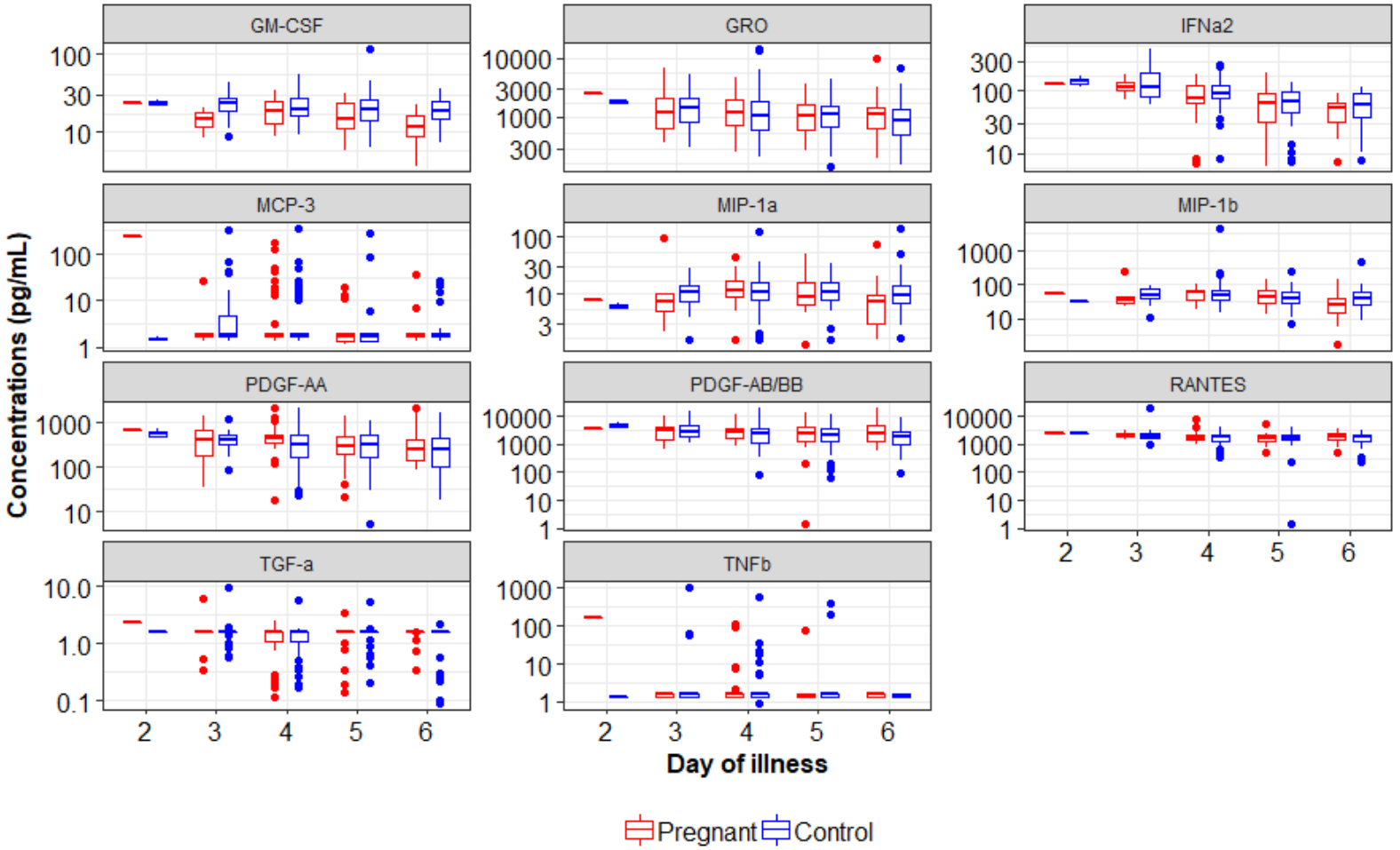


Figure 4.14: Second set of exploratory cytokines in 91 pregnant and 182 non-pregnant women with dengue

Names of cytokines are indicated at the top of each panel, and are listed alphabetically. Values under the lower limit of detection were replaced by half of the limit of detection.

Table 4.6: Statistical analyses for cytokines

Levels of individual cytokines were compared between pregnant and non-pregnant women with dengue, using the generalized-estimating-equation method. Non-pregnant patients were the reference. The order of cytokines was based on the analysis types and alphabet orders. Data for cytokines of primary analysis are highlighted in grey. Values under the lower limit of detection were replaced by half of the lower limit of detection.

	Mean difference in concentrations (95% CI)	p -value
Eotaxin	-11.86 (-18.63, -5.09)	**< 0.001**
FGF-2	1.63 (-8.44, 11.70)	0.75
IFNγ	-32.91 (-59.19, -6.62)	0.01
IL-10	-95.57 (-212.34, 21.21)	0.11
IL-12p70	0.97 (-3.20, 5.14)	0.65
IL-1a	-21.47 (-42.13, -0.83)	0.04
IL-1RA	-56.43 (-154.61, 41.75)	0.26
IL-6	-0.21 (-2.84, 2.42)	0.87
IL-8	-3.35 (-6.71, 0.02)	0.05
IP-10	-877.34 (-1661.85, -92.83)	0.03
MCP-1	-168.87 (-265.93, -71.82)	**< 0.001**
MDC	-73.40 (-118.36, -28.44)	**< 0.001**
sCD40L	185.76 (-31.05, 402.57)	0.09
TNFα	0.17 (-5.99, 6.33)	0.97
VEGF-A	-5.87 (-15.85, 4.10)	0.25
EGF	7.00 (-6.10, 20.11)	0.29
Flt-3L	2.19 (-4.45, 8.84)	0.52

Fractalkine	-81.96 (-183.87, 19.95)	0.11
G-CSF	8.68 (-3.03, 20.39)	0.15
IL-12p40	0.04 (-3.69, 3.76)	0.98
IL-13	4.03 (-6.82, 14.89)	0.47
IL-15	-1.00 (-2.59, 0.58)	0.22
IL-17A	-0.73 (-3.37, 1.92)	0.59
IL-1b	-0.93 (-2.40, 0.54)	0.21
IL-2	-0.12 (-1.04, 0.80)	0.80
IL-3	0.07 (-0.40, 0.55)	0.76
IL-4	2.95 (-39.40, 45.29)	0.89
IL-5	-0.50 (-1.55, 0.55)	0.35
IL-7	0.67 (-0.53, 1.87)	0.27
IL-9	0.27 (-3.09, 3.63)	0.88
GM-CSF	-3.58 (-5.73, -1.43)	0.001
GRO	333.11 (-208.97, 875.21)	0.23
IFNα2	-15.50 (-29.22, -1.78)	0.03
MCP-3	4.35 (-6.06, 14.77)	0.42
MIP-1a	0.29 (-3.02, 3.60)	0.86
MIP-1b	-25.85 (-81.03, 29.32)	0.36
PDGF-AA	134.49 (15.95, 253.03)	0.03
PDGF-AB/BB	1186.32 (208.52, 2164.12)	0.02
RANTES	44.32 (-175.10, 263.73)	0.69
TGF-α	0.05 (-0.14, 0.25)	0.59
TNFβ	-3.77 (-15.41, 7.86)	0.53

4.3.4 Viremia and NS1

In 541 patients with virologically confirmed dengue, viremia was highest at day 1-3 of illness, then reduced gradually. However, viremia did not differ significantly between pregnant and non-pregnant women with dengue (mean difference in $\log_{10}$ viremia [95% CI] = -0.28 [-0.50, -0.05], p = 0.02) (Figure 4.15). Due to the adjustment for multiple comparisons (Section 4.2.7.3), the p-value of 0.02 here was not statistically significant. I also found there are several patients with positive NS1 at 6-8 days of illness (Figure 4.16). However, the percentage of NS1 positive samples was not significantly different between the two arms (odds ratio [95% CI] = 1.24 [0.74, 2.07], p = 0.41). Collectively, these results suggest comparable viremia and NS1 antigenemia between pregnant and non-pregnant women with dengue.

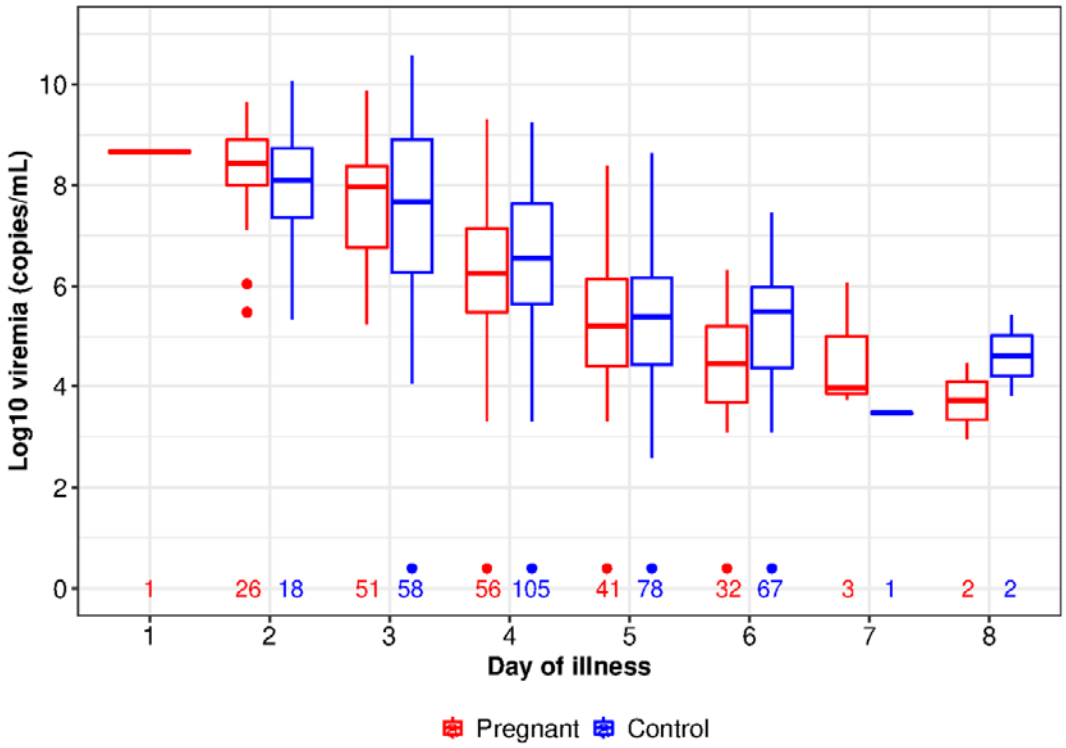


Figure 4.15: Viremia by day of illness in pregnant and non-pregnant dengue patients

Viremia was measured in enrolment samples. The number of pregnant and non-pregnant patients was indicated.

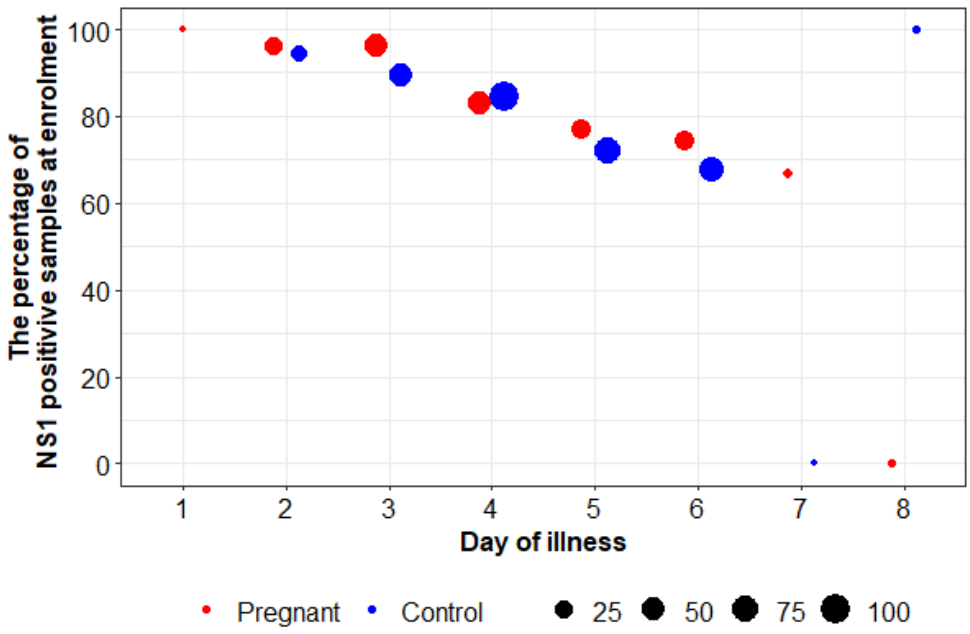


Figure 4.16: The percentage of NS1 positive samples across day of illness in pregnant and non-pregnant dengue patients

NS1 was assessed in enrolment samples. The size of the points shows the number of tested patients.

4.3.5 Immunological and virological factors in cord blood

We collected 112 cord-blood samples from babies born to 112 pregnant women in our studies. All of the 112 cord blood samples were PCR negative for DENV viremia and NS1. A total of 111 out of 112 cord blood samples were positive with DENV-reactive indirect IgG, confirming past exposure to a DENV, and the woman who delivered the baby with negative IgG was in the OFI group. With regard to DENV-reactive IgM, two cord blood samples were positive. These two cord-blood samples were from newborns of mothers who had VCD at 31 and 34 weeks of gestation, and then went on to deliver healthy babies at 40 and 39 weeks of gestation, respectively.

4.4 Discussion

In this study, I found the percentage of CD8+ CD27- CD45RA- T cells, and the concentrations of eotaxin, MCP-1 and MDC in pregnant women with dengue were lower than their non-pregnant counterparts. Although most other immunological and virological aspects of dengue were not measurably different between the two arms, these findings partially confirm the hypothesis that pregnant patients may have a less pronounced inflammatory immune response to dengue.

Here, pregnancy was associated with the lower percentage of CD8+ CD27- CD45RA- T cells. If these cells are shown to express CCR7 on the surface, they would be considered as EM CD8+ T cells, playing effector functions in the adaptive cellular immunity to dengue. A study that phenotyped subsets of CD8+ memory T cells in patients with DENV-2 in Brazil found the prevalent activation of CD8+ EM T cells during the acute period of dengue [197]. When examining DENV-specific CD8+ EM T cells, Weiskopf *et al.* showed their protective role in the blood of Nicaraguan general population who had experienced DENV infections in the past [198]. However, there is no study phenotyping DENV-specific CD8+ EM T cells in dengue-immune individuals. Unfortunately, I was not able to examine CCR7 as well as functional characteristics of the CD8+ CD27- CD45RA- T cells in my study, but in future work it would be interesting to perform functional studies using cytokine-expression markers or cytotoxic degranulation markers such as CD107a. If CD8+ EM T cells are shown to be protective, the lower percentage of CD8+ EM T cell responses may be one factor driving clinical severity of dengue during pregnancy.

While CCR7 was not examined in my study, the lower percentage of CD8+ CD27- CD45RA- T cells in pregnant women with dengue seems to be contradictory to a study in healthy individuals where the percentage of CD8+ EM T cells was similar between pregnant and non-pregnant women [199]. However, the findings here support the view that dengue during pregnancy may be associated with a slower increase in the breadth and magnitude of maternal CD8+ EM T cells, to provide a pathophysiological balance between controlling the infection and tolerating the fetus.

The approach I used to investigate subsets of CD8+ memory T cells is different from previous publications that revolved around transcriptomics [200, 201]. A limitation of transcriptomic approach is that levels of mRNA molecules are not directly proportional to levels of proteins those mRNAs encode. Therefore, lymphocyte phenotyping, which allows examination of cell-surface proteins directly, may be more useful and should be considered for subsequent studies investigating subsets of CD8+ memory T cells in dengue.

I also found that the percentages of CD8+ CD27+ CD45RA+ and CD8+ CD27+ CD45RA- T cells were prevalent among the four subsets of CD8+ memory T cells. If CCR7 was examined in my study, it would be likely that CD8+ CD27+ CD45RA+ cells and CD8+ CD27+ CD45RA- belongs to naïve and CM T cells, respectively. If this is true, my finding may be in contrast to a publication by Tian *et al.*, showing that CD8+ memory T cell

responses were dominated by CD8+ EM and EMRA T cells [200]. Here, I examined dynamics of CD8+ memory T cells during acute dengue while Tian *et al.* tested these cell subsets using blood from healthy donors who had had DENV infections in the past. During acute dengue, it is likely that more CD8+ naïve and CM T cells are generated. These two cell subsets then would give rise into CD8+ EM and EMRA T cells [202], playing a protective role against subsequent DENV infections. Testing this hypothesis, by continuously following participants of my study and phenotyping their CD8+ memory T cells over time, could help to elucidate the protective role for different subsets of CD8+ memory T cells after acute dengue.

In theory, the specificity of CD8+ T cells for DENVs can be investigated using tetramers that mimic HLA-A*11:01-restricted peptides. However, I failed to detect subsets of DENV-specific CD8+ memory T cells. Because 67% of the patients I tested were confirmed to have HLA-A*11:01 (contrary to my hypothesis), the tetramers I used might not been properly folded and thus might not have been able to bind to T cell receptors. Unfortunately therefore, whether DENV-specific CD8+ T cells differed between the pregnant and non-pregnant arms remains unanswered. Further studies should be performed to examine the specificity of CD8+ CD27- CD45RA- T cells and the cross-reactivity of CD8+ CD27- CD45RA- T cells to other DENV serotypes. This is particularly important because most of the pregnant women with dengue had secondary DENV infection (Table 4.3), and we do not know how pregnancy is associated with the dysregulation of DENV-specific CD8+ T cell response to secondary dengue. To this end, tetramers specific to each

DENV serotype could be used to characterize CD8+ CD27- CD45RA- T cell responses to the infecting serotype, and also to serotypes that patients were exposed to previously, as described before [115]. The serotype that patients exposed previously could be identified using PRNTs. Understanding CD8+ CD27- CD45RA- T cell responses to a previous DENV serotype, and to a contemporary DENV serotype, would broaden knowledge of 'original antigenic sin [118]' in pregnant women and help to understand whether this pathogenic mechanism is associated with pregnancy.

Here, I used tetramers which contain HLA-A*11:01-restricted peptides. However, DENV antigens can be restricted by any other HLA-A molecules that are common in Asian peoples such as HLA-A*24 [115]. Therefore, the investigation of DENV-specific CD8+ T cells may require tetramers covering other HLA-A alleles rather than just HLA-A*11:01 to capture a more comprehensive picture of the specificity.

The finding that 67% of tested patients were HLA-A*11:01 is interesting because this number is higher than those from two studies investigating HLA-A in heathy individuals and dengue patients in Vietnam. In the first study, HLA-A was genotyped from 170 healthy unrelated Vietnamese from 20 communes of Ha Noi and the frequency of HLA-A*11:01 was at 22.9% [203]. Another study, based in Children's Hospital No. 2 in Ho Chi Minh city from 2002 to 2005 found the percentages of HLA-A*11:01 were at 38.8% and 49.6% of patients with DSS and DHF, respectively [204]. Discrepancy in the percentage of HLA-A*11:01 may originate from several factors such as the study design, demographics of

studied population, and different methods to characterize HLA-A genotypes. Taken together, my study may highlight the importance of further investigation into HLA-A*11:01 in dengue.

I was unable to detect subsets of another group of adaptive immune cells (B cells), including memory B cells and plasmablasts. Therefore, I cannot comment on whether pregnancy is associated with percentages of those B cells subsets, as has been demonstrated in pregnant women suffering from atopic asthma. Pregnant women with and without atopic asthma presented higher B cell counts, and lower plasmablasts than healthy non-pregnant women [152]. Further studies should be performed to characterize B cell response to dengue during pregnancy.

The percentage of subsets of the remaining lymphocytes (NK cells) was not different between pregnant and non-pregnant dengue patients. The role for NK cells in dengue still remains controversial. On the one hand, the percentage of NK cells has been shown to be higher in DHF compared with DF cases [90, 105]. On the other hands, this positive association was not observed when cytotoxic and cytokine-producing NK cells were examined [106]. Therefore, more studies on NK cells in pregnant patients would clarify their features and roles in the context of dengue.

I found levels of three chemokines (Eotaxin, MCP-1, and MDC) were lower in pregnant women with dengue. Eotaxin is produced by a variety of cells such as epithelial cells,

endothelial cells, macrophages, and T cells to regulate the migration of eosinophils [205]. MCP-1 is also secreted by a variety of cells to recruit monocytes, memory T cells, and NK cells which are important for the immunological response to infection [206]. MDC, typically secreted by macrophages and T cells, bind to receptors on immune cells that play a role in the production of antibodies [207]. Because of the lower levels of the three chemokines, I hypothesize that pregnant dengue patients may have a less pronounced cytokine response. Testing this hypothesis would address whether the attenuated cytokine response is associated with clinical outcomes of dengue during pregnancy.

In phase-3 clinical trials of Dengvaxia, when cytokines were examined in participants who had breakthrough dengue after vaccination, 12 cytokines including eotaxin, MCP-1, and MDC were up-regulated during the acute period of dengue, compared with baseline levels (one month post vaccine dose 3) [86]. There are several other finding about MCP-1 in dengue. Notably, Friberg *et al.* found pre-infection levels of PBMC-derived MCP-1 were higher in individuals who later developed symptomatic dengue than those who later developed subclinical infections [96]. Moreover, in dengue patients, MCP-1 was lower in those without warning signs than in those with warning signs [97]. Taken together, these studies suggest a role for the MCP-1 response in dengue; my research also highlights the potential relevance of MCP-1 to dengue during pregnancy.

Although I found lower concentrations of pro-inflammatory eotaxin, MCP-1, and MDC in pregnant patients, those of anti-inflammatory cytokines (IL-10, IL-1RA) did not differ

between the two groups. This is in contrast to higher concentrations of anti-inflammatory cytokines such as IL-10, CD163 observed in pregnant women with Zika [159]. Variations in the exerimental designs, demographics of participants, and pathogenesis of ZIKV and DENVs may explain this heterogeneity.

Eotaxin, MCP-1, and MDC - three chemokines that were lower in pregnant dengue patients - are also thought to favor a type 2 response [205-207] which relates to an over-expression of T-helper 2 (Th2) cytokines such as IL-4, IL-8, IL-10. However, the levels of these cytokines did not differ between the two pregnancy arms. Similarly, T-helper 1 (Th1) cytokines (such as IFNγ, IL-2, IL-15) were not statistically different. Collectively, my study suggests that pregnancy may not link to a systemic and extensive changes in cytokine profile during dengue.

Combining the results for eotaxin, MCP-1, and MDC with data for the eight cytokines that had p-values ≤ 0.05, but not below 0.002, our predefined level of significance (GM-CSF, IFNα2, IFNγ, IL-1a, IL-8, IP-10, PDGF-AA, PDGF-AB/BB), I found both similarities and contrasts with the findings from Kraus *et al.*, who compared cytokines between pregnant and non-pregnant healthy women [153]. Both of the studies found pregnant subjects had lower levels of IP-10, and MCP-1. In contrast, while eotaxin, GM-CSF, IFNγ were down-regulated in my group of pregnant patients with dengue, these cytokines were up-regulated in healthy pregnant women. Pregnancy itself is linked to dysregulated cytokine responses

in the women, and my study implies another layer of modifications may occur in cytokine responses to dengue during pregnancy.

It is likely that the cytokine differences observed in this Chapter were not measurably relevant to the severity of dengue, but the number of severe dengue cases was small. In the cytokine population, 3/91 (3.30%) and 10/182 (5.49%) pregnant and non-pregnant patients had severe dengue (Table 4.3). Indeed, the severity of dengue is influenced by a variety of factors, particularly case management, so cytokine changes alone were not associated with this critical endpoint.

Among the 41 cytokines investigated, 33 cytokines had data for more than 50% of patients. This is in contrast to the Dengvaxia phase-3 trial where only 15 out of the 38 cytokines tested were detectable in the acute period of dengue (36 out of 38 cytokines were included in the 41-cytokine panel here) [86]. Although both of the studies examined cytokines in the acute period of dengue, and used the same technique to measure cytokines, variations in sampling time, limits of detection, the laboratories that performed the experiments and the technical procedures used may have contributed to these differences in the numbers of detectable cytokines. Furthermore, I measured cytokines only at enrolment, and not do so at discharge, so kinetics of cytokines during the acute dengue were unknown.

Cytokines are integral components of immune responses. Here, I compared not only immunological but also virological factors between pregnant and non-pregnant women

with dengue. I found pregnancy was not associated with viremia or the proportion of patients with NS1 antigenemia at the time of enrolment. The dynamics of viremia and NS1 were similar to other studies [29, 181] where viremia declined to become undetectable towards the end of the first week of illness. In contrast, NS1 persisted in patients' blood for longer, possibly due to slower clearance of the non-structural protein as opposed to viral RNAs. Because NS1 was detectable in a large number of VCD cases at enrolment, further NS1 experiments could be carried out using blood samples obtained at discharge to compare the clearance rate of NS1 between the two groups. A hypothesis is that the clearance rate of NS1 would be slower in pregnant than non-pregnant patients.

Previous studies have shown that viremia and NS1 are detectable in cord blood when women are infected with dengue late in their pregnancy [36, 208]. The lack of DENV viremia and NS1 detectable in the cord blood samples tested, which is in keeping with the timing of the maternal dengue. In the VCD population, delivery occurred at a median (IQR) of 17 (10, 25) weeks after maternal dengue. This suggests newborns did not experience DENV infection near the time before delivery. Previous studies that identified viremia and NS1 in cord blood involved women who had experienced dengue in late pregnancy [36, 208], so in this study, DENVs and their proteins were probably cleared if *in utero* DENV infection occurred.

DENV-reactive IgG was detectable in 111/112 cord blood samples, which is consistent with the finding of effective transfer of this antibody class from pregnant patients with

ZIKV to their fetuses [209]. In contrast, I detected DENV-reactive IgM in only 2/112 cord blood samples. This finding is similar to the result from a large prospective cohort in which DENV-specific IgM was detected in cord blood in 63/2,531 (2.5%) cases [210]. In addition to the possibility of false positive IgM results [211], one explanation for detecting DENV-reactive IgM in cord blood is that maternal IgM may cross the placental barrier. However, it should be noted that IgM is not generally transported across the placental barrier due to the large size of this immunoglobulin [212]. Therefore, another explanation relates to establishment of an immune response of the fetus that might produce IgM against DENVs, which would then be present in the cord blood. The detection of DENV-reactive IgM in cord blood was probably an evidence suggesting fetal response to DENV infection *in utero*.

It is important to acknowledge one limitation of the way women were enrolled to the study. Pregnant patients were generally less severe than non-pregnant women at study enrolment, demonstrated by the lower percentage of pregnant women with at least one warning sign at that time (Table 4.3). Pregnant patients are more likely to seek medical care if they experience intercurrent problems, due to concerns regarding fetal health. Medical doctors are also more likely to hospitalize pregnant patients for close monitoring of obstetric complications, even if they do not display warning signs. Thus the bias in the severity of dengue at enrolment should be taken into account when considering the findings of my study. Although I adjusted all of my comparisons for the presence of warning signs, the enrolment bias in the severity of dengue cannot be neglected. Further cohort studies that enroll and follow all pregnant women, and monitor their infections or diseases during

pregnancy, could help reduce this bias; however capturing the non-pregnant population with illness of similar severity could be difficult since many of these women might attend for healthcare at lower level facilities and are less likely to be admitted and/or visible to research staff.

Another limitation of the study is that I was unable to stratify my findings for CD8+ CD27- CD45RA- T cells, eotaxin, MCP-1, and MDC by gestational stage due to the small number of pregnant dengue patients. Because maternal immunity appears to be driven by gestational stages [147], these immune components may show different responses at different stages; it is possible that some parameters may be elevated if pregnant women have dengue in the first gestational stage, then may be suppressed if the disease occurs in the second gestational stage, and may return to an elevation upon delivery. However, to address this hypothesis, based on the means and standard deviations observed for MCP-1 in this study, I estimate a total of approximately one million pregnant women with VCD would be required. Therefore, it is not feasible to enroll such a high number of patients.

Despite these limitations, the statistical analyses for this Chapter were based on *a priori* plan. In the literature, to my knowledge, previous studies have not described *a priori* analysis plans, suggesting many were data driven. Here, I explored and compared immunological and virological factors, taking into considerations the issue of multiple-testing. The lower percentage of CD8+ CD27- CD45RA- T cells, and levels of eotaxin,

MCP-1, MDC may suggest subtle differences exist in the immune response to DENVs in pregnant versus non-pregnant women.

4.5 Conclusions

Three major findings of this Chapter are:

1. The percentage of CD8+ CD27- CD45RA- T cells in pregnant women with dengue was lower than among non-pregnant patients with the disease, suggesting pregnant women with dengue have attenuated CD8+ effector memory T cell responses compared with non-pregnant patients.
2. The levels of eotaxin, MCP-1, and MDC in pregnant women with dengue were also lower, which may demonstrate subtle decrease in the cytokine response in pregnant patients compared with non-pregnant patients.
3. DENV-reactive IgM were detectable in some cord blood samples, implying a fetal response to DENV infection *in utero*.

These findings do contribute to the understanding of immunological and virological factors of dengue during pregnancy. Further studies are necessary to investigate these factors more comprehensively and to avoid the bias toward lower severity of dengue in pregnant patients upon enrolments.

Chapter 5

Discussion and future directions

I investigated the pathogenesis of dengue in Vietnam with a focus on immunological and virological aspects of the disease. Chapter 2 revolves around the development of a ViBNA, and the use of this assay to assess the potency of a panel of mAbs, which were previously characterized as virus neutralizing antibodies in cell-culture assays. Chapter 3 further investigated antiviral profiles of a subset of these mAbs that had been classified as neutralizing using the ViBNA. The main importance of this work were to support the development of alternative therapeutic approaches for treatment of dengue and to inform the development of vaccines that can elicit desirable virus-neutralizing antibody responses.

Not only antibodies but also other components of the immune response were investigated in my thesis. In Chapter 4, humoral and cellular immune components, along with virological factors, were compared between pregnant and non-pregnant women with dengue. The backdrop and partial motivation for this work was the global emergence of the Zika virus in 2016 and its impact on pregnant women and their newborns. That the Zika virus could cause severe congenital infections prompted several research questions about potential similar issues that have been poorly investigated in the context of dengue - a) Might dengue be associated with complications for expectant mothers and their newborns? b) Does pregnancy alter the immune response to dengue? And c) Does pregnancy alter the virological features of dengue?

5.1 Human mAbs against DENVs

I led the development of the ViBNA and, using this assay, I re-characterized a panel of 12 human-derived mAbs. The results show that five antibodies were unable to neutralize DENVs at all, two mAbs moderately neutralized the virus, while seven inhibited DENV infection of mosquitoes. Each category of tested mAbs is discussed below.

Five mAbs, (1C19, 1M7, 22.3, 82. 11, 87. 1, 1M7), failed to neutralize any serotypes of DENV in the ViBNA. Differences between components used in the cell-culture assays and the ViBNA may be one explanation for the change in the neutralization capacity of the five mAbs [136, 140]. Cell-culture systems use cultured DENVs, usually maintained for many passages *in vitro*, potentially allowing for some degree of virus adaptation to the cultured cell type and temperature conditions. This concept is supported by a recent publication by Raut *et al.*, showing that *in vitro* cultured DENV-1 was less mature and more easily neutralized by antibodies than DENV virions in human plasma [134]. Further studies to investigate variations in the characteristics of virions as they exist *in vitro* in cultured cells, compared to *in vivo* in infected patients, are important for the design of dengue vaccines. In particular, it is critical to ensure that dengue vaccines can elicit long-lived antibodies and B memory responses that can neutralize all four DENV serotypes.

Two mAbs, 1F4 and 5J7, were grouped as moderately neutralizing antibodies in my study. The neutralization capacity of DENV-1 specific 1F4 was lower when the target virus had a N52D substitution in the E protein, as revealed by the sequencing of the tested DENV-1

strains. A possible explanation for this observation is that the affinity and avidity of 1F4 against its epitope on DENV-1 may be reduced when amino acid N at residue 52 of the E protein is substituted by D. To address this, cryo-electronic microscopy could be used to examine the crystal structure of the interaction interface formed by 1F4 and the two variants of DENV-1. Cultured DENV-1 strains with N52 and D52 could be isolated from patients with dengue, (or produced synthetically [213]), and then incubated with 1F4 so that the interaction interfaces could be compared between the two strains. Results of these studies might demonstrate the influence of viral amino acid substitutions on the antiviral profiles of these antibodies, as observed previously in relation to DENVs [189], and also in hepatitis C infection [214].

It is widely accepted that natural infection with one DENV serotype provides life-long clinical immunity to that serotype, suggesting that single viral amino acid substitutions do not drive virus escape from the immune response. However, the immune response to natural DENV infections may differ from the response induced by vaccination. If circulating DENVs evolve by acquiring mutations that lead to changes in viral amino acid residues, and if these changes occur at the binding site of vaccine-elicited neutralizing antibodies, then steric interference may alter antibody binding and potentially reduce the antibody-mediated efficacy of the vaccine. To overcome this problem, variations in the molecular epidemiology of circulating DENVs, especially at the binding sites of vaccine-elicited neutralizing antibodies, should be investigated extensively and frequently so that new mutations that may undermine vaccine efficacy can be detected early, similar to the

approach used for influenza. This concept could be applied to further studies assessing the long-term efficacy of Dengvaxia, to investigate whether DENVs evolve to escape from being neutralized by vaccine-elicited antibodies.

Besides non-neutralizing and moderately neutralizing mAbs, I found five strongly neutralizing mAbs (14c10, 2D22, 1L12, 747(4)B7, and 753(3)C10). Because these five mAbs neutralized DENVs in both the ViBNA and cell-culture systems [82, 139, 142], they have potential to be used therapeutically as antivirals for dengue. To this end, I defined the EC_{50} for three of the neutralizing mAbs (there were insufficient data to evaluate EC_{50} values for the two remaining mAbs effectively). The calculated EC_{50} values ranged from 0.013 μg/mL to 1.289 μg/mL, suggesting that mAbs are able to neutralize DENVs at low microgram per mL concentrations in the ViBNA. This information might be valuable to help determine dosing strategies for therapeutic mAbs, if intervention trials are planned to evaluate their efficacy and safety in humans. To this end, three main considerations will need to be addressed. The first is that viremia at the time of antibody administration should be high enough for DENV-reactive mAbs to have a measurable effect. This represents a challenge because viremia is usually low by the time dengue patients seek medical care. Second, since the cost of producing mAbs is extremely high (as described in the Discussion of Chapter 3), these products should be reserved for administration to patients who have a high risk of developing severe dengue. Unfortunately, since a reliable point-of-care algorithm to predict severe dengue is not available as yet, developing a cost-effective strategy for deployment of therapeutic mAbs that aim to reduce viremia and mitigate the

risk of developing severe dengue remains challenging. Finally, although several parameters (such as maximum % hemoconcentration, severity of bleeding, highest hepatic transaminase levels, fever clearance time, time to resolution of viremia) have been identified as relevant clinical and virological endpoints for clinical trials of dengue therapeutics [215], these events are relatively uncommon and thus a large sample size is needed to achieve robust results. Additionally, severe endpoints occur more frequently in children, further complicating trial design.

A therapeutic mAb antiviral should have the crystallization fragment (Fc) disengaged to eliminate Fc-mediated pathogenic pathways in dengue. Many approaches enable the disengagement of Fc. One of them relates to the use of an intact Fc with low affinity to bind to the receptor [216]. This low affinity relies on binding of the antibody binding fragment (Fab) at a flat angle (lying parallel to the virus surface) that hinders access to the Fc receptors [217]. Another approach is the use of LALA variants in which the function of Fc is disabled by two point mutations. However, there is a concern that the potency of such mAbs might be reduced by Fc disengagement. Contrary to this concern, I found the potency of an Fc-disabled therapeutic candidate (14c10-LALA) was similar to its wildtype, 14c10, implying the 14c10-mediated neutralization was independent of Fc engagement. This is in contrast to a study in which the engagement of Fc was found to increase the antiviral potency of mAbs against Ebola virus [218]. One explanation for the disparity between these two studies is that I examined the activity of Fc in the context of viremic blood samples. Although viremic blood consists of a variety of immune cells, this fluid

compartment may not be the main reservoir of FcR-bearing immune cells. Indeed, these FcR-bearing immune cells are largely present in primary and secondary lymphoid organs such as bone marrow, spleen, and lymph nodes. Therefore, it would be worth investigating the engagement of Fc in these lymphoid organs in animal models. Animal models do not mimic the symptoms of dengue as in humans, but such studies may allow extensive assessment of the effects of Fc engagement in controlling DENV infections.

Besides the neutralization of 14c10-LALA, I also investigated another functional antiviral property of 14c10 when I observed reduced potency of the mAb against DENV-1, produced by mosquito cells and secreted into the saliva of mosquitoes. The continuum of DENV maturation states [190] may explain this observation. 14c10 binds to quaternary epitopes on mature DENVs. If the maturation state of DENV-1 in the saliva of mosquitoes is lower than in viremic blood, the number of epitopes of 14c10 may be lower in saliva of mosquitoes. Therefore, one hypothesis is that 14c10 may bind with lower occupancy to a DENV-1 virion in saliva of mosquitoes than to a virion in viremic blood. Testing this hypothesis using electronic microscopy might improve our understanding about how the continuum of maturation states, as well as the structure of epitopes, drives the antiviral profile of neutralizing mAbs. This is particularly interesting for studies on cross-reactivity between DENVs and ZIKV. Because the two flaviviruses share some similarity in viral amino acid sequences [219], it is possible that DENV-neutralizing mAbs also bind to ZIKV. Interestingly, Zimmerman *et al.* found DENV-neutralizing mAbs bound to but did not neutralize ZIKVs *in vitro* [83, 220, 221], highlighting the involvement of other factors

such as the occupancy of antigen-antibody interactions, in the antiviral profiles of these cross-reactive mAbs.

When I collated results from the ViBNA with those from previously published structural studies of tested mAbs and their epitopes [82, 138, 139, 142, 222], I found that six out of the seven mAbs (5 neutralizing and 2 moderately-neutralizing) bind to quaternary epitopes on E proteins. The importance of quaternary epitopes as binding sites for neutralizing antibodies has been highlighted not only in DENVs, but also in ZIKV [223, 224] and human-immunodeficiency-virus infection [225] when most quaternary-epitope-binding mAbs neutralized these viruses effectively. Other quaternary-epitope-binding mAbs that neutralize the infectivity of DENVs in cell-culture systems [226, 227] should be tested for their antiviral potency using the ViBNA. Although antibodies produced by different people in response to infection vary, the ultimate aim of a dengue vaccine is to elicit potently neutralizing antibodies such as those that bind to quaternary epitopes. Vaccine-elicited neutralizing antibodies are conventionally measured by cell-based assays, such as PRNTs. Results of these assays are considered as the primary correlate of protective immunity in dengue [130, 133]. However, whether cell-based assays adequately measure quaternary-epitope-binding antibodies remains unknown.

I found discrepancies in characterizing neutralizing mAbs between these cell-based assays and the ViBNA. The cell-based assay (PRNT) also failed to predict vaccine efficacy in clinical trials of Dengvaxia [53, 54]. In particular, while vaccine-induced immunogenicity

was high, vaccine efficacy was low, particularly for DENV-2 [52]. All of these findings suggest that cell-based assays may fail to discriminate between antibodies that can successfully neutralize virus *in vivo* and those that cannot. Therefore, results from cell-based assays may not perform well as correlates of protective immunity *in vivo*. Such a hypothesis could be investigated directly by comparing the performance of the ViBNA and PRNTs side-by-side, using plasma from participants in the clinical trials of Dengvaxia to estimate the vaccine-elicited immunogenicity. Although the two assays assess different antibody responses, vaccine-elicited immunogenicity can be estimated by the two assays. Should it be possible to access these plasma samples, my expectation is that the vaccine-elicited immunogenicity assessed using the ViBNA would be lower than when assessed using PRNTs. This would suggest the suboptimal value of the PRNT, and indicate the need for improved assays that more accurately measure immunological correlates of protection from dengue. Having a more robust and reliable neutralization assay would directly support the assessment of vaccine-elicited immunogenicity, and thus contribute to the development of effective dengue vaccines.

Although vaccine-induced immunogenicity could be compared between the ViBNA and the PRNT, there are two main reasons why the ViBNA is not suited to be an alternative assay to the PRNT for dengue vaccine trials. The first relates to the potential for residual infectivity of DENVs that is undetectable using the ViBNA; I detected residual infectivity of DENV-1 after testing against a high concentration of 14c10 (10 μg/mL). To my knowledge, there is no evidence for the possibility of residual DENVs, undetected by cell-

based neutralization assays. Secondly, in the ViBNA, access to each serotype is driven by the serotype prevalence among patients attending the research center during the period of investigation, i.e. the studied patient population. Thus, a considerable amount of time might be required to obtain complete data about the four DENV serotypes if they are not circulating at equal frequency. These two reasons may undermine the use of the ViBNA as a substitute for the PRNT to measure correlates of protective immunity in dengue.

However, my ViBNA results do indicate the type of DENV-reactive neutralizing antibodies that a second-generation DENV neutralization assay should aim to measure. They are 14c10, 2D22, 747(4)B7, 753(3)C10 - i.e. quaternary-epitope-binding and potently-neutralizing mAbs. These mAbs, after being labelled, could be measured in a competitive ELISA. Specifically, vaccine-elicited or naturally-acquired antibodies could compete with these mAbs for quaternary epitopes. An increase in the number of labelled unbound mAbs would indicate an increase in the concentration of competing antibodies, either elicited by vaccination or by natural DENV infections. Such a competition ELISA could also be used to examine whether individuals have been exposed to DENVs before Dengvaxia vaccination, to improve the efficacy of the vaccine [228].

Two primary considerations should be taken into account when developing such a competition ELISA. First, the conditions for optimal interactions between antigens and antibodies should be investigated. Antibodies are often incubated with antigens under standard conditions (37°C for 30 minutes). However, the binding affinity between DENV

antigens and mAbs may increase when they are incubated at 40°C compared with 37°C [229]. This is of pathophysiological relevance because the body temperature of patients with dengue often exceeds 37°C. Therefore, further studies may be necessary to identify the optimal conditions for incubating antibodies and DENV antigens. Secondly, in areas with other flavivirus circulation, the cross-reactivity of antibodies, as measured by the competition ELISA, should be investigated carefully. Although DENVs are antigenically-distinct, they share high protein sequence homology with other flaviviruses such as ZIKV, JEV, YFV, and tick-borne encephalitis virus [230]. As such, antibodies generated by other flavivirus infections or vaccinations may be measured by the competition ELISA and wrongly characterized as neutralizing antibodies against DENVs [231]. Although a competition ELISA is expected to be epitope-specific, to what extent measured antibodies cross-react among flaviviruses is unknown.

In summary, Chapter 2 highlighted a discrepancy in the antiviral profiles of a panel of mAbs when assessed in the ViBNA compared to cell-culture systems. The difference in maturation state of DENVs used in the two assays may be an explanation for this observation. Chapter 3 elaborated several anti-viral properties of mAbs that neutralized DENVs effectively in both the ViBNA and cell-culture systems. Notably, I provided EC_{50} values that might inform the dosing strategy of therapeutic mAbs for dengue. Other antiviral properties of these neutralizing mAbs, such as quaternary-epitope binding, should also be considered in the design of future dengue vaccines. Finally, my experiments indicate the importance of having a more robust assay, such as a competition ELISA, to

measure DENV-neutralizing antibodies that may play a role as one correlate of protective immunity for dengue studies. An appropriate correlate of protective immunity to dengue may rely not only on neutralizing antibodies but also on other components of the immune system such as NK cells, and CD8+ T cells [130].

5.2 Immunological and virological characteristics of dengue during pregnancy

In my comparisons of immunological and virological factors between pregnant and non-pregnant female dengue patients, I found the percentage of CD8+ CD27- CD45RA- T cells, and the concentrations of eotaxin, MCP-1, MDC were lower in pregnant patients. Although viremia and NS1 antigenemia were not measurably different between the two groups, my findings suggests pregnant women may have a less pronounced inflammatory response to dengue, and highlight the need for further studies into immunological and virological features of dengue during pregnancy.

Because CD8+ EM T cells play effector functions in the adaptive cellular immune response to dengue, they are thought to maintain a balance between tolerating and rejecting the half-allogenic fetus [202]. While CCR7 was not examined in this study, the lower percentage of CD8+ CD27- CD45RA- T cells in pregnant patients highlights the need for further studies to investigate the impact of changes in CD8+ EM T cells on obstetric outcomes, by comparing between pregnant women with dengue and those without the disease. The lower percentage of CD8+ CD27- CD45RA- T cells also leads to questions around how T cell subsets manifest when pregnant women acquire other flaviviral diseases, such as Zika virus

infection. Answers to this question could help elucidate the immunopathology associated with ZIKV, and potentially help to explain the occurrence of microcephaly among babies born to some mothers who have Zika virus infection during pregnancy.

Both ZIKV and DENV belong to the flavivirus genus, so the specificity and cross-reactivity of detected CD8+ T cells should be investigated, given the possible implications for subsequent flaviviral infections for dengue patients who live in areas where DENVs and ZIKV are co-circulating. In mouse models, pre-existing CD8+ memory T cells to DENVs protected mice from ZIKV infection [232]. However, whether DENV-specific CD8+ memory T cells confer protective immunity against subsequent ZIKV infections in humans is poorly understood. Understanding this aspect of flavivirus pathogenesis could lead to the incorporation of cross-reactive CD8+ T cells as targets of pan-flavivirus vaccines [230].

CD8+ memory T cells are important for both pathogenic and protective roles in secondary DENV infections, while CD4+ memory T cells function in protecting against DENV infections in humans [109]. This protection is probably achieved by CD4+ EMRA T cells [233] that are suppressed during normal pregnancy [199]. Unfortunately, CD4+ T cells were not assessed in my study, and further attempts to examine subsets of CD4+ memory T cells in pregnant women with dengue might be necessary.

NK cells are another group of lymphocytes that I investigated and found no difference in the responses to dengue between the pregnant and non-pregnant participants. However, I was not able to examine other innate cellular components such as macrophages or monocytes due to technical constraints. Thus, whether differentiation of monocytes is skewed toward suppressive macrophages in the context of dengue during pregnancy (as has been demonstrated with Zika virus infection in pregnancy [234]) remains unknown. Further studies using more sophisticated flow cytometry, for example also including antibody markers for monocytes and macrophages (CD14, CD16), could provide a more complete picture of the innate cellular immune responses of pregnant women with dengue.

Both cellular and humoral immunity are involved in the inflammatory pathways against dengue. Cytokines, a major humoral component of the immune response, function in communicating between immune cells, and my study shows that eotaxin, MCP-1 and MDC levels in pregnant women was lower than in non-pregnant patients. The lower concentrations of these pro-inflammatory in pregnant patients suggest a less pronounced inflammatory response to dengue during pregnancy. Further studies on how pregnancy influences cytokine responses to other flaviviral diseases during pregnancy would allow comparisons and contrasts to those occurring with pregnant dengue patients.

Combining my findings regarding the lower concentrations of MCP-1, MDC and the lower percentage of CD8+ CD27- CD45RA- T cells in pregnant patients, another hypothesis is that plasma CD8+ CD27- CD45RA- T cells may present receptors of MCP-1 and MDC,

and thus migrate to other tissues at a higher rate in pregnant patients. CD8+ T cells can express chemokine receptors such as skin-homing markers [235], to allow their migration to other tissues. However, reasons for the increased migration of these T cells in pregnant patients are not understood.

Plausibly, the less pronounced inflammatory response in the pregnant women with dengue might be associated with higher acute viremia and NS1 antigenemia. However, contrary to this hypothesis, I found both viremia and the percentage of NS1-positive patients at enrolment did not differ between pregnant and non-pregnant women with dengue, after adjusting for a variety of baseline confounding factors. However, I did not test these virological factors in other physiological compartments, such as the spleen and lymph nodes, where DENVs transiently persist even after viremia resolves in animal models [236]. Future studies may be carried out in pregnant-animal models to examine virological and immunological factors in these lymphoid tissues. Although there is no appropriate animal model for dengue, these studies might contribute to the knowledge about characteristics of DENV-intrinsic factors during pregnancy.

To my knowledge this is the first study that compares immune responses and virus-intrinsic factors between pregnant and non-pregnant women with dengue, and provides some insight into the influence of pregnancy on dengue pathogenesis. This research was motivated, in part, by the global re-emergence of Zika. The impact of pregnancy on Zika pathogenesis remains poorly understood with the literature revolving mainly around how

ZIKV infection influences the immunity of pregnant women [160, 234, 237]. Since no studies primarily compare immunological and virological factors between pregnant and non-pregnant women with Zika virus infection, it is not possible to compare my findings with those of Zika during pregnancy. Zika virus can cause microcephaly and other fetal neurological complications while dengue during pregnancy has been reported to be associated with both maternal and fetal complications such as miscarriage, preterm birth, low birth weight [38, 39], stillbirth [40, 41], and maternal death [37]. These findings highlight the need for further studies into the pathogenesis of both dengue and Zika during pregnancy.

What I can compare about the effects of DENV and ZIKV infections during pregnancy is the detection of immunological and virological factors in the cord blood of the babies born to women experiencing these infections. In my study, DENV RNA and NS1 antigen were not detectable in any of the 112 cord blood samples, while DENV-reactive IgM was detected in only 2/112 specimens. Although this finding could reflect a leaky maternal-fetal barrier, the detection of DENV-reactive IgM in cord blood suggests a fetal response to DENV infection may sometimes occur *in* utero. In any case, the maternal-fetal transmission rate of DENVs appears to be substantially lower compared with ZIKV. In one prospective cohort enrolling pregnant women with ZIKV infections at any pregnancy stage, the maternal-fetal transmission rate was calculated at 26% (76/291 babies), using RT-PCR and IgM ELISA [238]. One explanation for this variation in transmission rates between the two flaviviruses is that while interactions between DENVs and placental cells

are unknown, ZIKV is known to target a variety of human placental cells such as cytotrophoblasts, endothelial cells, Hofbauer cells, and amniotic epithelial cells. The high susceptibility of placental cells to ZIKV suggests an effective route for maternal-fetal transmission, and this helps explain the congenital anomalies that are known to be associated with Zika virus infection during pregnancy.

5.3 Concluding comments

Five major results of this thesis are as follows:

1. Five mAbs were characterized as non-neutralizing antibodies using the ViBNA, in contrast to the results obtained using cell-culture systems, suggesting the need for another gold-standard DENV neutralization assay.
2. Seven mAbs were characterized as neutralizing antibodies, highlighting species of "transmission-blocking" antibodies that dengue vaccines should aim to elicit.
3. The calculated EC50 values for three of the neutralizing mAbs ranged from 0.013 μg/mL to 1.289 μg/mL. These data have implications for the dosing strategy of mAbs if used as therapeutic antiviral agents.
4. Pregnant women with dengue had lower percentages of CD8+ CD27- CD45RA- T cells, and lower concentrations of eotaxin, MCP-1, and MDC than non-pregnant patients, implying a less pronounced inflammatory immune response to dengue compared with non-pregnant patients.

5. I found no virological evidence for DENV infection in infants, born to women who experienced dengue during pregnancy, in contrast to the high maternal-fetal transmission rate that is known to occur in association with ZIKV infections.

These results deepen our general knowledge regarding dengue pathogenesis. Moving forward, additional questions that would be interesting to address through further research are suggested below:

1. How does the maturation state of DENVs influence the binding of antibodies?
2. What are the structural characteristics of virions propagated by cultured cells *in vitro*, compared with those propagated in infected patients?
3. What is the influence of Fc engagement in lymphoid organs in animal models?
4. To what extent do maturation states differ between DENVs in viremic blood and those in the saliva of mosquitoes?
5. Are CD4+ T cells, monocytes, macrophages, etc. dys-regulated in the context of dengue during pregnancy?
6. Is there an increase in the migration of plasma CD8+ CD27- CD45RA- T cells that present receptors of MCP-1 and MDC to tissues?
7. What are the kinetics of viremia and NS1 antigenemia in various lymphoid tissues of pregnant DENV-infected animal models?

Answering these questions will advance the development of dengue vaccines and therapeutics as well as the understanding of pathogenesis of dengue during pregnancy.

REFERENCES

1. Bhatt, S., et al., *The global distribution and burden of dengue.* Nature, 2013. **496**(7446): p. 504-507.
2. Clapham, H.E., D.A.T. Cummings, and M.A. Johansson, *Immune status alters the probability of apparent illness due to dengue virus infection: Evidence from a pooled analysis across multiple cohort and cluster studies.* Plos Neglected Tropical Diseases, 2017. **11**(9).
3. Bich, T.D., et al., *A pregnant woman with acute cardiorespiratory failure: dengue myocarditis.* Lancet, 2015. **385**(9974): p. 1260-1260.
4. Kusuma, N.A. and A.A.N.J. Kusuma, *Dengue hemorrhagic fever in pregnancy with placenta abruption and vertical transmission: a case report.* Bali Medical Journal, 2017. **6**(3): p. S100-S104.
5. Gubler, D.J., et al., *Dengue and Dengue Hemorrhagic Fever, 2nd Edition*. 2014: CABI.
6. Shepard, D.S., et al., *The global economic burden of dengue: a systematic analysis.* Lancet Infectious Diseases, 2016. **16**(8): p. 935-941.
7. WHO, *Dengue and severe dengue: Fact sheet*. 2017.
8. Brady, O.J., et al., *Refining the Global Spatial Limits of Dengue Virus Transmission by Evidence-Based Consensus.* Plos Neglected Tropical Diseases, 2012. **6**(8).
9. Stanaway, J.D., et al., *The global burden of dengue: an analysis from the Global Burden of Disease Study 2013.* Lancet Infectious Diseases, 2016. **16**(6): p. 712-723.

10. Kraemer, M.U.G., et al., *The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus.* Elife, 2015. **4**.

11. Lourenco, J. and M. Recker, *The 2012 Madeira Dengue Outbreak: Epidemiological Determinants and Future Epidemic Potential.* Plos Neglected Tropical Diseases, 2014. **8**(8).

12. Franco, L., et al., *Molecular epidemiology suggests Venezuela as the origin of the dengue outbreak in Madeira, Portugal in 2012-2013.* Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 2015. **21**(7): p. 713.e5-8.

13. Neumayr, A., et al., *Sentinel surveillance of imported dengue via travellers to Europe 2012 to 2014: TropNet data from the DengueTools Research Initiative.* Eurosurveillance, 2017. **22**(1): p. 39-47.

14. Cuong Hoang, Q., et al., *Synchrony of Dengue Incidence in Ho Chi Minh City and Bangkok.* Plos Neglected Tropical Diseases, 2016. **10**(12).

15. Guo, C., et al., *Global Epidemiology of Dengue Outbreaks in 1990-2015: A Systematic Review and Meta-Analysis.* Frontiers in Cellular and Infection Microbiology, 2017. **7**.

16. Region, W.W.P., *Dengue situation update number 576*, WHO, Editor. 2019.

17. Hung, T.M., et al., *The Estimates of the Health and Economic Burden of Dengue in Vietnam.* Trends Parasitol, 2018. **34**(10): p. 904-918.

18. WHO, *Dengue: guidelines for diagnosis, treatment, prevention and control*. 2009.

19. Simmons, C.P., et al., *Dengue.* New England Journal of Medicine, 2012. **366**(15): p. 1423-1432.

20. Gubler, D.J., *Dengue and dengue hemorrhagic fever.* Clinical Microbiology Reviews, 1998. **11**(3): p. 480-496.

21. Wilder-Smith, A., et al., *Dengue.* Lancet, 2019. **393**(10169): p. 350-363.

22. Hadinegoro, R.S., *The revised WHO dengue case classification: does the system need to be modified?* Paediatrics and International Child Health, 2012. **32**: p. 33-38.

23. Kien, D.T.H., et al., *Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma.* Journal of Virological Methods, 2011. **177**(2): p. 168-173.

24. Hang, V.T., et al., *Diagnostic Accuracy of NS1 ELISA and Lateral Flow Rapid Tests for Dengue Sensitivity, Specificity and Relationship to Viraemia and Antibody Responses.* Plos Neglected Tropical Diseases, 2009. **3**(1).

25. Mansfield, K.L., et al., *Flavivirus-induced antibody cross-reactivity.* Journal of General Virology, 2011. **92**: p. 2821-2829.

26. Lam, P.K., et al., *Clinical Characteristics of Dengue Shock Syndrome in Vietnamese Children: A 10-Year Prospective Study in a Single Hospital.* Clinical Infectious Diseases, 2013. **57**(11): p. 1577-1586.

27. Whitehorn, J., et al., *Dengue Therapeutics, Chemoprophylaxis, and Allied Tools: State of the Art and Future Directions.* Plos Neglected Tropical Diseases, 2014. **8**(8).

28. Vianney, T., et al., *A Randomized Controlled Trial of Chloroquine for the Treatment of Dengue in Vietnamese Adults.* Plos Neglected Tropical Diseases, 2010. **4**(8).

29. Nguyen, N.M., et al., *A Randomized, Double-Blind Placebo Controlled Trial of Balapiravir, a Polymerase Inhibitor, in Adult Dengue Patients.* Journal of Infectious Diseases, 2013. **207**(9): p. 1442-1450.

30. Whitehorn, J., et al., *Lovastatin for the Treatment of Adult Patients With Dengue: A Randomized, Double-Blind, Placebo-Controlled Trial.* Clinical Infectious Diseases, 2016. **62**(4): p. 468-476.

31. Low, J.G., et al., *Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): a phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial.* Lancet Infectious Diseases, 2014. **14**(8): p. 706-715.

32. Tam, D.T.H., et al., *Effects of Short-Course Oral Corticosteroid Therapy in Early Dengue Infection in Vietnamese Patients: A Randomized, Placebo-Controlled Trial.* Clinical Infectious Diseases, 2012. **55**(9): p. 1216-1224.

33. Nguyen, T.H.T., et al., *Corticosteroids for Dengue - Why Don't They Work?* Plos Neglected Tropical Diseases, 2013. **7**(12).

34. Marques, V.D., et al., *Neurological Complications of Congenital Zika Virus Infection.* Pediatric Neurology, 2019. **91**: p. 3-10.

35. Cugola, F.R., et al., *The Brazilian Zika virus strain causes birth defects in experimental models.* Nature, 2016. **534**(7606): p. 267-271.

36. Basurko, C., et al., *Estimating the Risk of Vertical Transmission of Dengue: A Prospective Study.* American Journal of Tropical Medicine and Hygiene, 2018. **98**(6): p. 1826-1832.

37. Paixao, E.S., et al., *Dengue in pregnancy and maternal mortality: a cohort analysis using routine data.* Scientific Reports, 2018. **8**.

38. Paixao, E.S., et al., *Dengue during pregnancy and adverse fetal outcomes: a systematic review and meta-analysis.* Lancet Infectious Diseases, 2016. **16**(7): p. 857-865.

39. Nascimento, L.B., et al., *Symptomatic dengue infection during pregnancy and livebirth outcomes in Brazil, 2007-13: a retrospective observational cohort study.* Lancet Infectious Diseases, 2017. **17**(9): p. 949-956.

40. Paixao, E.S., et al., *Symptomatic dengue infection during pregnancy and the risk of stillbirth in Brazil, 2006-12: a matched case-control study.* Lancet Infectious Diseases, 2017. **17**(9): p. 957-964.

41. Basurko, C., et al., *A prospective matched study on symptomatic dengue in pregnancy.* Plos One, 2018. **13**(10).

42. Carroll, I.D., S. Toovey, and A. Van Gompel, *Dengue fever and pregnancy - a review and comment.* Travel medicine and infectious disease, 2007. **5**(3): p. 183-188.

43. Pang, T., T.K. Mak, and D.J. Gubler, *Prevention and control of dengue-the light at the end of the tunnel.* Lancet Infectious Diseases, 2017. **17**(3): p. e79-e87.

44. WHO, *Dengue control*. 2017.

45. Curtis, C.F. and J.D. Lines, *Should DDT be banned by international treaty?* Parasitology Today, 2000. **16**(3): p. 119-121.

46. Ooi, E.E., K.T. Goh, and D.J. Gubler, *Denque prevention and 35 years of vector control in Singapore.* Emerging Infectious Diseases, 2006. **12**(6): p. 887-893.

47. Achee, N.L., et al., *A Critical Assessment of Vector Control for Dengue Prevention.* Plos Neglected Tropical Diseases, 2015. **9**(5).

48. Moreira, L.A., et al., *A Wolbachia Symbiont in Aedes aegypti Limits Infection with Dengue, Chikungunya, and Plasmodium.* Cell, 2009. **139**(7): p. 1268-1278.

49. Zabalou, S., et al., *Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(42): p. 15042-15045.

50. Alphey, L., et al., *Genetic control of Aedes mosquitoes.* Pathogens and Global Health, 2013. **107**(4): p. 170-179.

51. Qsim, M., et al., *Genetically Modified Aedes aegypti to Control Dengue: A Review.* Critical Reviews in Eukaryotic Gene Expression, 2017. **27**(4): p. 331-340.

52. Sabchareon, A., et al., *Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial.* Lancet, 2012. **380**(9853): p. 1559-1567.

53. Capeding, M.R., et al., *Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial.* Lancet, 2014. **384**(9951): p. 1358-1365.

54. Villar, L., et al., *Efficacy of a Tetravalent Dengue Vaccine in Children in Latin America.* New England Journal of Medicine, 2015. **372**(2): p. 113-123.

55. Hadinegoro, S.R., et al., *Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease.* New England Journal of Medicine, 2015. **373**(13): p. 1195-1206.

56. Sridhar, S., et al., *Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy.* The New England journal of medicine, 2018. **379**: p. 327-340.

57. WHO, *Dengue vaccine: WHO position paper*, WHO, Editor. 2018.

58. Kirkpatrick, B.D., et al., *The live attenuated dengue vaccine TV003 elicits complete protection against dengue in a human challenge model.* Science Translational Medicine, 2016. **8**(330).

59. Durbin, A.P., et al., *A 12-Month-Interval Dosing Study in Adults Indicates That a Single Dose of the National Institute of Allergy and Infectious Diseases Tetravalent Dengue Vaccine Induces a Robust Neutralizing Antibody Response.* The Journal of infectious diseases, 2016. **214**(6): p. 832-835.

60. Kirkpatrick, B.D., et al., *Robust and Balanced Immune Responses to All 4 Dengue Virus Serotypes Following Administration of a Single Dose of a Live Attenuated Tetravalent Dengue Vaccine to Healthy, Flavivirus-Naive Adults.* Journal of Infectious Diseases, 2015. **212**(5): p. 702-710.

61. Saez-Llorens, X., et al., *Safety and immunogenicity of one versus two doses of Takeda's tetravalent dengue vaccine in children in Asia and Latin America: interim*

results from a phase 2, randomised, placebo-controlled study. Lancet Infectious Diseases, 2017. **17**(6): p. 615-625.

62. Smith, S.A., et al., *Dengue Virus prM-Specific Human Monoclonal Antibodies with Virus Replication-Enhancing Properties Recognize a Single Immunodominant Antigenic Site.* Journal of Virology, 2016. **90**(2): p. 780-789.

63. Screaton, G., et al., *New insights into the immunopathology and control of dengue virus infection.* Nature Reviews Immunology, 2015. **15**(12): p. 745-759.

64. Zhang, X.C., et al., *Structures and Functions of the Envelope Glycoprotein in Flavivirus Infections.* Viruses-Basel, 2017. **9**(11).

65. Zhang, Q., et al., *The Stem Region of Premembrane Protein Plays an Important Role in the Virus Surface Protein Rearrangement during Dengue Maturation.* Journal of Biological Chemistry, 2012. **287**(48): p. 40525-40534.

66. Mackenzie, J.M., M.K. Jones, and Y.R. Young, *Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication.* Virology, 1996. **220**(1): p. 232-240.

67. Avirutnan, P., et al., *Vascular leakage in severe dengue virus infections: A potential role for the nonstructural viral protein NS1 and complement.* Journal of Infectious Diseases, 2006. **193**(8): p. 1078-1088.

68. Avirutnan, P., et al., *Binding of Flavivirus Nonstructural Protein NS1 to C4b Binding Protein Modulates Complement Activation.* Journal of Immunology, 2011. **187**(1): p. 424-433.

69. Xie, X.P., et al., *Membrane Topology and Function of Dengue Virus NS2A Protein.* Journal of Virology, 2013. **87**(8): p. 4609-4622.

70. Arias, C.F., F. Preugschat, and J.H. Strauss, *DENGUE-2 VIRUS NS2B AND NS3 FORM A STABLE COMPLEX THAT CAN CLEAVE NS3 WITHIN THE HELICASE DOMAIN.* Virology, 1993. **193**(2): p. 888-899.

71. Rodriguez-Madoz, J.R., et al., *Inhibition of the Type I Interferon Response in Human Dendritic Cells by Dengue Virus Infection Requires a Catalytically Active NS2B3 Complex.* Journal of Virology, 2010. **84**(19): p. 9760-9774.

72. Aguirre, S., et al., *Dengue virus NS2B protein targets cGAS for degradation and prevents mitochondrial DNA sensing during infection.* Nature Microbiology, 2017. **2**(5).

73. Gebhard, L.G., S.B. Kaufman, and A.V. Gamarnik, *Novel ATP-Independent RNA Annealing Activity of the Dengue Virus NS3 Helicase.* Plos One, 2012. **7**(4).

74. Zou, J., et al., *Characterization of Dengue Virus NS4A and NS4B Protein Interaction.* Journal of Virology, 2015. **89**(7): p. 3455-3470.

75. Miller, S., et al., *The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner.* Journal of Biological Chemistry, 2007. **282**(12): p. 8873-8882.

76. Umareddy, I., et al., *Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA.* Journal of General Virology, 2006. **87**: p. 2605-2614.

77. Tan, B.H., et al., *Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity.* Virology, 1996. **216**(2): p. 317-325.

78. Benmansour, F., et al., *Discovery of novel dengue virus NS5 methyltransferase non-nucleoside inhibitors by fragment-based drug design.* European Journal of Medicinal Chemistry, 2017. **125**: p. 865-880.

79. Mazzon, M., et al., *Dengue Virus NS5 Inhibits Interferon-alpha Signaling by Blocking Signal Transducer and Activator of Transcription 2 Phosphorylation.* Journal of Infectious Diseases, 2009. **200**(8): p. 1261-1270.

80. Hasan, S.S., et al., *Structural biology of Zika virus and other flaviviruses.* Nature Structural & Molecular Biology, 2018. **25**(1): p. 13-20.

81. Mukhopadhyay, S., R.J. Kuhn, and M.G. Rossmann, *A structural perspective of the Flavivirus life cycle.* Nature Reviews Microbiology, 2005. **3**(1): p. 13-22.

82. Dejnirattisai, W., et al., *A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus.* Nature Immunology, 2015. **16**(2): p. 170-177.

83. Slon Campos, J.L., J. Mongkolsapaya, and G.R. Screaton, *The immune response against flaviviruses.* Nat Immunol, 2018. **19**(11): p. 1189-1198.

84. Playfair, J.H.L. and G.J. BanCroft, *Infection and Immunity*. Second edition ed. 2004: Oxford University Press.

85. Zhang, J.-M. and J. An, *Cytokines, inflammation, and pain.* International anesthesiology clinics, 2007. **45**(2): p. 27-37.

86. Harenberg, A., et al., *Cytokine Profile of Children Hospitalized with Virologically-Confirmed Dengue during Two Phase III Vaccine Efficacy Trials.* Plos Neglected Tropical Diseases, 2016. **10**(7).

87. Xavier Ferreira, R.A., et al., *Circulating cytokines and chemokines associated with plasma leakage and hepatic dysfunction in Brazilian children with dengue fever.* Acta Tropica, 2015. **149**: p. 138-147.

88. Rathakrishnan, A., et al., *Cytokine Expression Profile of Dengue Patients at Different Phases of Illness.* Plos One, 2012. **7**(12).

89. Green, S., et al., *Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity.* Journal of Infectious Diseases, 1999. **179**(4): p. 755-762.

90. Chau, T.N.B., et al., *Dengue in Vietnamese infants--results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity.* The Journal of infectious diseases, 2008. **198**(4): p. 516-524.

91. Srikiatkhachorn, A., et al., *Virus-induced decline in soluble vascular endothelial growth receptor 2 is associated with plasma leakage in dengue hemorrhagic fever.* Journal of Virology, 2007. **81**(4): p. 1592-1600.

92. Soo, K.M., et al., *Meta-analysis of biomarkers for severe dengue infections.* Peerj, 2017. **5**.

93. Kao, Y.T., M.M.C. Lai, and C.Y. Yu, *How Dengue Virus Circumvents Innate Immunity.* Frontiers in Immunology, 2018. **9**.

94. La Braga, E., et al., *Detection of circulant tumor necrosis factor-alpha, soluble tumor necrosis factor p75 and interferon-gamma in Brazilian patients with dengue fever and dengue hemorrhagic fever.* Memorias Do Instituto Oswaldo Cruz, 2001. **96**(2): p. 229-232.

95. Bethell, D.B., et al., *Pathophysiologic and prognostic role of cytokines in dengue hemorrhagic fever.* Journal of Infectious Diseases, 1998. **177**(3): p. 778-782.

96. Friberg, H., et al., *Protective versus pathologic pre-exposure cytokine profiles in dengue virus infection.* Plos Neglected Tropical Diseases, 2018. **12**(12).

97. Rathakrishnan, A., et al., *Cytokine Expression Profile of Dengue Patients at Different Phases of Illness.* Plos One, 2012. **7**(12).

98. Aziz, N., et al., *Stability of cytokines, chemokines and soluble activation markers in unprocessed blood stored under different conditions.* Cytokine, 2016. **84**: p. 17-24.

99. Mathew, A., *Defining the role of NK cells during dengue virus infection.* Immunology, 2018. **154**(4): p. 557-562.

100. Laoprasopwattana, K., et al., *Antibody-dependent cellular cytotoxicity mediated by plasma obtained before secondary dengue virus infections: Potential involvement in early control of viral replication.* Journal of Infectious Diseases, 2007. **195**(8): p. 1108-1116.

101. Drews, E., et al., *Upregulation of HLA-E by dengue and not Zika viruses.* Clinical & Translational Immunology, 2018. **7**(9).

102. Beltrame, L.M., et al., *Influence of KIR genes and their HLA ligands in susceptibility to dengue in a population from southern Brazil.* Tissue Antigens, 2013. **82**(6): p. 397-404.

103. Petitdemange, C., et al., *Longitudinal Analysis of Natural Killer Cells in Dengue Virus-Infected Patients in Comparison to Chikungunya and Chikungunya/Dengue Virus-Infected Patients.* Plos Neglected Tropical Diseases, 2016. **10**(3).

104. McKechnie, J.L., et al., *HLA Upregulation During Dengue Virus Infection Suppresses the Natural Killer Cell Response.* Frontiers in Cellular and Infection Microbiology, 2019. **9**.

105. Green, S., et al., *Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever.* Journal of Infectious Diseases, 1999. **180**(5): p. 1429-1435.

106. Keawvichit, R., et al., *Differences in activation and tissue homing markers of natural killer cell subsets during acute dengue infection.* Immunology, 2018. **153**(4): p. 455-465.

107. Khor, C.C., et al., *Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1.* Nature Genetics, 2011. **43**(11): p. 1139-U134.

108. Libraty, D.H., et al., *Circulating Levels of Soluble MICB in Infants with Symptomatic Primary Dengue Virus Infections.* Plos One, 2014. **9**(5).

109. Weiskopf, D., et al., *Dengue virus infection elicits highly polarized CX3CR1(+) cytotoxic CD4(+) T cells associated with protective immunity.* Proceedings of the

National Academy of Sciences of the United States of America, 2015. **112**(31): p. E4256-E4263.

110. Tian, Y., et al., *Human T Cell Response to Dengue Virus Infection.* Frontiers in Immunology, 2019. **10**.

111. Haltaufderhyde, K., et al., *Activation of Peripheral T Follicular Helper Cells during Acute Dengue Virus Infection.* The Journal of infectious diseases, 2018.

112. Rivino, L., et al., *Differential Targeting of Viral Components by CD4(+) versus CD8(+) T Lymphocytes in Dengue Virus Infection.* Journal of Virology, 2013. **87**(5): p. 2693-2706.

113. Grifoni, A., et al., *Characterization of Magnitude and Antigen Specificity of HLA-DP, DQ, and DRB3/4/5 Restricted DENV-Specific CD4+T Cell Responses.* Frontiers in Immunology, 2019. **10**.

114. Wu, S.J.L., et al., *Human skin Langerhans cells are targets of dengue virus infection.* Nature Medicine, 2000. **6**(7): p. 816-820.

115. Simmons, C.P., et al., *Early T-cell responses to dengue virus epitopes in Vietnamese adults with secondary dengue virus infections.* Journal of Virology, 2005. **79**(9): p. 5665-5675.

116. Duangchinda, T., et al., *Immunodominant T-cell responses to dengue virus NS3 are associated with DHF.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(39): p. 16922-16927.

117. Mongkolsapaya, J., et al., *T cell responses in dengue hemorrhagic fever: Are cross-reactive T cells suboptimal?* Journal of Immunology, 2006. **176**(6): p. 3821-3829.

118. Mongkolsapaya, J., et al., *Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever.* Nature Medicine, 2003. **9**(7): p. 921-927.

119. Nguyen, T.P.D., et al., *Timing of CD8(+) T Cell Responses in Relation to Commencement of Capillary Leakage in Children with Dengue.* Journal of Immunology, 2010. **184**(12): p. 7281-7287.

120. Priyamvada, L., et al., *B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts.* Journal of Virology, 2016. **90**(12): p. 5574-5585.

121. Rodrigo, W., et al., *Dengue virus neutralization is modulated by IgG antibody subclass and Fc gamma receptor subtype.* Virology, 2009. **394**(2): p. 175-182.

122. Halstead, S.B. and E.J. Orourke, *Antibody-enhanced dengue virus-infection in primate leukocytes.* Nature, 1977. **265**(5596): p. 739-741.

123. Halstead, S.B., et al., *Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes.* Lancet Infectious Diseases, 2010. **10**(10): p. 712-722.

124. Halstead, S., *Recent advances in understanding dengue.* F1000Research, 2019. **8**.

125. Dejnirattisai, W., et al., *Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans.* Science, 2010. **328**(5979): p. 745-748.

126. Katzelnick, L.C., et al., *Antibody-dependent enhancement of severe dengue disease in humans.* Science, 2017. **358**(6365): p. 929-932.

127. Salje, H., et al., *Reconstruction of antibody dynamics and infection histories to evaluate dengue risk.* Nature, 2018. **557**(7707): p. 719-723.

128. Katzelnick, L.C., et al., *Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort.* Proceedings of the National Academy of Sciences of the United States of America, 2016. **113**(3): p. 728-733.

129. Barba-Spaeth, G., et al., *Structural basis of potent Zika-dengue virus antibody cross-neutralization.* Nature, 2016. **536**(7614): p. 48-53.

130. Katzelnick, L.C., E. Harris, and P. Participants in the Summit on Dengue Immune Correlates of, *Immune correlates of protection for dengue: State of the art and research agenda.* Vaccine, 2017.

131. WHO, *Serological diagnosis of influenza by microneutralization assay*. 2010.

132. Nguyen, T.H.T., et al., *Methods to discriminate primary from secondary dengue during acute symptomatic infection.* Bmc Infectious Diseases, 2018. **18**.

133. Buddhari, D., et al., *Dengue Virus Neutralizing Antibody Levels Associated with Protection from Infection in Thai Cluster Studies.* Plos Neglected Tropical Diseases, 2014. **8**(10).

134. Raut, R., et al., *Dengue type 1 viruses circulating in humans are highly infectious and poorly neutralized by human antibodies.* Proceedings of the National Academy of Sciences of the United States of America, 2018. **116**(1): p. 227-232.

135. de Alwis, R., et al., *Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(19): p. 7439-7444.

136. Beltramello, M., et al., *The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing Activity.* Cell Host & Microbe, 2010. **8**(3): p. 271-283.

137. Marston, H.D., C.I. Paules, and A.S. Fauci, *Monoclonal Antibodies for Emerging Infectious Diseases - Borrowing from History.* New England Journal of Medicine, 2018. **378**(16): p. 1469-1472.

138. Fibriansah, G., et al., *A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface.* Embo Molecular Medicine, 2014. **6**(3): p. 358-371.

139. Fibriansah, G., et al., *Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers.* Science, 2015. **349**(6243): p. 88-91.

140. Smith, S.A., et al., *The Potent and Broadly Neutralizing Human Dengue Virus-Specific Monoclonal Antibody 1C19 Reveals a Unique Cross-Reactive Epitope on the bc Loop of Domain II of the Envelope Protein.* Mbio, 2013. **4**(6).

141. Fibriansah, G., et al., *A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins.* Nature Communications, 2015. **6**.

142. Teoh, E.P., et al., *The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody.* Science Translational Medicine, 2012. **4**(139).

143. Wahala, W., et al., *Recombinant Dengue Type 2 Viruses with Altered E Protein Domain III Epitopes Are Efficiently Neutralized by Human Immune Sera.* Journal of Virology, 2012. **86**(7): p. 4019-4023.

144. Messer, W.B., et al., *Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1.* Journal of Virology, 2016. **90**(10): p. 5090-5097.

145. Widman, D.G., et al., *Transplantation of a quaternary structure neutralizing antibody epitope from dengue virus serotype 3 into serotype 4.* Scientific Reports, 2017. **7**.

146. Gallichotte, E.N., et al., *A New Quaternary Structure Epitope on Dengue Virus Serotype 2 Is the Target of Durable Type-Specific Neutralizing Antibodies.* Mbio, 2015. **6**(5).

147. Mor, G., P. Aldo, and A.B. Alvero, *The unique immunological and microbial aspects of pregnancy.* Nature Reviews Immunology, 2017. **17**(8): p. 469-482.

148. Morelli, S.S., et al., *The maternal immune system during pregnancy and its influence on fetal development.* Research and Reports in Biology, 2015. **6**: p. 171-189.

149. Medawar, P.B., *Some immunological and endocrinological problems raised the evolution of viviparity in veterbrates.* Symposia of the Society for Experimental Biology, 1953. **7**: p. 320-338.

150. Hanna, J., et al., *Decidual NK cells regulate key developmental processes at the human fetal-maternal interface.* Nature Medicine, 2006. **12**(9): p. 1065-1074.

151. Racicot, K., et al., *Cutting Edge: Fetal/Placental Type I IFN Can Affect Maternal Survival and Fetal Viral Load during Viral Infection.* Journal of Immunology, 2017. **198**(8): p. 3029-3032.

152. Martins, C., et al., *Pregnancy alters the circulating B cell compartment in atopic asthmatic women, and transitional B cells are positively associated with the development of allergy manifestations in their progeny.* American Journal of Reproductive Immunology, 2016. **76**(6): p. 465-474.

153. Kraus, T.A., et al., *Peripheral Blood Cytokine Profiling During Pregnancy and Post-partum Periods.* American Journal of Reproductive Immunology, 2010. **64**(6): p. 411-426.

154. Kraus, T.A., et al., *Characterizing the Pregnancy Immune Phenotype: Results of the Viral Immunity and Pregnancy (VIP) Study.* Journal of Clinical Immunology, 2012. **32**(2): p. 300-311.

155. Wu, Z.M., et al., *Pro-inflammatory cytokine-stimulated first trimester decidual cells enhance macrophage-induced apoptosis of extravillous trophoblasts.* Placenta, 2012. **33**(3): p. 188-194.

156. Graham, C., et al., *In vivo immune signatures of healthy human pregnancy: Inherently inflammatory or antiinflammatory?* Plos One, 2017. **12**(6).

157. Lim, S., et al., *Nuclear Factor Kappa B Activation Occurs in the Amnion Prior to Labour Onset and Modulates the Expression of Numerous Labour Associated Genes.* Plos One, 2012. **7**(4).

158. Kourtis, A.P., J.S. Read, and D.J. Jamieson, *Pregnancy and Infection.* New England Journal of Medicine, 2014. **370**(23): p. 2211-2218.

159. Foo, S.-S., et al., *Asian Zika virus strains target CD14+ blood monocytes and induce M2-skewed immunosuppression during pregnancy.* Nature microbiology, 2017.

160. Kam, Y.W., et al., *Specific Biomarkers Associated With Neurological Complications and Congenital Central Nervous System Abnormalities From Zika Virus-Infected Patients in Brazil.* Journal of Infectious Diseases, 2017. **216**(2): p. 172-181.

161. Perret, C., et al., *Dengue infection during pregnancy and transplacental antibody transfer in Thai mothers.* Journal of Infection, 2005. **51**(4): p. 287-293.

162. Nujum, Z.T., et al., *Seroprevalence of dengue infection in pregnant women and placental antibody transfer.* Medical journal, Armed Forces India, 2019. **75**(1): p. 90-95.

163. Basurko, C., et al., *Estimating the Risk of Vertical Transmission of Dengue: A Prospective Study.* The American journal of tropical medicine and hygiene, 2018.

164. Paixao, E.S., et al., *Dengue during pregnancy and adverse fetal outcomes: a systematic review and meta-analysis.* Lancet Infectious Diseases, 2016. **16**(7): p. 857-865.

165. Halstead, S.B., S. Nimmannitya, and S.N. Cohen, *Observations related to pathogenesis of dengue hemorrhagic fever. 4. Relation of disease severity to*

antibody response and virus recovered. Yale Journal of Biology and Medicine, 1970. **42**(5): p. 311-28.

166. Whitehead, S.S., et al., *Prospects for a dengue virus vaccine.* Nature Reviews Microbiology, 2007. **5**(7): p. 518-528.

167. Whiteman, M.C., et al., *Virus Reduction Neutralization Test: A Single-Cell Imaging High-Throughput Virus Neutralization Assay for Dengue.* The American journal of tropical medicine and hygiene, 2018. **99**(6): p. 1430-1439.

168. Nguyen, N.M., et al., *Host and viral features of human dengue cases shape the population of infected and infectious Aedes aegypti mosquitoes.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(22): p. 9072-9077.

169. Kanagal-Shamanna, R., *Emulsion PCR: Techniques and Applications*, in *Clinical Applications of Pcr, 3rd Edition*, R. Luthra, R.R. Singh, and K.P. Patel, Editors. 2016. p. 33-42.

170. Herrera, B.B., et al., *T Cell Responses to Nonstructural Protein 3 Distinguish Infections by Dengue and Zika Viruses.* Mbio, 2018. **9**(4).

171. Smith, S.A., et al., *Isolation of Dengue Virus-Specific Memory B Cells with Live Virus Antigen from Human Subjects following Natural Infection Reveals the Presence of Diverse Novel Functional Groups of Antibody Clones.* Journal of Virology, 2014. **88**(21): p. 12233-12241.

172. Vaughn, D.W., et al., *Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity.* Journal of Infectious Diseases, 2000. **181**(1): p. 2-9.

173. Park, S., et al., *Use of structural equation models to predict dengue illness phenotype.* Plos Neglected Tropical Diseases, 2018. **12**(10).

174. Salazar, G., et al., *Antibody therapies for the prevention and treatment of viral infections.* Npj Vaccines, 2017. **2**.

175. Ramadhany, R., et al., *Antibody with an engineered Fc region as a therapeutic agent against dengue virus infection.* Antiviral Research, 2015. **124**: p. 61-68.

176. Rathore, A.S., A. Sarker, and R.D. Gupta, *Designing antibody against highly conserved region of dengue envelope protein by in silico screening of scFv mutant library.* Plos One, 2019. **14**(1).

177. Ritz, C., et al., *Dose-Response Analysis Using R.* Plos One, 2015. **10**(12).

178. Rouvinski, A., et al., *Recognition determinants of broadly neutralizing human antibodies against dengue viruses.* Nature, 2015. **520**(7545): p. 109-113.

179. Kumar, V., et al., *Intravenous immunoglobulin for severe thrombocytopenia in secondary dengue.* BMJ case reports, 2018.

180. Subbiah, A., et al., *Intravenous immunoglobulin therapy for dengue capillary leak syndrome in a renal allograft recipient.* BMJ case reports, 2018.

181. Duyen, H.T.L., et al., *Kinetics of Plasma Viremia and Soluble Nonstructural Protein 1 Concentrations in Dengue: Differential Effects According to Serotype and Immune Status.* Journal of Infectious Diseases, 2011. **203**(9): p. 1292-1300.

182. Davey, R.T., et al., *A Randomized, Controlled Trial of ZMapp for Ebola Virus Infection.* New England Journal of Medicine, 2016. **375**(15): p. 1448-1456.

183. Park, T., S.K. Griggs, and D.C. Suh, *Cost Effectiveness of Monoclonal Antibody Therapy for Rare Diseases: A Systematic Review.* Biodrugs, 2015. **29**(4): p. 259-274.

184. Hung, T.M., et al., *The Estimates of the Health and Economic Burden of Dengue in Vietnam.* Trends in Parasitology, 2018. **34**(10): p. 904-918.

185. Aguas, R., et al., *Cross-serotype interactions and disease outcome prediction of dengue infections in Vietnam.* Scientific Reports, 2019. **9**.

186. Tuan, N.M., et al., *An Evidence-Based Algorithm for Early Prognosis of Severe Dengue in the Outpatient Setting.* Clinical Infectious Diseases, 2017. **64**(5): p. 656-663.

187. Tanner, L., et al., *Decision Tree Algorithms Predict the Diagnosis and Outcome of Dengue Fever in the Early Phase of Illness.* Plos Neglected Tropical Diseases, 2008. **2**(3).

188. Nikolayeva, I., et al., *A Blood RNA Signature Detecting Severe Disease in Young Dengue Patients at Hospital Arrival.* Journal of Infectious Diseases, 2018. **217**(11): p. 1690-1698.

189. Gallichotte, E.N., et al., *Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization.* Cell Reports, 2018. **25**(5): p. 1214-1224.

190. Kuhn, R.J., et al., *Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion.* Cell, 2002. **108**(5): p. 717-725.

191. Pazos, M., et al., *The influence of pregnancy on systemic immunity.* Immunologic Research, 2012. **54**(1-3): p. 254-261.

192. Machain-Williams, C., et al., *Maternal, Fetal, and Neonatal Outcomes in Pregnant Dengue Patients in Mexico.* Biomed Research International, 2018.

193. Ribeiro, C.F., et al., *Dengue infection in pregnancy and its impact on the placenta.* International Journal of Infectious Diseases, 2017. **55**: p. 109-112.

194. Basurko, C., et al., *A prospective matched study on symptomatic dengue in pregnancy.* PloS one, 2018. **13**(10): p. e0202005-e0202005.

195. Paixao, E.S., et al., *Dengue during pregnancy and live birth outcomes: a cohort of linked data from Brazil.* Bmj Open, 2019. **9**(7).

196. Langers, I., et al., *Natural killer cells: role in local tumor growth and metastasis.* Biologics : targets & therapy, 2012. **6**: p. 73-82.

197. de Matos, A.M., et al., *CD8+T Lymphocyte Expansion, Proliferation and Activation in Dengue Fever*. Plos Neglected Tropical Diseases, 2015. **9**(2).

198. Weiskopf, D., et al., *Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8(+) T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(22): p. E2046-E2053.

199. Kieffer, T.E.C., et al., *Pregnancy persistently affects memory T cell populations.* Journal of Reproductive Immunology, 2017. **119**: p. 1-8.

200. Tian, Y., et al., *Dengue-specific CD8+ T cell subsets display specialized transcriptomic and TCR profiles.* The Journal of clinical investigation, 2019. **130**: p. 1727-1741.

201. Waickman, A.T., et al., *Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling.* Nature Communications, 2019. **10**.

202. Kieffer, T.E.C., et al., *Memory T Cells in Pregnancy.* Frontiers in Immunology, 2019. **10**.

203. Hoa, B.K., et al., *HLA-A, -B, -C, -DRB1 and -DQB1 alleles and haplotypes in the Kinh population in Vietnam.* Tissue Antigens, 2008. **71**(2): p. 127-134.

204. Lan, N.T.P., et al., *Protective and Enhancing HLA Alleles, HLA-DRB1*0901 and HLA-A*24, for Severe Forms of Dengue Virus Infection, Dengue Hemorrhagic Fever and Dengue Shock Syndrome.* Plos Neglected Tropical Diseases, 2008. **2**(10).

205. Gelfand, E.W., *Eosinophils in Human Disease Introduction*. Eosinophils in Health and Disease, ed. J.J. Lee and H.F. Rosenberg. 2013.

206. Deshmane, S.L., et al., *Monocyte Chemoattractant Protein-1 (MCP-1): An Overview.* Journal of Interferon and Cytokine Research, 2009. **29**(6): p. 313-326.

207. Mantovani, A., et al., *Macrophage-derived chemokine (MDC).* Journal of Leukocyte Biology, 2000. **68**(3): p. 400-404.

208. Phongsamart, W., et al., *Dengue virus infection in late pregnancy and transmission to the infants.* Pediatric Infectious Disease Journal, 2008. **27**(6): p. 500-504.

209. Singh, T., et al., *Efficient transplacental IgG transfer in women infected with Zika virus during pregnancy.* Plos Neglected Tropical Diseases, 2019. **13**(8).

210. Tan, P.C., et al., *Dengue infection in pregnancy - Prevalence, vertical transmission, and pregnancy outcome.* Obstetrics and Gynecology, 2008. **111**(5): p. 1111-1117.

211. Hunsperger, E.A., et al., *Evaluation of Commercially Available Anti-Dengue Virus Immunoglobulin M Tests.* Emerging Infectious Diseases, 2009. **15**(3): p. 436-440.

212. Jauniaux, E., et al., *Materno-fetal immunoglobulin transfer and passive immunity during the first trimester of human pregnancy.* Human Reproduction, 1995. **10**(12): p. 3297-3300.

213. Wimmer, E., et al., *Synthetic viruses: a new opportunity to understand and prevent viral disease.* Nature Biotechnology, 2009. **27**(12): p. 1163-1172.

214. Keck, Z.Y., et al., *A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope.* Journal of Virology, 2008. **82**(12): p. 6067-6072.

215. Simmons, C.P., et al., *Therapeutics for Dengue: Recommendations for Design and Conduct of Early-Phase Clinical Trials.* Plos Neglected Tropical Diseases, 2012. **6**(9).

216. Renner, M., et al., *Characterization of a potent and highly unusual minimally enhancing antibody directed against dengue virus.* Nature Immunology, 2018. **19**(11): p. 1248-1256.

217. Morrone, S.R. and S.-M. Lok, *Structural perspectives of antibody-dependent enhancement of infection of dengue virus.* Current opinion in virology, 2019. **36**: p. 1-8.

218. Gunn, B.M., et al., *A Role for Fc Function in Therapeutic Monoclonal Antibody-Mediated Protection against Ebola Virus.* Cell Host & Microbe, 2018. **24**(2): p. 221-233.

219. Huber, R.G., et al., *Structure mapping of dengue and Zika viruses reveals functional long-range interactions.* Nature Communications, 2019. **10**.

220. Langerak, T., et al., *The possible role of cross-reactive dengue virus antibodies in Zika virus pathogenesis.* Plos Pathogens, 2019. **15**(4).

221. Zimmerman, M.G., et al., *Cross-Reactive Dengue Virus Antibodies Augment Zika Virus Infection of Human Placental Macrophages.* Cell Host & Microbe, 2018. **24**(5): p. 731-742.

222. Fibriansah, G., et al., *A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins.* Nature Communications, 2015. **6**: p. 6341.

223. Collins, M.H., et al., *Human antibody response to Zika targets type-specific quaternary structure epitopes.* Jci Insight, 2019. **4**(8).

224. Long, F., et al., *Structural basis of a potent human monoclonal antibody against Zika virus targeting a quaternary epitope.* Proceedings of the National Academy of Sciences of the United States of America, 2019. **116**(5): p. 1591-1596.

225. Gorny, M.K., et al., *Identification of a new quaternary neutralizing epitope on human immunodeficiency virus type 1 virus particles.* Journal of Virology, 2005. **79**(8): p. 5232-5237.

226. Fernandez, E., et al., *Human antibodies to the dengue virus E-dimer epitope have therapeutic activity against Zika virus infection.* Nature Immunology, 2017. **18**(11): p. 1261-9.

227. Lu, J.S., et al., *Potent Neutralization Ability of a Human Monoclonal Antibody Against Serotype 1 Dengue Virus.* Frontiers in Microbiology, 2018. **9**.

228. Arien, K.K. and A. Wilder-Smith, *Dengue vaccine: reliably determining previous exposure.* Lancet Global Health, 2018. **6**(8): p. E830-E831.

229. Stan, R.C., et al., *Febrile temperatures increase in vitro antibody affinity for malarial and dengue antigens.* Plos Neglected Tropical Diseases, 2019. **13**(4).

230. Wen, J. and S. Shresta, *Antigenic cross-reactivity between Zika and dengue viruses: is it time to develop a universal vaccine?* Current opinion in immunology, 2019. **59**: p. 1-8.

231. Maeki, T., et al., *Analysis of cross-reactivity between flaviviruses with sera of patients with Japanese encephalitis showed the importance of neutralization tests for the diagnosis of Japanese encephalitis.* Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy, 2019.

232. Wen, J.S., et al., *Identification of Zika virus epitopes reveals immunodominant and protective roles for dengue virus cross-reactive CD8(+) T cells.* Nature Microbiology, 2017. **2**(6).

233. Patil, V.S., et al., *Precursors of human CD4(+) cytotoxic T lymphocytes identified by single-cell transcriptome analysis.* Science Immunology, 2018. **3**(19): p. 13.

234. Foo, S.S., et al., *Asian Zika virus strains target CD14(+) blood monocytes and induce M2-skewed immunosuppression during pregnancy.* Nature Microbiology, 2017. **2**(11): p. 1558-1570.

235. Rivino, L., et al., *Virus-specific T lymphocytes home to the skin during natural dengue infection.* Science Translational Medicine, 2015. **7**(278).

236. St John, A.L. and A.P.S. Rathore, *Adaptive immune responses to primary and secondary dengue virus infections.* Nature reviews. Immunology, 2019.

237. Castanha, P.M.S., et al., *Perinatal analyses of Zika- and dengue virus-specific neutralizing antibodies: A microcephaly case-control study in an area of high dengue endemicity in Brazil.* Plos Neglected Tropical Diseases, 2019. **13**(3).

238. Pomar, L., et al., *Maternal-fetal transmission and adverse perinatal outcomes in pregnant women infected with Zika virus: prospective cohort study in French Guiana.* Bmj-British Medical Journal, 2018. **363**.

APPENDIX

PERFORMING DENGUE NS1 Ag STRIP

1. Purpose

To describe the procedure for performing the Dengue NS1 Ag Strip test, from BioRad using patient-derived samples.

2. Principle

The BioRad Dengue NS1 strip test is used as a diagnostic for dengue virus infection in humans. It uses a sample of human serum or plasma to rapidly and qualitatively detect the dengue virus NS1 antigen. The use of this test aids in the diagnosis of acute dengue infection faster than other available tests (eg. MAC-ELISA).

3. Reference

Dengue NS1 Ag Strip – instruction manual.

4. Responsibility

Lab technicians have responsibility for performing testing following defined procedures. The test result have to record into "Dengue NS1 Ag strip result" *FM-RR-DNG-033.*

5. Definition

- DENV Dengue virus
- NS1 Non-structural protein 1

6. Specimen used

Human plasma or serum. (In order to obtain this sample, refer to SOP *TP-SM-DNG-002* "Separation of plasma from blood by centrifugation").

7. Materials/Equipments used

- Dengue NS1 Ag strip Biorad Cat.No: 70700
- Sterile high plastic tube
- Pipette
- Centrifuge

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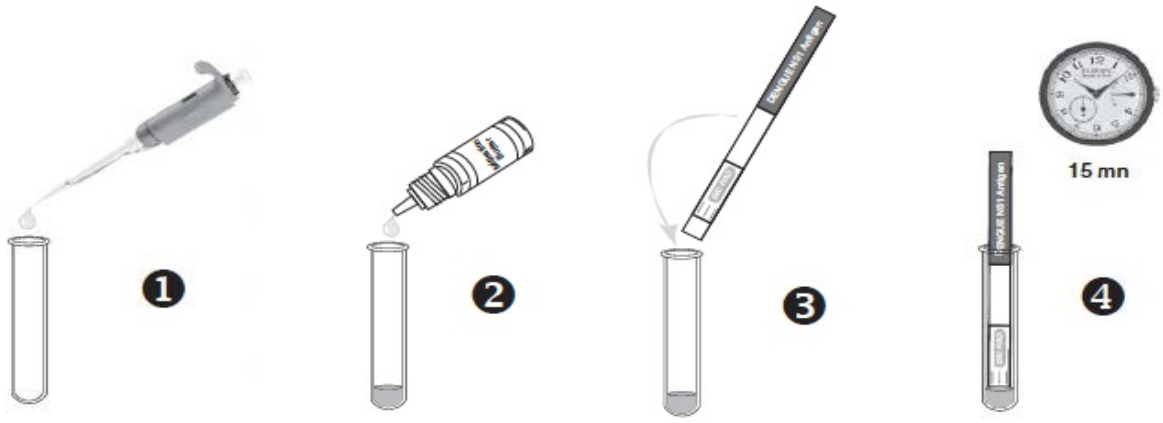
8. Procedures

Schematic illustrations of the procedures are indicated below, as provided by the manufacturer of the kit.

1) Add 50µl of plasma to a 10 ml test tube with lid.
2) Add 1 drop of the migration buffer in the kit to the tube. Gentlty agitate the tube to ensure the plasma sample is adequately mixed with the buffer.
3) Add the NS1 strip to the tube. Make sure the bottom of the strip is submerged in the liquid, and it is correctly oriented.
4) Wait for 15 mins for the test to develop.
5) The lines on the strip will indicate the results of the test.
6) Record the result in the "Dengue NS1 Ag strip result" *FM-RR -DNG-033*



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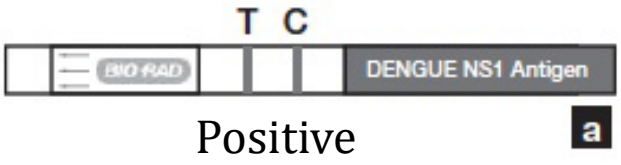
9. Interpretation and report

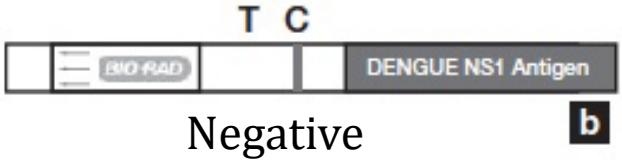
Lines on the strip indicate the result of the test.

The control line (C) must be visible for any test to be considered valid.



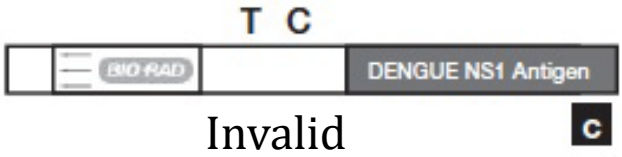
Positive a

The appearance of a blue or purple line at the test line (T) and the control line (C) indicates a positive result (as above).



Negative b

The appearance of a blue or purple line at the control line (C) only indivates a negative result (as above).



Invalid c

If the control line (C) is negative, the test is invalid (as above) and should be retested with a fresh aliquot of the sample and a new strip.

10. Safety

- Universal precautions should be used when working with clinical specimens as they may contain pathogens that can infect the staff member.
- Appropriate PPE should be worn at all times to ensure the staff member is protected from infection. This includes gloves, safety glasses (to protect the eyes from splashes), and a lab coat.

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11. Supplementary information

- The plasma/serum suspension should be clear and free of any particles in suspension
- The NS1 strip test always be stored from 2-8ºC in a fridge before and after use. Doing so will allow the test to be used up to the expiration date.
- Do not touch the reaction zone with finders.
- Wear disposable gloves when performing the test.

12. Risk assessment and risk management

Department Dengue group **Location of work** OUCRU-HCM Laboratory	**Persons involved** Dengue staff
Description of procedure The procedure describes how to perform an DENV NS1 strip test.	**Hazardous Substances used** Plasma from human blood
Quantities used <5ml	**Frequency of use** Daily
Hazards identified Potential exposure to infectious pathogens and bacteria that are infected within the plasma sample being tested	**Could a less hazardous substance be used instead?** NO.
What measures have you taken to control risk? Universal precautions are to be used when handling any human blood sample. PPE must be work, including lab coat, gloves, safety glasses, mask.	
Checks on control measures Supervisors to intermittently check that ensure all staff are trained in performing the procedure and make sure the records for testing are maintained appropriately	
Is health surveillance required? No	**Training requirements:** i. Handling of human biological samples. ii. Waste disposal iii. Accident reporting procedures iv. Spill clean up procedures v. Sign and symptoms of infection
Emergency procedures: i. Contact supervisor ii. Contact lab management group	**Waste disposal procedures**: All waste to be autoclaved at 121°C for 30 minutes.

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13. Related documents:

Document name	Document code
Dengue NS1 Ag strip result	*FM-RR-DNG-033*
BioRad NS1 Ag strip Manual	N/A

14. Document history:

Revision no	Effective date	Details of amendment
00	01 May 2010	New issue
001	14 Aug 2015	Converted to new OUCRU SOP template, added Principle, improved description of procedures, identified safety concerns, added Risk Assessment and Supplementary notes from BioRad manual. – Lauren Carrington
002	27 Mar 2018	Transferred to new OUCRU SOP template. Checked for accuracy, general editing. – Huynh Le Phuong Thuy & Lauren Carrington

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1. Purpose:

To provide a qualitative procedure for RNA extraction using MagNA Pure 96 DNA and Viral NA Small Volume Kit

2. Principle

This assay is used to extract nucleic acids from either human samples, or from mosquitoes. It is a high-throughout assay, providing high quality extractions for downstream PCRs, sequencing and other purposes as necessary. Most of the assay is automated – the raw sample is added to the plate, and the extracted sample is returned ~60 mins later. The only preparation required is to prepare the reagents and add them to the system correctly.

The Viral NA Small Volume Kit is specifically designed to be used with the MagnaPure 96 system. The benefits of this kit are that it can obtain high yields of pure genomic DNA or viral DNA and RNA, with a short processing and purification time (<60 min for 96 samples). It can be used with a broad range of sample materials, and can save time during instrument setup using a minimal number of ready-to-use reagents. Finally, it can minimize pipetting steps and contamination risk using reagents prefilled in containers.

The nucleic acid isolation procedure is based on the MagNA Pure Magnetic Glass Particle (MGP) Technology. The key steps of a MagNA Pure 96 NA isolation procedure are:

1. Sample material is lysed, nucleic acids are released, and nucleases are denatured.
2. Nucleic acids bind to the silica surface of the added MGPs due to the chao-tropic salt conditions and the high ionic strength of the lysis/binding buffer.
3. MGPs with bound nucleic acids are magnetically separated from the residual lysed sample.
4. Unbound substances, such as proteins, cell debris, and PCR inhibitors are removed by several washing steps.
5. Purified nucleic acids are eluted from the MGPs

3. Scope and responsibilities:

Lab technicians have responsibility for performing testing following defined procedures.

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4. Reference

MagNA Pure 96 DNA and Viral NA Small Volume Kit protocol

5. Definition

- NA = Nucleic acid
- RNA = Ribonucleic acid
- DNA = deoxyribonucleic acid
- DENV = Dengue Virus
- EAV = Equine Arteritis Virus

6. Specimen requirement

- Mammalian whole blood
- Serum
- Plasma
- Urine
- Sputum
- Swabs
- Cerebrospinal fluid
- Bacterial cell culture supernatant
- Mosquito homogenates

7. Materials/Equipment used

7.1 Kit contents

MagNA Pure 96 DNA and Viral NA Small Volume Kit Cat. No: 06543588001

Component	Label	Content
Tray 1	**Reagent Tray 1**	**3 trays**
Container 1	Wash Buffer I	• 1 container, 160ml *for removing impurities*

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Container 2	Wash Buffer I	• 1 container, 80ml *for removing impurities*
Container 3	Lysis/Binding Buffer	• 1 container, 80ml *for cell/virus lysis and binding of NA*
Tray 2	**Reagent Tray 2**	**3 trays**
Container 1	Empty	
Container 2	Proteinase K	• 1 container, 15ml *for digestion of proteins*
Container 3	Elution Buffer	• 1 container, 80ml *for elution NA*
Container 4	Wash Buffer III	• 1 container, 160ml *for removing impurities*
Bottle 1	Magnetic Glass Particles	• 6 vials,18ml MGP suspension each *for binding NA*

7.2 Equipment

- MagNA Pure 96 Extractor — Roche
- MagNA Pure 96 System Fluid — Roche
- MagNA Pure 96 Sealing Foil — Roche
- Extra-long filter tip — Thermo scientific F110-R204S-
- 8 -Strip tubes & Ind Caps Natural — Alpha LW2510

8. Procedures

8.1. Sample preparation

- Make a plate layout using sample list. Thaw samples and an internal control (EAV) tube (**in case of clinical samples used for extraction**)
- Prepare a processing cartridge and a box of extra-long filter tip
- Spin samples before open the lids, then add 140µl of each of plasma samples and 20µl of EAV (**if extraction of clinical samples**) into each well of the processing cartridge as the plate layout.
- In every extraction batch, a negative control should be included. The negative control is 140 µl of molecular grade water and 20 µl of EAV.

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- In every extraction batch an external positive control should be included. The external positive/specificity controls are culture supernatant from interested targets (e.g DENV, CHIKV, ZIKV…). The standard volume for the External controls is 140µl of each culture supernatant and 20 µl of EAV.
- Both negative and external controls are processed in an identical fashion to clinical samples.

8.2. Start Instrument, Software and run Daily Maintenance:

- Start the MagNA Pure 96 instrument.
- Start the MagNA Pure 96 Software. Click *Logon*
- Enter the User ID and Password. The options in the *Overview* tab are enabled.
- Perform daily maintenance the MagNA Pure 96 instrument before stating work day by clicking *Maintenance* on the *Overview* screen.
- In the Purification Run group, click *Ordering*, then *Enter Order*
- Load the run template "*Dengue RNA Extraction template"*.
- Enter a Sample Name for each sample and enter Sample Notes (optional) in the sample table.
- Save isolation experiment with the Batch file number from the Batch file index. In the toolbar, click *SAVE*
- Click *Next* button

8.3. Set Up Instrument Stage/ Start Run:

- Open Load Flap of the MagNA Pure96 Instrument.
- Use the information of the *Stage Setup* sub-tab to place all disposables, trays and bottles necessary for the run on the stage of the instrument (followed instructions on the screen of the system).
- Load the Processing Cartridge and other cartridges on the stage of the instrument.
- Close the Load Flap of the MagNA Pure96 Instrument. The barcode check starts.
- If material and reagents are in place the color of the respective positions on the stage is displayed green in the *Stage* sub-tab and the *Start* button is enabled.
- In the global action bar, click Start.

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The system will do extraction automatically using the set up program as the RNA viral kit suggested.

8.4. Collect extracted RNA

- After ~60 mins, move extracted plate out to collect RNA
- Transfer RNA from extracted plate to 8 -Strip tubes
- Store RNA at -80° C freezer for future analysis

9. Quality Control

Internal control: use of EAV

To assess the uniformity of RNA extraction and PCR, a standard amount of equine arteritis virus (EAV) is added to patient's plasma samples prior to RNA extraction .A negative control using nucleic acid free water should be included in each extraction performance to control contamination.

External controls: cultured DENV-1 to DENV-4.

In every batch of RNA extraction cultured DENV-1 to DENV-4 are used as positive controls and controls for specificity, e.g. the DENV-1 PCR should be negative on DENV-2, DENV-3 and DENV-4 culture supernatants.

10. Safety

- Universal precautions should be used when working with clinical specimens. When working with chemicals, always wear a suitable lab coat, disposal gloves, and protective goggles.
- Place any waste in the clinical waste bins provided.

11. Supplementary notes

- The UV light in MagNA Pure96 Instrument has to turn on at the end of working day at least 15min to sterilize the machine.
- System fluid needs to be changed after 2 isolation batches.

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- Tip cracks need to be changed and cleaned after isolation running batch.
- Clean waste bin after 2 isolation running batches.

12. Risk assessment and risk management:

Department = Dengue group **Location of work** = Room 215A (oucru lab)	**Persons involved** Dengue staff
Description of procedure This protocol describe RNA extraction procedurefor RNA extraction using MagNA Pure 96 DNA andViral NA Small Volume Kit	**Hazardous Substances used** NO
Quantities used 140µLplasma/sample	**Frequency of use** Daily or weekly (depend on the studies)
Hazards identified Human specimens may contain infectious virus, bacteria or other pathogens.	**Could a less hazardous substance be used instead?** NO.
What measures have you taken to control risk? i. Safety glasses, ii. Gloves, iii. Lab coat iv. Staff training about risks and management	
Checks on control measures • Supervision and observation by senior staff. • Equipment maintenance and calibration. • Spill kit is checked for content regularly	
Is health surveillance required? Hepatitis B serology	**Training requirements:** Task specific training for all staff, plus training in good laboratory practices
Emergency procedures: i Biological and chemical spill cleanup procedure *PR-HS-ALL-005*	**Waste disposal procedures**: OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

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13. Related Documents:

Document name	Document code
MagNA Pure 96 DNA and Viral NA Small Volume Kit protocol	N/A

14. Document History:

Revision #	Effective date	Details of Amendment
00	01 January 2011	New issue
01	7 April 2018	Transferred to new OUCRU SOP template, checked for accuracy. Added information about specimens, quality control. Added risk assessment and risk management table. Removed obsolete forms associated with this procedure. – Huynh Le Anh Huy & Lauren Carrington

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1. Purpose

To describe the procedure for the detection of dengue virus RNA by *quantitative RT-PCR* using RNA as template.

2. Principle:

This procedure both quantifies the amount of DENV in a sample, as well as detecting what the infecting serotype is. It is a one-step multiplex RT-PCR protocol, that uses RNA as the starting template, and it allows the standard two-step Reverse Transcription + Polymerase Chain Reaction proedures to be performed within a single tube. This process cuts down on time, effort and consumables.

To determine the infecting serotype of a given sample, a single sample must be run in two independent reactions. The first reaction is designed to amplify both DENV-1 and DENV-3, while the second reaction amplifies both DENV-2 and DENV-4. The addition of serotype-specific fluorgenic probes to the mastermix allows the detection of the infecting serotype – a sample will be negative for all serotypes other than the one present in the template.

3. Scope and responsibilities:

Lab technicians have responsibility for performing testing following this defined procedure. For each PCR batch, the procedure steps must be documented into the "Dengue Viral Load Calculation template" *FM-RR-DG-032*. This worksheet provides a step-by-step recording system for the PCR set-up and analysis. Any changes to the procedure, e.g. different probe concentration, must be recorded in the worksheet.

4. Reference

Hue Kien Duong Thi, Tuan Trung Vu, Hanh Tien Nguyen Thi, Cameron P Simmons et al. (2011). Validation of an internally-controlled one-step real time RT-PCR assay for the detection and quantification of dengue virus RNA in plasma. *Journal of Virological Methods*. 177(2): 168-73.

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5. Definition

Nucleic acids

- A = Adenine
- T = Thymine
- G = Guanine
- C = Cytosine
- Y = C, T
- R = A, G

Other

- DENV = dengue virus
- EAV = equine arteritis virus
- Fwd = Forward
- Rev = Reverse
- LOD = limit of detection
- CSF = Cerebral Spinal Fluid

6. Specimen requirement

- Homogenised mosquito tissue (protocol *TP-TO-DNG-057*)
- Human plasma/serum samples (protocol *TP-SM-DNG-002*)
- CSF samples

7. Materials/Equipments used

DENV-1 – 4 primers	Sigma – Proligo
DENV-1 – 4 probes	TIB MolBiol
EAV primer	Sigma – Proligo
EAV probe	Sigma – Proligo
RNA Master Hydrolysis Probes	Roche – Cat. No: 04 991 885 001
PCR plate and Sealing foil	Roche – Cat. No: 04 729 757 001
Filter tip	

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- Pipette
- Centrifuge

Primer sequences

Serotype	Primer sequences (5'-3')	Position	Product size (# bases)
DENV-1 – Fwd	ATCCATGCCCAYCACCAAT	9865-9883	100
DENV-1 – Rev	TGTGGGTTTTGTCCTCCATC	9945-9964	
DENV-2 – Fwd	TCCATACACGCCAAACATGAA	9859-9879	125
DENV-2 – Rev	GGGATTTCCTCCCATGATTCC	9963-9983	
DENV-3 – Fwd	TTTCTGCTCCCACCACTTTC	9591-9610	118
DENV-3 – Rev	CCATCCYGCTCCTTGAGA	9691-9708	
DENV-4 – Fwd	GYGTGGTGAAGCCYCTRGAT	9587-9606	178
DENV-4 – Rev	AGTGARCGGCCATCCTTCAT	9744-9763	
EAV – Fwd	CATCTCTTGCTTTGCTCCTTAG	1847-1868	133
EAV – Rev	AGCCGCACCTTCACATTG	1980-1997	

Probe sequences

Target	Probe Sequence
DENV-1	5'(FAM) -TCAGTGTGGAATAGGGTTTGGATAGAGGAA- 3'(BHQ-1)
DENV-2	5'(FAM) -AGGGTGTGGATTCGAGAAAACCCATGG- 3'(BHQ-1)
DENV-3	5'(Cyan500) -AAGAAAGTTGGTAGTTCCCTGCAGACCCCA- 3'(BHQ-1)
DENV-4	5'(Cyan500) -ACTTCCCTCCTCTTYTTGAACGACATGGGA- 3'(BHQ1)
EAV	5'(Cy5) -CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG- 3'(BHQ3)

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8. Procedures

Sample preparation

1) The starting material is treated the same, whether or not it is human plasma or serum, CSF, or mosquito tissue.
2) To each sample that will be processed (i.e., experimental samples, positive and negative controls), spike 20µl of EAV into the 140µl of the biological sample. EAV serves as the internal positive control, which is prepared according to the *TP-TC-DNG-006* protocol.
3) RNA is then extracted from the sample according the *TP-TC-DNG-003* protocol, using the QIAamp Viral RNA mini kits. This extracted RNA then goes directly into the PCR.

PCR master mixes

4) To perform the PCR for each of the four DENV serotypes, two PCRs must be conducted for each sample. Each PCR is a multiplex reaction, amplifying 3 independent targets.
   - 1st multiplex DENV-1, DENV-3, EAV
   - 2nd multiplex DENV-2, DENV-4, EAV

Master mix for multiplex #1	**Working conc.**	**µl/reaction**
Mix of DENV-1 Fwd + Rev primer	20µM	1.00
Mix of DENV-3 Fwd + Rev primer	20µM	1.00
Mix of EAV Fwd + Rev primer	20µM	0.20
DENV-1 probe	10µM	0.28
DENV-3 probe	10µM	0.28
EAV probe	10µM	0.08
Activator		1.40
Enhancer	20x	1.00
RNA master mix	2.7	7.40
H_2O		1.36
RNA from sample (DNA from standard curve)		6.00
Total		*20.00*

* *RNA is added in the Extraction Room only* and should not be added in the "*Mix Room*"

Note: This is a CONTROLLED document. Any documents in paper form not stamped "Controlled copy" are not controlled and should be checked against the master document.

Master mix for multiplex #2	Working conc.	µl/reaction
Mix of DENV-2 Fwd + Rev primer	20µM	1.00
Mix of DENV-4 Fwd + Rev primer	20µM	1.00
Mix of EAV Fwd + Rev primer	20µM	0.20
DENV-2 probe	10µM	0.28
DENV-4 probe	10µM	0.28
EAV probe	10µM	0.08
Activator		1.40
Enhancer	20x	1.00
RNA master mix	2.7	7.40
H_2O		1.36
RNA from sample (DNA from standard curve)		6.00
Total		*20.00*

** RNA is added in the Extraction Room only* and should not be added in the "*Mix Room*"

Plate set up

5) After preparing the PCR mix, transfer 14µl PCR mix to each well in the PCR plate. This is done in the *Mix room* only.
6) When ready, transfer the PCR plate containing the PCR mix to the *Extraction room* and add 6µl RNA from samples, external controls and standard curve.
7) Each standard in the standard curve is performed in duplicate. The samples and external controls (negative, positive and specificity) are performed each using a single well.
8) After RNA has been added, centrifuge at 3000rpm for 2min in the centrifuge available in the extraction room to collect all droplets in the bottom of the tubes.
9) Once the plate has been spun, take the plate to the *PCR Room* and turn on the Roche Light Cycler II PCR machine.
10) On the software interface, apply the "one-step Dengue Multiplex PCR" template. Name the file for that batch with the Batch file number from the Batch file index.

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PCR programme

11) The samples are converted to cDNA and amplified in the one tube, by sequentially incubating the samples at the appropriate temperatures. The pre-programmed template follows the temperatures in the table below:

Number	Step	Temperature	Time	# Cycles
1	Reverse transcription	61°C	10 mins	x 1
2	Denaturation	95°C	2 mins	x 1
3	Amplification	95°C	15 secs	x 45
		60°C	30 secs*	
4	Cooling	37°C	1 min	x 1

** The fluorescence reading is taken at the end of the 30 sec elongation step, after each round of amplification.*

9. Quality Control

Internal control: use of EAV

To assess the uniformity of RNA extraction and PCR, a standard amount of equine arteritis virus (EAV) should be added to patient's plasma samples prior to RNA extraction. The characteristics of each batch of EAV will be checked and optimized before using.

External controls: cultured DENV 1- 4.

In every batch of RNA extraction, and PCR, the investigator should include control samples. The control samples serve to confirm the performance of the DENV-specific PCR assays and also tests specificity, i.e. culture supernatant from DENV 2-4 should be negative in the DENV-1 PCR.

Calibration of the Light Cycler machine

In a multicolour reaction, the wavelengths of light emitted by the dyes may overlap, causing one channel to pick up signals from a dye that should be measured by another channel. This bleed-

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over of fluorescence signal can result in incorrect data. To correct for this optical crosstalk, a color compensation assay is run to eliminate the fluorescence crosstalk every 6 months or when the filters are changed on the Light Cycler.

10. Calculations

Converting sample measurements in copies per reaction to RNA copies per ml of sample:

DENV RNA equivalents per ml can be quantified and results lower than the LOD should be regarded as <LOD. The Light Cycler software automatically reports results in the units of the standard curve, in this case, it is copies per reaction. DENV RNA equivalents per ml can be quantified and results lower than the LOD should be regarded as such, and considered as negatives. The convert step is performed in "Dengue Viral Load Calculation template" *FM-RR-DNG-032*. LOD of each DENV serotype is summarized in the table below.

	DENV-1	**DENV-2**	**DENV-3**	**DENV-4**
LOD (copies/ reaction)	5	1	5	10

11. Interpretation of results and report

The results of concentration of DENV in samples should be reported as either *Copies/mL* for human fluid, or *Copies/tissue* for mosquito tissue.

Determining Ct values

The software associated with the Roche Light Cycler determines the threshold cycle value by algorithmic means. In practice, this means the cycle threshold value (Ct) is set automatically.

The Internal EAV control; criteria for acceptability

The Internal control (EAV) serves as a qualitative control for extraction, and PCR. Failure of the internal control may reflect inhibition or reaction failure in any of the steps leading to and including the PCR. An acceptable Ct value for EAV in samples or controls is within 10% of the mean value determined in the optimization experiments. However, if the EAV Ct value falls outside the allowable range for any sample, then the result must be examined following the criteria in the table below.

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	EAV/ DENV			
Result	(+/+)	(+/-)	(-/+)	(-/ -)
Outcome	Pass	Pass	Pass	Fail

The range or mean of Internal control in each batch of samples and controls should be recorded in the report file of "Dengue Viral Load Calculation template" *FM-RR-DNG-032.*

The External Negative control: criteria for acceptability

The External Negative control is molecular grade water that has been processed, starting with RNA extraction, in the same manner as samples and other external controls. The external control should be negative for any PCR batch run. If the result is positive then all results are invalid and contamination of reagents or the environment must be considered.

The External positive control: criteria for acceptability

The External Positive control serves to confirm the validity of the RNA extraction, cDNA sythesis and PCR. The permissible range for External Positive control Ct values is dependent on the batch. During PCR optimization of each new External Positive control batch, the mean Ct value for that batch is determined according to the "Optimizing Internal-External controls for PCR" protocol (*TP-TO-DNG-008*), with results recorded in the "Optimizing Internal and External Control Result" form (*FM-TO-DNG-024*) and the Culture Control Batch Number Index for each of the four serotypes:

- DENV-1 Culture control batch number index *FM-TO-DNG-019*
- DENV-2 Culture control batch number index *FM-TO-DNG-020*
- DENV-3 Culture control batch number index *FM-TO-DNG-021*
- DENV-4 Culture control batch number index *FM-TO-DNG-022*

An acceptable Ct value for External Positive control is within 10% of the mean value determined in the optimization experiments. If the External Positive control Ct value falls outside the allowable range, then the result must be examined. The range or mean of External Positive control in each batch of samples should be recorded in the report file of "Dengue Viral Load Calculation template" *FM-RR-DNG-032*.

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The External Specificity Controls: criteria for acceptability

The External Specificity controls are DENV culture supernatants that are not matched to the PCR reagents being used for that particular run. For example, if the PCR target is DEN-2, then DENV-1, -3 and -4 serve as specificity controls. The External Specificity controls should be positive for any PCR batch run. If the result is positive for any non-target serotype then all results are invalid and contamination of reagents or the environment must be considered.

12. Reference range and critical value:

The Standard Curve: criteria for acceptability

The standard curve for quantification of DENV concentration in the samples is prepared according the protocol "Preparation of standard curve for dengue PCR" (*TP-TO-DNG-009*). After amplification of the standard curve in the same run as the samples, the Light Cycler system automatically determines the line of best fit for the standard curve Ct values using a polynomial equation.

The standard curve must be checked for PCR efficiency (the slope of the curve); efficiency must be >1.7. The accuracy of the standard must be checked (e.g. how well do the duplicates correspond); accuracy must be <0.2. If the standard curve fails one of these measures then the assay batch is not valid for quantification (but may be OK for serotype detection) and must be repeated.

13. Safety

Universal precautions are used when working with clinical specimens. When working with chemicals, always wear a suitable lab coat, disposal gloves, and protective goggles. Always use PERSET and Ethanol 70% to clean pipette and working area. Place any waste in the clinical waste bins provided.

14. Risk assessment and risk management:

Department	**Persons involved**
Dengue group	Dengue staff

Location of work Room215A+215B (oucru lab)	
Description of procedure The procedure describes how to serotype DENV by using quantification RT-PCR	**Substances used** N/A
Quantities used N/A	**Frequency of use** Daily
Hazards identified N/A	**Could a less hazardous substance be used instead?** Yes / NO.
What measures have you taken to control risk? **PPE:** i. Designated gown for BSCII work ii. Gloves iii. Cover shoe	
Checks on control measures N/A	
Is health surveillance required? NO.	**Training requirements:** i. BSCII. ii. Centrifuge iii. Waste disposal iv. Accident reporting procedures v. Spill clean up procedures vi. Sign and symptoms of infection
Emergency procedures: i. Contact supervisor ii. Contact lab management group i Biological and chemical spill cleanup procedure	**Waste disposal procedures**: 1% Presept for 30 minutes then autoclave 121°C/30 minutes. OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

15. Related Documents:

Document name	Document code
Mosquito homogenisations	*TP-TC-DNG-058*
Separation of plasma from blood by centrifugation	*TP-SM-DNG-002*
RNA extraction using QIAamp Viral RNA mini kits	*TP-TC-DNG-003*
Preparation of EAV for internal control in dengue PCR	*TP-TC-DNG-006*
Preparation of standard curve for dengue PCR	*TP-TO-DNG-009*

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Optimizing internal and external controls for dengue PCR	*TP-TO-DNG-008*
Preparation of Primers & Probes	*TP-TO-DNG-078*
Preparation of EAV Primers & Probes	*TP-TO-DNG-014*
Optimizing internal and external control results	*FM-TO-DNG-024*
Dengue Viral Load Calculation template	*FM-RR -DNG-032*
DENV-1 Culture control batch number index	*FM-TO-DNG-019*
DENV-2 Culture control batch number index	*FM-TO-DNG-020*
DENV-3 Culture control batch number index	*FM-TO-DNG-021*
DENV-4 Culture control batch number index	*FM-TO-DNG-022*

16. Document History:

Revision #	Effective date	Details of amendment
00	18-DEC-2009	New issue
001	01-JUN-2010	Using new DENV2 primer, probe. Separate DENV2 and DENV4 PCR assay. Correct volume using for each reaction. Use the Dengue Viral load calculation template
002	01-JAN-2011	Changed the criteria for acceptability of internal control and external control. Added the LOD value of each DENV standard curve. Combine the 2nd and 3rd multiplex assay. Deleted quality control charts because no longer used: FM-TO-DNG-012, FM-TO-DNG-013, FM-TO-DNG-014, FM-TO-DNG-015
003	20-JUN-2013	Corrected primer sequences.
004		Reformatting, add reference.
005	07-AUG-2014	Reformatted and edited language. Corrected primer sequence for DENV-1 reverse primer. Removal of section on calibration of colour compensation, to become a stand-alone SOP. Added steps on sample

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		preparation prior to PCR. Updated all reference SOPs – Lauren Carrington.
006	01-OCT-2015	Added Risk Assessment, updated Principle – Lauren Carrington
007	29-MAR-2018	Transferred to new OUCRU SOP template, update document code. Checked for accuracy. – Huynh Le Anh Huy & Lauren Carrington

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1. Purpose

This procedure describes how to hatch, rear and maintain colonies of *Aedes* mosquitoes, derived from either the field or already established colonies, for the development of eggs for both experimental purposes and further colony maintenance.

2. Principle:

Eggs entering the laboratory may be derived from field material, or originate from collaborators' insectaries. This SOP outlines the two approaches to rearing these mosquitoes, with appropriate consideration of associated safety issues.

For colonized mosquitoes, *Aedes* eggs are hatched and reared to adulthood where they are blood fed daily, in order to produce eggs for the subsequent generation. The procedure describes the steps required for maintaining and producing large, healthy individuals at each life stage, and collection of eggs in order to maintain a colony with multiple generations.

For field-derived material, the process of establishing a colony (which is confirmed to be virus-free) from the field is outlined. Some parts of this procedure vary slightly from the standard rearing method (including the blood feeding method), and so changes are outlined here in detail where they differ.

Note: This procedure applies to rearing both *Aedes aegypti* and *Aedes albopictus*

3. Scope and responsibilities:

This procedure is to be carried out within the OUCRU insectary, HCMC. People performing the work will be trained by senior insectary staff from the Dengue Group. Insectary staff are responsible for making sure all container labelling is accurate and that mosquitoes are suitable for use in experiments. Senior insectary members are responsible for making sure all directives are followed by staff members and worksheets are completed as required with good laboratory practice.

4. Definition

- OUCRU: Oxford University Clinical Research Unit
- HCMC: Ho Chi Minh City

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- G_0, G_1, G_2… Generation 0, 1, 2…
- F_0 mosquitoes Larvae or adult mosquitoes directly from the field
- F_1 mosquitoes Mosquitoes that are the first generation reared under laboratory conditions
- Out crossing The practice of regular introduction of field-derived males into a breeding line. This is performed avoid inbreeding (reduced genetic diversity) in the population, and keep the genetics of the colony as representative as possible of that in the field.
- Backcrossing The process of crossing a hybrid with one of its parents (or an individual genetically similar to its parent), in order to achieve offspring with a genetic identity which is closer to that of the parent. With each subsequent generation of backcrossing, the genetic similarity of the progeny will be closer and closer to that of the target parental line that it has been crossed with. After 5 generations, the progeny will share >95% of the nuclear genetics with the parental line.

5. Specimen requirement

- From field collections: *Aedes aegypti* and *Aedes albopictus* eggs, larvae or adult mosquitoes, collected from the field (either within HCMC, or field sites). Any field material entering the lab is considered the founding generation (F_0) of the colony.
- From the OUCRU Insectary: *Aedes aegypti* and *Aedes albopictus* eggs laid by a parental generation, already maintained as a colony within the lab.
- From collaborators: *Aedes* eggs on filter paper.

6. Materials/ Equipment used

- Fresh water
- Larval food (pork liver, shrimp, dried dog food)
- Plastic trays for larval rearing
  - Large: 23 cm x 13cm x 8 cm (Volume ~ 2.4L)
  - Small: 15 cm x 9 cm x 8 cm (Volume ~ 1.2L)

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- Plastic cups for pupal collections (Volume ~ 200 ml)
- Mesh squares
- Rubber bands
- Cotton squares
- Sucrose/glucose solutions
- Incubator (set at 28°C, light 12:12, humidity 70-80%)
- Adult mosquito cages (30cm x 30 cm x 30cm)

7. Procedures

7.1 For strains already maintained in the lab

Note: All stages of immature development and adult maintenance of these mosquitoes is performed in the clean room (Incubators #1 and #2 only), with a temperature of 28°C, 12:12hr light:dark cycle and 70-80% relative humidity.

7.1.1 Floating eggs and rearing larvae

1) Estimate the number of viable mosquitoes eggs on a strip of paper, based on strain and storage time (1-3 months) due to the hatching rate of eggs. After 3 months, the viability of eggs drops significantly, so all eggs should be hatched before this time.

Storage time	**Hatching rate (%)**	
	WT	**wMel**
< 1 month	~ 80%	~ 75%
1 month - 2 months	~ 70%	~ 50%
3 months	~ 50%	~ 10%

2) Filter papers containing eggs are floated into fresh water, based on the size and the volume of water.
   - Large size: 1.5 L water (~10000 eggs)
   - Small size : 0.75 L water (~5000 eggs)
3) Labelling the tray accordingly. An example, and its explanation follows:

"Number of generation, tray, strain of mosquitoes, date"

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Example: *Generation 23, Tray #1, Aedes aegypti from HCMC, infected with Wolbachia, floating on 25-10-2018* would be written as:

Generation # *23*	Mosquito strain *15*
Tray # *I*	Floating date *25 / 10 / 2018*

4) The table below summarizes the amount of food given to larvae at each development stage.

Development Stages	Condition	Food	Note
Day 1: Floating Eggs	Put the filter paper containing mosquito eggs in 1.5 L water (~10000 eggs) = ~ 7 eggs/ml	Do not need any food (because the eggs have not yet hatched and food will not be eaten, leading to the water becoming dirty and smelly)	It takes ~24 – 48hr for hatching, depend on the storage time of eggs, and the strains of the mosquitoes being reared
Day 2: Hatching Eggs		100mg tablet of dog food per tray	Take the filter paper containing hatched eggs out of the tray
Day 3 and 4: Controlling larval density	Split into 3 trays (density ~ 3300 - 3500 larvae / 1.5L = 1 larvae/0.45 ml of water)	100mg tablet of dog food per tray	
Day 5 and 6: Control the larvae density	Continuously split and control the density of larvae (each tray contains ~ 500 larvae in 1.5L water = 1 larvae / 3 mL water	~200mg boiled shrimp + ~200mg boiled pork liver each day	

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Day 7 to 10: Pupae collecting		Add small amount of food (~50mg boiled shrimp + ~50mg boiled pork) to maintain the remaining larvae because pupae no longer feed	The males will pupate first, followed by the females, ~ 24-36 hrs later

5) At all times, every tray needs to be covered by the net to prevent mosquito escape, as well as any loose mosquitoes in the incubator laying fresh eggs in the trays.
6) Water needs to be changed daily the fresh water daily, except the first 2-3 days because of small larvae. To do this, gently remove ¾ the amount of old water, pouring it into the sink, carefully keeping the larvae and pupae in the corner of the tray (they will migrate to corner of the tray naturally). Re-filled the tray with fresh water, to the same approximate volume as previous.
7) The larvae are maintaining in the incubator #1 or #2 in the clean room at 28°C, with a 12:12hr light: dark cycle, humidity 70-80%

7.1.2 Collecting pupae

8) Females develop slower than males, and are larger in size. For this reason, it is normally easy to determine the sex of the mosquito before they emerge as adults.
9) After ~7-10 days, larvae start to pupate. The timing of pupation will depend on temperature, amount of food and density of mosquitoes. When larvae start to become pupae, it is not necessary to add any additional food, because the pupae no longer feed.
10) The following table describes how to collect pupae, depending on the future purpose of the mosquitoes.

Purpose	Preparation	Example
Experimental work (i.e., blood feeding or inoculation)	Transfer the pupae into the plastic cup with 20ml of water and cover by the net.	**For inoculation:** 45 female pupae/cup **For membrane feeding:**

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	The number of pupae depends on different experiment, but in general, will not normally exceed 45 females per cup.	45 female pupae + 2 male[1] pupae/cup
For colony maintenance: Closed colonies	Mosquitoes can be sexed at the pupal stage. Transfer the required number of pupae of each sex in the same generation into mosquito cage (400-500 pupae/gender) and maintain mosquitoes for 4 weeks by both blood directly feeding and 10% sucrose solution.	For producing F_2 closed colony from F_1 mosquitoes: - 400 female pupae of F_1 - 400 male pupae of F_1
For colony maintenance: Backcrossing (To achieve offspring with a genetic similar to its parent, normally performed for backcrossing Wolbachia onto a new background)	Females = 100% from the (hybrid) Wolbachia-infected population. Males = 100% from the target population for the crossing. Let these emerge into the same cage. They will mate, and the resulting population will be closer in genetics to the male population than previous. This is repeated, with each generation of progeny becoming more similar to the target wild type population. Feed with direct blood feeding.	Collect 500 female pupae from the Wolbachia-infected population, and 500 male pupae from the wild type population.

[1] Adding 2 male pupae into the cup to increase the engorgement rate of mosquitoes

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For colony maintenance: Outcrossing (To keep genetic diversity in the population, we perform outcrossing with 10% of male mosquitoes in the field)	Assuming the population is already backcrossed, or genetically diverse, outcrossing maintains that diversity. All females will be from the same population. Count how many male pupae are available from that same population. Prepare 10-20% MORE males, from the F_0/F_1 wild type population. Merge these two sets of males together for emergence. The inclusion of wild males allow the females to mate with them. This is repeated, every 2 generations, to avoid inbreeding/laboratory adaptation. Feed with direct blood feeding.	Collect 500 female and 450 male pupae from the Wolbachia-infected population. Collect 50 male pupae from the wild type F0/F1) population[2]. Let these individuals emerge into the same cage.

7.1.3 Emergence of adults

11) Pupae emerge into adult mosquitoes after ~48 hrs (this will be slightly longer for females).
12) If pupae are being used for experimental purposes, they will be in a cup. Once all adults have emerged, remove the water inside the cup. To do this, simply tip the water through the mesh screen.
    - The exuviae (pupal casings) will remain in the netting.
    - The interior of the cup will soon dry.
    - The adults remain inside the cup.

[2] It is important that the males from both populations are the same age when they are released into the cages, and that are released synchronously. This is because of the timing and frequency of male mating. Males are ready to mate sooner than females, and so if they are released at different times, or are different ages when released, they may have a disadvantage in mating. This will mean that the outcrossing processing was not effective.

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13) If pupae are being used to start a new generation of a colony, they will be in a cage. Once all the adults have emerged, remove the cup containing the water and remaining pupal casings. *Be careful not to let mosquitoes escape when removing the cup from the cage.*
14) Adults are fed with: one cotton ball wet with 10% sucrose solution; one cotton ball wet with water.

7.1.4 Maintaining adult mosquitoes

7.1.4.1 Mosquito maintenance for experiment

15) Mosquitoes are normally maintained with wet cotton wool, (10% sucrose solution, and water).
16) We use mosquitoes at 1-2 days age for the experiments.
    - If mosquitoes will definitely be used for experiments the next day, just give water.
    - If mosquitoes will not be used in experiments yet, give mosquitoes 10% sucrose and water. Be sure to remove the sucrose from the mosquitoes 24 hrs before they will eventually be used in experiments. This is to ensure they are starved, and will be able to feed/receive inoculum in intrathoracic inoculations.

7.1.4.2 Blood feeding of mosquito colonies:

17) For colony maintenance, mosquitoes will be provided with access to the arm or fingers of a volunteer for feeding. Precise details of this procedure are in the Direct Blood Feeding SOP (*TP-TO-DG-053*). In addition to this, they will have access to water and 10% sucrose *ad libitum*.
18) Normally, the mosquito colonies are fed ~3 times/week.
19) The number of mosquito eggs laid will depend on the frequency of blood feeding. If maximal egg output is required[3], the frequency of feeding can be increased to every day.
20) Volunteers help perform direct blood feeding, because *Aedes* mosquitoes are well adapted to human blood, and have higher fecundity when feeding on human blood than other blood sources.

[3] For experimental purposes or to build up mosquito population size of recently collected field females for example

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21) One single volunteer is responsible for feeding a single cage for the duration of the cage maintenance. Their name will be labeled on the cage clearly, and **no one else is to feed a cage that does not have their name on it**. More details about volunteer feeding and the steps taken to avoid cross-feeding can be found in "Informed consent form for blood feeding of mosquitoes by healthy volunteers".

Cage # 1	Generation G23	Mosquito strain *15*
Emergence date *25 / 10 / 2018*	*Volunteer name* *LONG*	

22) Before feeding for the first time, volunteers must sign an informed consent form before feeding. Every time the volunteer feeds, they must ensure their tympanic temperature is <37°C, and this is to be recorded in the form named "*Tympanic temperature recording for volunteers suppling blood to mosquitoes*", placed in "*Volunteer Feeding mosquitoes*" folder in the insectary document cabinet (in the clean room).

7.1.4.3 Collecting eggs for the next generation

23) The length of the gonotrophic cycle is about 2-3 days. Normally *Aedes aegypti* mosquitoes feeds >1 time per gonotrophic cycle, therefore mosquitoes should be offered a blood meal about every 2-3 days (~3 times/week)
24) Strips of filter paper are placed inside the colony cages, inside a cup containing about 7 mL of water. The water should be ~1/2 way up the height of the paper. The mosquitoes will lay their eggs just above the water line.
25) These eggs are collected after every 2 days, by carefully reaching into the cage, and pulling out the cup. It is important here not to let any mosquitoes escape the cage when removing the cup.
26) Remove the strip of filter paper and replace it with a fresh strip. Fill up the water in the cup as needed. Replace the cup back into the cage to collect more eggs.
27) The old strip of filter paper (that contains the eggs) are dried at room temperature for 1 day in the labelling individual box to avoid eggs of different mosquito stain contaminating each other

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28) Then we kept the filter paper containing the eggs in a small ziplock bag, which sits inside a closed plastic box. Keep these ziplock bag open. Inside the box with a small wet cotton wool, to maintain humidity in the box.
29) The wool is re-wet every ~2 days for the duration of the eggs storage. If the box is not humid inside, the mosquito eggs will dry out, and not be viable to hatch.
30) Each box will be labelled based with the mosquito strain, generation number, the date the eggs were collected, and who fed the adult mosquitoes.

Generation G23 Mosquito strain *15*
Date eggs collected *25 / 10 / 2018*

7.2 For strains being colonized from field collections

Note: All stages of immature development and adult maintenance of these mosquitoes is performed in the clean room (Incubators #1 and #2 only), with a temperature of 28°C, 12:12hr light:dark cycle and 70-80% relative humidity.

7.2.1 F0 generation

1) After collection of larvae from the field, they are brought into the insectary. All larvae (of all life stages) caught fresh from field into a plastic tray filled with 0.5L fresh water. Each strain is maintained in different tray, with a different label[4].
   - These are F_0 mosquitoes.
2) Do NOT change the water daily (unlike in normal colony rearing). This is because mosquitoes will be in variable stages of development, and it can be easy to lose the small larvae when we change water. Change the water[5] only after a few days, when it is clear that no small larvae will be lost.

[4] If mosquitoes are thought to come from different populations, these should be maintained in different trays (for example, wMel and WT mosquitoes from Tri Nguyen and Nha Trang). Even if there may be some uninfected mosquitoes in the wMel population, setting up experiments with different strains is best done by separation.

[5] Some experiments require that water is not changed at all, and that no food is added to the trays. If this is the case, this will be clearly indicated in the experimental design, and must be followed.

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3) Add ~200mg boiled shrimp and ~200mg boiled pork liver to the larval trays if the density of larvae is about 1 larvae/ 3 ml. The amount of food depends on he density of larvae
4) Pupae are collected daily from larval trays, and adults emerge. These adults are F_0 adults.
5) Adults mosquitoes are to be checked that they are the correct, desired species for colonization. Unwanted species are to be discarded in accordance with the General Insectary SOP (*SD-MM-DNG-001)*.
6) All F_0 females **must be fed via membrane feeders** to avoid the risk of vertical transmission of viruses from field mosquitoes to their progeny. When mosquitoes are 3 days old, they are blood fed using human blood from a non-febrile, healthy person, through membrane feeders. Procedures for performing artificial membrane feeding are described in detail in *TP-TO-DG-051*.

7.2.2 F1 generation

7) Eggs are collected from the adults to establish the next F_1 generation. The F_1 progeny eggs of the field derived mosquitoes are reared to adults (see **Section 7.1** of this SOP), and again fed on human blood via membrane feeders.
8) The F_1 adult females are allowed to lay F_2 eggs, which are collected, for ~4 weeks.

7.2.3 F2 generation

9) F_2 eggs are hatched and reared to adults, as above.
10) When they emerge as adults, 10% of the adults will be sacrificed, pooled into groups of 40-50 mosquitoes, and screened for DENV, CHIKV and ZIKV by RT-PCR. This screening should be performed as soon as possible after emergence.
11) If the subset of F_2 mosquitoes are confirmed free of viruses, the remaining adults are now allowed to be used in direct feeding. F_2 females will mate and lay F_3 eggs, which will be used in subsequent experiments (for colonization or for experimental purposes, which may involve feeding on patients, if *approved* experimental designs dictate).

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8. Quality Control

- Individual data loggers are placed inside the insectary and within the rearing chambers to makes sure temperature is optimal for mosquito rearing. We check data logger every two weeks, but if we discover any problem with incubator, we check data loggers immediately and transfer mosquitoes to other incubators if the problem is serious and has been ongoing. If possible, check with the supervisor and report the problem before making any major movements of mosquito cages or trays.
- **Mosquito colonies are screened by RT-PCR every generation to check the Wolbachia infection status of mosquito strains**. A subset of 40 adults are collected after emergence and tested for infection in a standard duplex PCR (targetting *Aedes aegypti* & *Wolbachia*).
  - It is important that cross-contamination of Wolbachia mosquitoes into uninfected lines, or uninfected mosquitoes into Wolbachia infected lines DOES NOT HAPPEN. The integrity of our experiments depends on clean, uncontaminated lines, which means careful handling of eggs, larvae and adults both in the incubators and when feeding colonies in the main insectary room.
- To ensure eggs don't contaminate other lines, only one set of eggs should even be on the bench at any one time. When finishing work with that strain of eggs, remove the papers, the bags and all associated packaging. Wipe down the bench with ethanol so the bench is clean. Only then should another strain of mosquito eggs be brought to the work bench.

9. Limitations:

- The hatching rate of eggs will be influenced by how long the eggs are being stored. It is recommended to keep mosquito eggs for no longer than 3 months to maintain acceptable viability of the eggs.
- Keeping mosquitoes as closed colonies in the insectary for extended periods can lead to reduced genetic diversity in the population. To minimize the negative effects of this by outcrossing. This is performed in accordance with experimental requirements, which will be indicated to staff when a new experiment is being established. More detail about backcrossing is described in **Section 11: Supplementary Notes.**

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10. Safety

- Minimize mosquito escape by covering all trays and cups by net.
- When adding or removing objects or hands from a colony cage, be sure to carefully shake the netting to disturb any resting mosquitoes. They will fly away, and their escape should be minimal.
- Ensure no holes in the netting or wire mesh of the mosquito cages that will alow mosquitoes to escape. Any holes must be fixed immediately upon detection (if the cage is currently in use), or that cage is to be removed from operation until it is fixed permanently.
- If one or more mosquitoes are released, it is essential that all personnel in the insectary are alerted immediately to the situation. As a priority, all available personnel must:
  - Temporarily stop their work at the soonest possible moment, secure any mosquitoes they are working with themselves
  - Assist other staff in the room to find, and kill the escaped mosquito(es).
- Appropriate PPE (a minimum of lab coats, head cover, goggles, mask, socks and shoes, long trousers, and gloves) is to be worn at all times while in the insectary and undertaking these procedures.
- Any waste generated will be disposed of in the appropriate waste receptacle.
- Special care should be taken not to spill the large volumes of water that are used for larval rearing, as this presents both a slip hazard, and a potential loss of experimental material..
- The extra larvae/pupae are killed by adding 10% bleach in a cup. This should take a few hours for all immatures to be killed. Then put in the contamination bin

11. Supplementary notes

- The flowchart below summarizes backcrossing to produce a hybrid with one of its parents (or an individual genetically similar to its parent), in order to achieve offspring with a genetic identity which is closer to that of the parent.
  - The flowchart used as example of replacing the Wolbachia infection from mosquitoes from Australia onto a HCMC nuclear genetic background.

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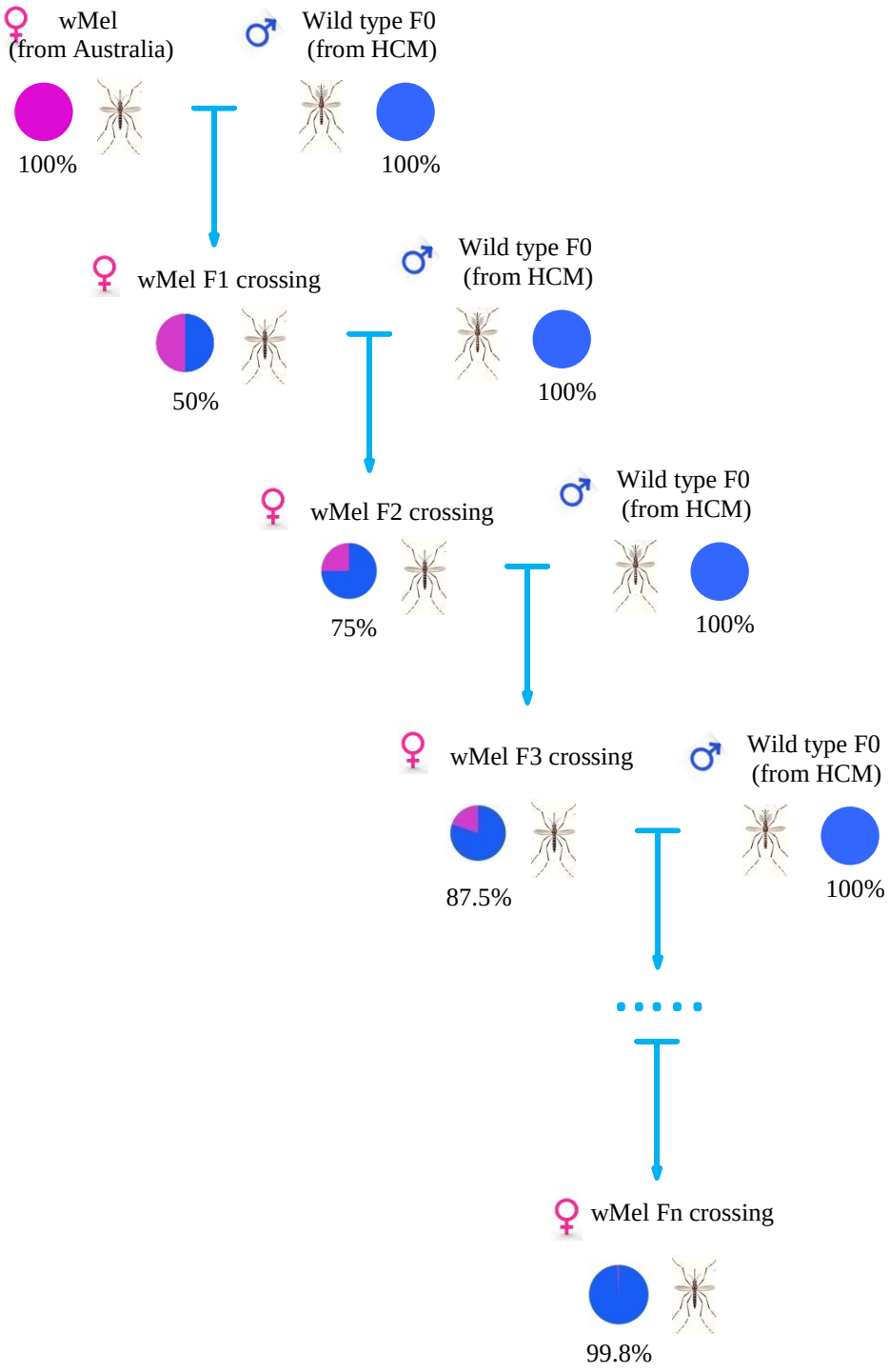

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12. Risk assessment and risk management:

<table>
<tr><td colspan="2">COSHH Risk Assessment COSHH risk assessment - University of Oxford COSHH Assessment Form</td></tr>
<tr><td>Description of procedure
Rearing Aedes Mosquitoes</td><td>Hazardous substances used
Mosquitoes – vectors of arboviruses</td></tr>
<tr><td>Quantities used
Many hundreds</td><td>Frequency of use
Daily</td></tr>
<tr><td>Hazards identified
Mosquitoes may bite humans (and will, as part of direct feeding for colony maintenance)
Mosquitoes from the field may contain infectious pathogens
Mosquito escape</td><td>Could a less hazardous substance be used instead?
No</td></tr>
<tr><td colspan="2">What measures have you taken to control risk?
Appropriate PPE provided (lab coat, gloves, eye protection; SOP of Universal precaution and Personnel Protective Equipment guidelines for working in the laboratories, PR-HS-AL-006).
Mosquitoes that are reared for colony maintenance but sourced recently from the field are fed on artificial sources for the first 2 generations, because of the risk of vertical transmission of mosquitoes (even though this risk is small). Before the second generation is fed directly on humans, a subset of the adults are screened for arboviruses (ZIKV, DENV and CHIKV) to check for infection. .Only when they are found clean of pathogens can the staff feed the remaining mosquitoes from that generation for colony maintenance.
Staff are trained in procedures to minimize escape, and all understand the importance of direct and indirect blood feeding at the appropriate generations after colonization.
Staff are trained in the special mosquito handling instructions, as indicated in the Safety section above,</td></tr>
<tr><td colspan="2">Checks on control measures
Regular screening of mosquitoes, with the record of these screens logged.</td></tr>
<tr><td>Is health surveillance required?
Yes, Hepatitis B serology.</td><td>Training requirements:
Good laboratory practice
Specific training in this procedure
(with associated documentation of training by staff and supervisor).</td></tr>
<tr><td>Emergency procedures:
Spills: follow “biological and chemical spill clean up procedure” PR-HS-ALL-005.
Staff must report incidents to supervisors immediately.</td><td>Waste disposal procedures:
OUCRU BSL2 manual of waste management SD-HS-ALL-009.</td></tr>
</table>

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13. Related Documents:

Document name	Document code
Mosquito egg hatching and pupae collection	*FM-TC-DNG-024*
General Insectary SOP	*SD-MM-DNG-001*
Direct Blood Feeding	*TP-TC-DNG-053*
Artificial membrane feeding	*TP-TC-DNG-051*
RT-PCR targeting DENV, Wolbachia and *Aedes aegypti*	*TP-TC-DNG-074*
Informed consent form for blood feeding of mosquitoes	
Volunteer mosquito feeding – temperature recordings	

14. Document History:

Revision #	Effective date	Details of amendment
00	20 Mar 2011	New issue
01	01 Nov 2012	Modify the protocol for general usage
02	18 Aug 2013	Modifications for clarity and improved detail, addition of food schedule. Altered title – Lauren Carrington
03	16 Sep 2014	Removed unnecessary headings, updated Materials and Equipment used, added section of management of risks – Lauren Carrington
04	15 May 2015	Added Risk Assessment and reformatted – Lauren Carrington
05	28 Mar 2018	**Principle:** More detail provided about the steps involved in the procedure itself, and the fact that this differs slightly between already-colonised material, and that which is field-derived. **Procedures:** Created separate, detailed descriptions for mosquitoes already in colony *vs.* those recently colonized from the field. Highlighted the importance and necessity of

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		indirect blood feeding for F0 and F1 mosquitoes. Clarified feeding schedule of mosquitoes in accordance with developmental stage. General improvement of level of detail. **Quality control:** Added points on quality control for screening strains and to avoid contamination of linces. Supplementary notes: Described outcrossing, how and when it should be used for colony maintenance. **Safety:** Changed the screening procedures for virus screening from JEV to ZIKV (still in addition to DENV + CHIKV). **Related documents:** Added additional related documents in line with more detailed procedural information above. – Lauren Carrington

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MOSQUITO HOMOGENISATIONS

1. Purpose

This procedure describes the process of the mosquito homogenisation (both whole mosquitoes and dissected tissues) to prepare mosquito homogenates, that can be used for subsequent RNA/DNA extraction and PCR, or in some instances for inoculation into whole mosquitoes (after filtration).

2. Principle:

Whole mosquitoes or dissected tissues (abdomen, head/thorax) are harvested and distributed to individual wells of the 96-well plates or screw tube. A number of small beads are added to the tubes/wells, which will macerate the tissue during mechanical shaking. These samples can be stored at -80°C either before or after the homogenisation process. The samples must be centrifuged after homogenisation, in order to pellet the tissue debris in the tube. It is the supernatant after centrifugation that is used in subsequent PCRs and inoculation steps.

During homogenisation, the mosquito tissues are soaked in either mosquito diluent (MD) or extraction buffer which helps to preserve any genetic materials (DNA/RNA) of the interested targets. MD is used when preparing mosquitoes for purification of nucleic acids and when planning to inoculate that homogenate into naïve mosquitoes. A simple extraction buffer is used when mosquitoes will be crudely screened for DENV (or other arboviruses) or Wolbachia, for example.

3. Scope and responsibilities:

This procedure will be carried out by dengue group members who perform molecular biology work. Homogenisations are performed in Room 111, using the Qiagen Tissue Lyser II and Eppendorf 5810R centrifuge, both located within the room. Only trained people are allowed to implement the procedure. Senior staff are responsible for ensuring this procedure is strictly followed.

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4. Reference

- Anderson, S.L., Richards, S. L., Smart, C. T. (2010). A simple method for determining arbovirus transmission in mosquitoes. Journal of the American Mosquito Control Association 26:108-11.
- SOP for Adult mosquito screening using TaqMan assay (384-well format) of Eliminate Dengue Program, Monash University, Australia, 2016.

5. Definition

- MD: Mosquito Diluent
- BSL-2: Biosafety Level 2
- FCS: Fetal Calf Serum
- DENV: Dengue virus
- Squash buffer: Reagent used for DNA extraction process. It contains TRIS which is a reagent to maintain a stable pH for cell lysis.
- Proteinase K: Proteinase K is used to digest the proteins present in the sample while extracting the DNA. These proteins contaminate the DNA.

6. Specimen requirement

Any mosquitoes (whole or partial) that are to be subsequently used in PCR or inoculation procedures. Normally, the samples are Aedes mosquitoes that:

- have been exposed to potentially infectious blood samples; or
- those collected from either the field or laboratory colonies, for routine screening.

Samples will be obtained either as fresh mosquito tissues or they will have been stored in the freezer prior to processing. It is to be assumed that all mosquitoes are infected and therefore samples are to be treated as infectious materials at all times, as a safety precaution.

7. Materials/Equipments used

- Tissue Lyser — Qiagen, Cat. No. 85300
- 1mm Zirconica/Silica beads — Biospec, Cat. No. 11079110Z
- 2.5mm Zirconica/ Silica beads — Biospec, Cat. No. 11079125Z

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o Centrifuge	Eppendorf
o Cell culture medium	RMPI-1640 (Sigma, Cat. No. R0883-500ml)
o Penicillin-Streptomycin	Invitrogen, Cat. No. 15140122
o Gentamycin	Sigma, Cat. No. G1397-100ml
o Fungizone	Gibco, Cat. No. 15290-018
o Heat-inactivated Fetal Calf Serum	Sigma, Cat. No. F7524 (Inactivated at 56°C for 30 minutes)
o Glutamine	Invitrogen, Cat. No. 25030032
o TRIS base (MW 121.14)	
o EDTA (MW 372.24)	
o NaCl (MW 58.44)	
o Proteinase K	(it is available from MagNA Pure 96 DNA and Viral NA Small Volume Kit) Cat. No: 05467497001

8. Procedures

We are using two kinds of diluents which are classified by our purpose:

- MD is used for: high-quality DNA/RNA extractions & inoculations
- Extraction buffer: crude screening of mosquitoes

8.1. Preparation of mosquito diluent (MD)

This diluent must be prepared in a safety cabinet (BSL-2) to avoid the contamination and then stored at 4°C for long-time usage.

1) Take a fresh bottle of 500mL RPMI
2) Remove 26mL of the media
3) Add to that same bottle:
    - 10mL Heat-inactivated FCS
    - 5mL Glutamine
    - 5mL Fungizone
    - 5mL Penicillin-Streptomycin
    - 1mL Gentamycin

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8.2. Preparation of extraction buffer (Squash buffer + Proteinase K)

This buffer is prepared with proteinase K and squash buffer in a 1:160 ratio and then stored at 4ºC for up to 1 week.

1) To make the Squash buffer, mix together in a bottle:
   - 0.485g TRIS base (MW 121.14)
   - 0.149g EDTA (MW 372.24)
   - 1.169g NaCl (MW 58.44)
2) Make up in 400 mL of water and adjust pH to 8.2, then autoclave.
3) To prepare the final Extraction Buffer, Proteinase K needs to be added to the Squash buffer, in a ratio of 1:160.
   - For example, to make 200mL squash buffer, 1.25 mL proteinase K is added,

8.3. Homogenisations

Mosquito homogenisations can be processed whether mosquitoes were collected into the individual wells of a 96-well plate, or in screw cap tubes. The plate format normally is set up for collection of the individual mosquitoes, dissected tissues; the tube format is applied to collect pooled samples (5-7 mosquitoes).

8.3.1. Sample homogenisations in plate format

- Bring the mosquito sample plates to room temperature if they were stored in freezers.
- Before open the 8-strip optically clear flat caps, make sure that all samples are in the bottom of the wells by quickly centrifuge the plates and use a pen to label the order numbers of the caps.
- Open the caps and lay them separately on a clear tissue, add carefully 5-7 of small beads (1mm) into each of the wells (marked as a beaded plate). Do not touch on the well during adding beads. If the big beads (2.5mm) are used, we just add one bead into each well because if there are many beads they can be stuck in the caps while homogenisation. It is better to use small beads for plate homogenisation.

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- Prepare MD or extraction buffer followed the above instructions (sections 8.1 and 8.2). In a falcon tube, prepare enough buffer for the number of mosquitoes to be extracted plus 10% more.
- Each mosquito samples (abdomen, head/thorax or whole mosquito) is extracted in 100µL MD/extraction buffer. Aliquot 100µL buffer per well into the beaded plate.
- Re-seal plates with caps carefully and store in the 4°C until required for extraction (it should be extracted within the same day otherwise PCR signals might degrade).
- Place the plates into adapters formatted for plate and then insert the adapters in the clamps. Screw the clamps tightly. Always make a balance between the left and right clamps of tissue lyser machine during homogenisation.
- Turn on the tissue lyser machine and adjust the homogenisation program (30 Hz, 5 mins) and then start the program.
- After homogenised, move the plates out the clamps and centrifuge at maximum speed for 1-2 minutes to remove liquid from caps. Look at the plates carefully to make sure that all samples are homogenised. If any sample still not be homogenised, the plate should be run the second time.
- There are 2 kinds of buffer are using: MD or extraction buffer.
    - If **extraction buffer** is used, the homogenised sample plate is incubated at 56°C for 10 minutes, then 98°C for another 15 minutes to deactivate the proteinase K, then cool down to 12°C. All of this is performed in a 96-well thermocycler. Then, stored at 4°C for up to 3 days for running the PCR.
    - If **MD** is used to homogenise the mosquitoes, the inactivation of Proteinase K will not be performed, because the homogenates will be used for purified DNA/RNA extraction or inoculation procedures in later step.
- Label plate as “heated” then store the plates in 4°C fridge until PCR for up to 3 days. After that the homogenates should be stored at -80°C.

8.3.2. Sample homogenisation in tube format

- Bring sample tubes to room temperature if they were stored in freezers

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- Use a pen to label the numbers on the screw caps to reduce the confusion for the order of the tubes.
- Add 5-7 of big beads into each tube.
- Then add 800µL MD/extraction buffer in each tube.
- Ensure that all tubes are securely tightened, then place the tubes in order into the adapters which formatted for tubes. Each adapter can be used for 24 tubes and then attach into the clamps of the tissue lyser. Screw the clamps tightly. **Always make sure the two arms of the tissue lyser are balanced (the weight of the plates between the left and right clamps of machine must be even).**

  Note: If processing an odd number of samples, prepare a spare tube, with the same volume of liquid, and the same number of beads to use as a weigh t balance.
- Turn on the tissue lyser machine, set the program (30Hz, 5mins) and start.
- After homogenised, the tubes are move out and spin them down to pellet the debris. If the mosquito is still recognisable, homogenise the sample again for a further 2 mins.
- After the 2nd cycle of homogenisation, spin the samples down again, and double check to see that the mosquitoes are macerated and unrecognisable.
- After homogenised, 50µL of homogenates are aliquoted into 96-well plates to deactivate proteinase K in the same way as section 8.3.1 if using extraction buffer; or the homogenates are used for purified DNA/RNA extraction if using MD buffer as section 8.3.1
- Label plate as “heated”, then store in 4°C fridge until PCR for up to 3 days. After that stored at -80°C for long-time usage.

8.4. Collected homogenates

- Mosquito homogenates extracted by extraction buffer are now ready for crude PCR screening procedures (see SOP *TP-TC-DNG-074*).
- Homogenates in MD are now available to inoculate mosquitoes and process nucleic acid isolation to collect purified DNA/RNA for PCR, positive controls and sequencing techniques (see associated SOPs *TP-TC-DNG-074* and *TP-TC-DNG-076*).
- If there is any delay for using purposes, the homogenates should be stored at -20°C until use.

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9. Quality Control

- Close the caps of the plates and tubes tightly during the homogenization to avoid spill and contamination between samples.
- Include an unexposed (virus negative) mosquito throughout the entire process to ensure there is no contamination of virus between samples from other sources.
- Include 1-2 wells/tubes for negative controls (only squash buffer/MD) during homogenisation.

10. Safety

- Place any waste in the appropriate waste bins with Hazard.
- Handling infectious tissue must be done inside BSL-2 cabinet.
- All samples should be assumed infectious and be treated/handled accordingly.
- Extra care must be taken to ensure the Tissue Lyser is balanced each and every time the plates are loaded into the hands of the machine. If this is not done, the plates may jump out during the shaking, and samples may SPILL. This means potential for infection of staff working on the procedure, as well as cross-contamination between samples.
- If there is spill, follow spillage procedure of OUCRU

11. Supplementary notes

TissueLyser Handbook, Qiagen, January 2008

12. Risk assessment and risk management

University of Oxford COSHH Risk assessment Form	
Description of procedure Mosquito Homogenisations	**Hazardous substances used** Potentially DENV, other arboviruses
Quantities used 100ul-individual samples 800ul-pooled samples	**Frequency of use** Frequent
Hazards identified • Potentially infectious virus in mosquito samples • Aerosolisation of pathogen during	**Could a less hazardous substance be used instead?** No

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homogenisation • Unbalance samples during high speed of homogenisation, as well as improper loading of samples into the hands othe Tissue Lyser machine. • Puregene Proteinase K Solution, RNase A Solution, Lytic Enzyme Solution in kits is a sensitizer, and irritant.	
What measures have you taken to control risk? PPE for staff performing the procedure. Spill kit to manage any spills during homogenization. Staff trained on the importance of proper balancing and loading of the samples into the Tissue lyser.	
Checks on control measures Supervision and observation by senior staff. Equipment maintenance and calibration. Spill kit is checked for content regularly.	
Is health surveillance required? Yes, Hepatitis B serology.	**Training requirements:** Task specific training for all staff, plus training in good laboratory practices.
Emergency procedures: Biological and chemical spill clean up procedure *PR-HS-ALL-005*	**Waste disposal procedures:** OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

13. Related Documents:

Document name	Document code
Dissecting mosquito legs and abdomens	*TP-TO-DNG-054*
Dissecting mosquito midguts and salivary glands	*TP-TC-DNG-055*
Intrathoracic inoculations	*TP-TC-DNG-058*
RNA Extraction using QIAamp Viral RNA Minikits	*TP-TC-DNG-003*
Serotyping and Quantification DENV Multiplex PCR	*TP-TC-DNG-010*
RNA Extraction using MagNA Pure 96 DNA and Viral NA Small Volume Kit	*TP-TC-DNG-050*

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14. Document History:

Revision #	Effective date	Details of amendment
00	01-11-2012	New issue
01	04-06-2012	Updated procedures for homogenisation and specimen requirements
02	18-07-2013	Editing, review of procedures. Altered title. – Lauren Carrington
03	11-09-2014	Updated Principle and Specimen requirements. – Lauren Carrington
04	09-10-2014	Added formal Risk Assessment table. – Lauren Carrington
05	28-03-2018	Converted to new OUCRU SOP template, and add more information the following sections: Purpose; Principle; Scope and responsibilities; Reference ; Definition; Specimen requirement; Materials/Equipment used; Procedures; Quality Control; Safety. – Lauren Carrington

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1. Purpose

To describe the procedure for detection of dengue virus (all serotypes), Wolbachia and *Aedes aegypti* DNA targets in a multiplex quantitative RT-PCR.

2. Principle:

Using extracted RNA/DNA from homogenised mosquito samples (either crudely, or with a refined extraction method), this procedure details the reverse transcription and amplficiation of three target sequences, within a single tube. These targets are DENV, Wolbachia and *Aedes aegypti*. The simultaneous amplification of three targets is possible with the use of multiple primers and fluorescent probes (specific for each target) included in the mastermix. Fluorescently-labelled probes (FAM, Cy5 and Hex) each emit different wavelengths of light that are received by different channels in the LightCycler™ system, as the target sequence amplifies.

This procedure is optimised for three targets, however if only two are of interest, the missing volume in the mastermix can simply be replaced with water. In all cases, the *Aedes aegypti* should be included in the reaction, as an internal control.

3. Scope and responsibilities:

Lab technicians have responsibility for performing this defined procedure as follows, and checking that the results obtained are logical, and replicable as necessary. Supervisors will intermittently check the results of assays to confirm results.

4. Reference

Dengue Quick Screening Protocol – EDP project – Monash University.

5. Definition

Nucleotides

- A = Adenine
- T = Thymine
- G = Guanine
- C = Cytosine
- Y = C, T
- R = A, G

Other

- LOD = limit of detection
- Fwd = Forward
- Rev = Reverse
- DENV = dengue virus
- DENV-G = name of oligonucleotides targetting all four serotypes of DENV
- Rps17 = name of oligonucleotides targetting *Aedes aegypti*
- wMel = Wolbachia strain *w*Mel (& name of oligonucleotides targetting *w*Mel)

6. Specimen requirement

- RNA/DNA from homogenised mosquitoes – see SOP TP-TO-DG-057 or TP-TO-DNG-050

7. Materials/Equipments used

Equipment

- PCR plate and Sealing foil Roche – Cat. No: 04 729 757 001
- Filter tips, pipettes
- Plate centrifuge

Reagents

- Light cycler multiplex RNA virus master mix — Roche – Cat. No: 7083173001
- Primers for each target, as outlined below — Sigma
- Probes for each target, as outlined below — Sigma

Primer sequences

Serotype	**Oligonucleotide sequences (5'-3')**
DENV-G Fwd	5'-AAGGACTAGAGGTTAGAGGAGACCC-3'
DENV-G Rev	5'-CGTTCTGTGCCTGGAATGATG-3'
Rps17 Fwd	5'-TCCGTGGTATCTCCATCAAGCT-3'
Rps17 Rev	5'-CACTTCCGGCACGTAGTTGTC-3'
wMel Fwd	5'-CAAATTGCTCTTGTCCTGTGG-3'
wMel Rev	5'-GGGTGTTAAGCAGAGTTACGG-3'

Probe sequences

Serotype	**Oligonucleotide sequences (5'-3')**
DENV-G Probe	5'-**FAM**-AACAGCATATTGACGCTGGGAGAGACCAGA-**BHQ1**-3'
Rps17 Probe	5'- **CY5**-CAGGAGGAGGAACGTGAGCGCAG-**BHQ1**-3'
wMel Probe	5'-**Hex**- TGAAATGGAAAAATTGGCGAGGTGTAGG-**BHQ1**-3'

8. Procedures

PCR master mixes

1) Prepare the mastermix as per the table below, calculating the volume required based on the number of samples to be tested.

Reagent	**Concentration**	**Volume/reaction**
Lightcycler multiplex RNA virus master	1x	2 µL
RT enzyme	1x	0.05 µL
DENV-G Fwd + DENV-G Rev	10 µM	0.50 µL
DENV-G Probe (FAM)	10 µM	0.25 µL
Rps17 Fwd	10 µM	0.10 µL
Rps17 Rev	10 µM	0.10 µL
Rps17 probe (Cy5)	10 µM	0.10 µL
wMel Fwd	10 µM	0.25 µL
wMel Rev	10 µM	0.25 µL
wMel probe (HEX)	10 µM	0.25 µL
ddH_2O		4.15 µL
RNA template *		2.00 µL
Total volume		**10 µL**

* *RNA is added in the Extraction Room only* and should not be added in the "*Mix Room*"

Plate setup

2) After preparing the PCR mix, transfer 8 µl PCR mix to each well in the PCR plate. To avoid contamination, this is done in the *Mix room* only.
3) When ready, transfer the PCR plate containing the PCR mix to the *Extraction room* and add 2 µl RNA from test samples, as well as the internal and external.
4) After RNA has been added, place the seal on the plate, and centrifuge the plate at 1500 rpm for 2 min in the centrifuge available in the *Extraction room* to collect all droplets in the bottom of the tubes.

5) Once the plate has been spun, take the plate to the *PCR Room* and turn on the Roche Light Cycler II PCR machine.

PCR programme

6) On the software interface, apply the "New Dengue Group one step PCR" template.
7) The RNA in the samples is first converted to cDNA, and then all the DNA is amplified in the one tube, by sequentially incubating the samples at the appropriate temperatures. The pre-programmed template follows the temperatures in the table below:

Number	Step	Temperature	Time	# Cycles
1	Reverse transcription	58°C	10 mins	x 1
2	Denaturation	95°C	5 mins	x 1
3	Amplification	95°C	3 secs	x 40
		60°C	30 secs*	
		72°C	1 sec	
4	Cooling	37°C	1 min	x 1

** The fluorescence reading is taken at the end of the 30 sec elongation step, after each round of amplification.*

9. Quality Control

Internal control: use of *Ae. aegypti* Rps17

To assess the uniformity of RNA/DNA extraction and PCR, Rps17 is used in every run, to target Ae, aegypti genomic sequence. Because every mosquito being tested is Ae. aegypti, every result should be positive, confirming that the reaction worked.

External positive control: cultured DENV 1-4

In every batch of RNA extraction, and PCR, the control samples should be included. These control samples serve to confirm the performance of the DENV-group PCR assay. All cultured virus samples should be positive in the DENV-group.

External negative control: ddH_2O

The external negative control is molecular grade water that has been processed, starting with RNA extraction, in the same manner as samples and other external controls. The external control should be negative for every PCR batch run. If the result is positive then all results are invalid and contamination of reagents or the environment must be considered.

10. Interpretation of results and report

Determining Ct values

The software associated with the Roche LightCycler determines the threshold cycle value by algorithmic means. In practice, this means the cycle threshold value (Ct) is set automatically.

The Internal *Aegypti* control: criteria for acceptability

The Internal control (*Ae. aegypti* Rps17) serves as a qualitative control for extraction, and PCR. Failure of the internal control may reflect inhibition or reaction failure in any of the steps leading to and including the PCR. All *Aegypti* internal control sample curves should be positive and have a clear, sigmoidal curve.

	Aegypti/DENV			
Result	**(+ / +)**	**(+ / -)**	**(- / +)**	**(- / -)**
Outcome	Pass	Pass	Pass	Fail

11. Safety

Universal precautions are used when working with clinical specimens. When working with chemicals, always wear a suitable lab coat, disposal gloves, and protective goggles.

Always use PRESEPT and Ethanol 70% to clean pipette and working area.

Place any waste in the clinical waste bins provided.

12. Risk assessment and risk management:

Department	**Persons involved**
Dengue group	Dengue staff

Location of work OUCRU-HCM Laboratory	
Description of procedure The procedure describes how to perform a multiplex, one-step PCR detecting DENV, wolbachia and Aedes aegypti targets.	**Substances used** N/A
Quantities used N/A	**Frequency of use** Daily
Hazards identified N/A	**Could a less hazardous substance be used instead?** NO.
What measures have you taken to control risk? **PPE:** i. Designated gown for BSCII work ii. Disposable gloves iii. Safety glasses	
Checks on control measures N/A	
Is health surveillance required? NO.	**Training requirements:** i. BSCII. ii. Centrifuge iii. Waste disposal iv. Accident reporting procedures v. Spill clean up procedures vi. Sign and symptoms of infection
Emergency procedures: i. Contact supervisor ii. Contact lab management group	**Waste disposal procedures**: 1% Presept for 30 minutes then autoclave 121°C/30 minutes.

13. Related Documents:

Document name	Document code
Dengue Quick Screening Protocol – EDP project – Monash University	N/A
Mosquito homogenisations	*TP-TO-DNG-057*
RNA Extraction using MagNA Pure 96 DNA and Viral NA Small Volume Kit	*TP-TO-DNG-050*

14. Document History:

Revision #	Effective date	Details of amendment
00	01 April 2017	New issue
01	06 April 2018	Change to the new OUCRU SOP template format. Shortened title. – Nguyen Minh Nguyet & Lauren Carrington

1. **Purpose**:

Analysis of expression samples by SDS-PAGE

2. **Scope and Responsibility**

Lab technicians have responsibility for performing testing following defined procedures

3. Definition or abbreviation:

IPTG : Isopropyl β-D-1-thiogalactopyranoside

SDS: Sodium dodexyl sulfate

4. Materials and instruments:

4.1. Instruments

4.1.1. Sonicator and Microtip probe

4.1.2. Centrifuge machine

4.1.3. Protein electrophoresis system

4.1.4. Thermocycler machine

4.1.5. Micro pipettes and pipettes tips

4.1.6. Ependorfs 1.5mL

4.1.7. Falcon 15 mL

4.2. Materials

4.2.1. 20 mM Tris-HCl (pH 7.5)

4.2.2. 1% SDS

4.2.3. Reducing agent: 1M DTT or 1M Beta-mercaptoethanol

4.2.4. SDS-PAGE running buffer (1X): 25 mM Tris-base ($C_4H_{11}NO_3$), 250 mM Glycine, 0.1% SDS

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4.2.5. Coomassie stain solution: 0.125% Coomassie brilliant blue R, 40% Ethanol, 7% Acetic acid

4.2.6. Destain solution: 30% Methanol, 10% Acetic acid

4.2.7. 4X loading buffer: 4 mM EDTA, 200 mM Tris-HCl (pH 6.8), 8% SDS, 40% Glycerol, 0.4% Bromophenol blue

4.2.8. SDS-PAGE gel ingredients: 40% Acrylamide mix, (store at 4ºC) 10 % SDS, 10 % APS (store at 4ºC), TEMED, 1.5 M Tris-HCl (pH 8.8) and 0.5 M Tris-HCl (pH 6.8) (buffers used specifically for SDS gel)

5. Specimen and interference:

5.1. Specimen types

1 mL uninduced and induced samples collected after IPTG induction of pET30a-SaoM containing *BL21* strain

(Overexpression of Sao M protein in *E. Coli BL21 DE3 plyS* – Protocol version 001)

6. Test or principle:

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, a technique widely used in biochemistry, genetics, molecular biology and biotechnology to separate proteins according to their electrophoretic mobility and no other physical feature. SDS is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

7. Procedure:

Sections	*Descriptions*	*Notes*
7.1.	**Soluble cytoplasmic fraction** 1. Completely resuspend the pellets from 1 mL of uninduced and induced sample in 100 uL of 20 mM	

	Tris-HCl (pH 7.5) and 11 uL of 10 mM PMSF 2. Sonicate on ice using a microtip with the power level of 3.5 for three 30-second bursts. Always keep the sample cool during sonication to avoid heat denaturation of proteins 3. Centrifuge at 13000 rpm for 5 min (4ºC), keep the supernatant as soluble fraction at -20ºC. Save the insoluble pellet fraction on ice for processing	
7.2.	**Insoluble cytoplasmic fraction** Wash the insoluble pellet by resuspending in 750 ul of 20 mM Tris-HCl (pH 7.5). Centrifuge at 13000 rpm, 5 min, remove the supernatant and repeat the wash step. Resuspend the final pellet in 100 uL of 1% SDS with heating and vigorous mixing or sonication, if necessary.	
7.3.	**Running SDS-PAGE gel** Making SDS-PAGE gel ✓ Running gel: prepare gel buffer in 15 mL Falcon (follow the recipe below)	

Running gel 10%	
ELGA	2.3 mL
40% Acrylamide mix	1.35 mL
1.5 M Tris (pH 8.8)	1.3 mL
10% SDS	50 uL
10% APS	50 uL
TEMED	3 uL

Total	5 mL

- ✓ mix the buffer evenly before and after adding TEMED
- ✓ pour gel buffer into the plate with only one run
- ✓ cover the gel surface with isopropanol.
- ✓ wait around 30 min for running gel to cool, pour out the isopropanol layer
- ✓ Stacking gel: prepare the stacking gel buffer as recipe below

Stacking gel 5%	
ELGA	1500 uL
40% Acrylamide mix	312 uL
0.5 M Tris (pH 6.8)	625 uL
10% SDS	25 uL
10% APS	25 uL
TEMED	2.5 uL
Total	2.5 mL

- ✓ Insert the comb and wait about 15 min for gel cooling

Sample preparation

- ✓ Mix 15 uL sample with 5 uL 4X loading buffer and 2 uL reducing agent
- ✓ Heat at 95°C for 5 min

Run gel at 150V for 1 hour

(Note, fill in empty wells with a volume of 1X loading buffer equal to that of the sample)

Stain gel with Coomassie stain solution for 1h

	and then destain in 1h	

8. Quality control:

N/A

9. Calculation: N/A

10. Normal/Reference range, Critical value, Limit of detection (LOD), Detection range:

N/A

11. Interpretation and reporting:

Batch number: ……. …….Date operation:……..

Stored: ……Notes: ……

Samples' order:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

12. Further reading or reference:

pET system manual – TB055 8th Edition 02/99 (Novagen)

13. Health and safety issues:

13.1. Safety issues including PPE

When working with chemicals, always ware a suitable lab coat, disposal gloves, and protective goggles.

13.2. Emergency management

If there is any accident while working with this test report to supervisor or Management department

13.3. Waste disposal

Place any waste in the clinical waste bins provided.

13.4. Vaccination need: N/A

14. Approval and competency: N/A

15. Annex or appendix:N/A

16. Risk assessment

17. Related documents

Document name	Document code
Analysis of expression samples by SDS-PAGE	SS-010

18. Document history

Revision no	Effective date	Details of Amendment
2	26March2013	Modify from the previous version(SS-010)

1. Purpose

To provide a qualitative procedure for cDNA synthesis from RNA

2. Principle:

N/A

3. Scope and responsibilities:

Lab technicians have responsibility for performing testing following defined procedures

4. Reference

N/A.

5. Definition

cDNA = complementary Deoxyribonucleic acid

RNA = Ribonucleic acid

MW = Molecular water

6. Specimen requirement

RNA

7. Materials/Equipments used

7.1 Random Hexamer (10ng/µl) (or specific primer)	Roche	Cat. No: 11034731001
7.2 dNTPs (10mM)	Roche	Cat. No: 03622614001
7.3 DTT	Invitrogen	Cat. No: 18080 - 085
7.4 5x buffer	Invitrogen	Cat. No: 18080 - 085
7.5 Rnase Inhibitor	Invitrogen	Cat. No: 10777-019
7.6 Superscript III reverse transcriptase	Invitrogen	Cat. No: 18080 - 085
7.7 Molecular Water	Sigma	Cat. No: W4502

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7.8 Filter tip

7.9 Pipette

7.10 Sterile eppendorf tube

7.11	8 -Strip tubes & Ind Caps Natural	Alpha	LW2510
7.12	PCR tube crack	Alpha	LW5880AS

7.13 Microcentrifuge (with rotor for 1.5 ml or 2 ml tubes)

8. Procedures

Each sample in the cDNA synthesis batch should be labeled with sample No according to batch number derived from the batch number index (electronic file). The cDNA master mix is prepared in the Mix room. The cDNA master mix is transferred to the extraction room when it is time to add sample RNA. The cDNA synthesis is performed on a PCR block in the extraction room.

<table>
<tr><th>Section</th><th>Description</th><th>Person(s) Responsible</th></tr>
<tr><td>1. Reaction preparation:</td><td>
<table>
<tr><th>Mix 1</th><th>ul/ reaction</th></tr>
<tr><td>MW</td><td>2</td></tr>
<tr><td>Random hexamer (10ng/ul) and</td><td>0.2</td></tr>
<tr><td>Reverse specific primer</td><td>0.4</td></tr>
<tr><td>dNTP (10mM)</td><td>0.5</td></tr>
<tr><td>5x Buffer</td><td>1</td></tr>
<tr><td colspan="2">Distribute 3uL of Mix 1 to PCR plate/ tube</td></tr>
<tr><td>Vrial RNA</td><td>8</td></tr>
</table>
Vol of Mix 1 14
<table>
<tr><th>Mix 2</th><th>ul/ reaction</th></tr>
<tr><td>MW</td><td>2</td></tr>
</table>
</td><td>Assigned Laboratory Staff</td></tr>
</table>

	5x Buffer	2	
	DTT	1	
	RNAse Out	0.5	
	Transcriptase	0.5	
	Vol of Mix 2	**6**	
	Mix well and briefly centrifuge. Heat mixture at 65ºC for 5 min and quickly chill on ice for at least 1 min. Add mix 2 into mix 1.		
2. cDNA synthesis programme	Run at 25^0C for 5 minutes followed by 50ºC for 1hour then inactivate the reaction at 70ºC for 15 minutes.		Assigned Laboratory Staff

9. Quality Control

N/A

10. Calculations

N/A

11. Interpretation of results and report

N/A

12. Reporting of results

N/A

13. Reference range and critical value:

N/A

14. Limitations:

N/A

15. Safety

Universal precautions should be used when working with clinical specimens.

When working with chemicals, always ware a suitable lab coat, disposal gloves, and protective goggles.

Place any waste in the clinical/ infectious waste bins provided.

16. Supplementary notes

Do not bring plasma, DNA, RNA, cDNA into PCR Mix room

17. Risk assessment and risk management:

N/A

18. Related Documents:

Document name	Document code

19. Document History:

Revision no.	Effective date	Details of amendment
001	02-06-2009	New issue
002	12-05-2011	Delete cDNA storage record form *FM-TO-DG-009* Delete cDNA synthesis batch number index form *FM-TO-DG-008.* Delete cDNA synthesis from RNA form *FM-TO-DG-007* Change the Reagent preparation section.
003	01-06-2013	Use the new SOP format and coding.
004	14-01-2014	Change the reaction volume from 20ul to 10ul
005	12-10-2015	Change the reaction volume from to 20ul Use reverse specific primer

1. Purpose

To provide a qualitative procedure for preparing amplicon library for 454 sequencing.

2. Principle:

N/A

3. Scope and responsibilities:

Lab technicians have responsibility for performing testing following defined procedures

4. Reference

N/A.

5. Definition

A	= Adenine
T	= Thymine
G	= Guanine
C	= Cytosine
Y	= C, T
R	= A, G
Fw	= Forward
Rv	= Reverse
cDNA	= complementary Deoxyribonucleic acid
PCR	= Poly Chain Reaction
NGS	= Next-Generation Sequencing
MID	= Multiplex Identifiers
emPCR	= emulsion PCR

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6. Specimen requirement

cDNA and PCR product

7. Materials/Equipments used

7.1	dNTPs (10mM)	Roche - 03622614001
7.2	FastStart High-Fidelity DNA polymerase	Roche - 03533361001
7.3	1st round DENV PCR primers (Appendix 1)	
7.4	Primer A and B key (Appendix 2)	
7.5	AMPure XP	Beckman Courter - A63882
7.6	Agencourt SPRIPlate 96 Ring Super Magnet Plate	Beckman Courter - A32782
7.7	QIAquick PCR Purification Kit (50)	QIAgen – 28104
7.8	Quant-iT™ PicoGreen® dsDNA Assay Kit	Invitrogen - P7589
7.9	Molecular Water	Sigma - W4502
7.10	TE buffer	Sigma – T9285
7.11	96 –Well Half Skirt PCR*Plates	Labcon - 3979-520
7.12	Optically Clear Strip Caps	Labcon - 3951-850
7.13	Filter tip	

8. Procedures

Using the next-generation sequencing (NGS) to generate the sequence data. It is able to generate up to 70.000 amplicon reads with average read lengths between 400–500 bases. This long-read sequencing performance, in combination with a sample pooling strategy that uses ‘‘bar-coded’’ amplicons for parallel analysis of pooled samples. 2 Sequencing of PCR fragments by 454 sequencing technology requires certain adaptations to the DNA fragments. For the emµlsion PCR (emPCR), the single strand DNA fragments require binding sequences, called adapters, at the 5’ end for immobilization onto specifically designed DNA capture beads. There exist two kinds of such adapters, A and B, allowing the bidirectional sequencing of the target sequence. In addition, each fragment requires a four-nucleotide ‘key’ sequence that is recognized by the 454 software and indicates the starting point of a sequence read.

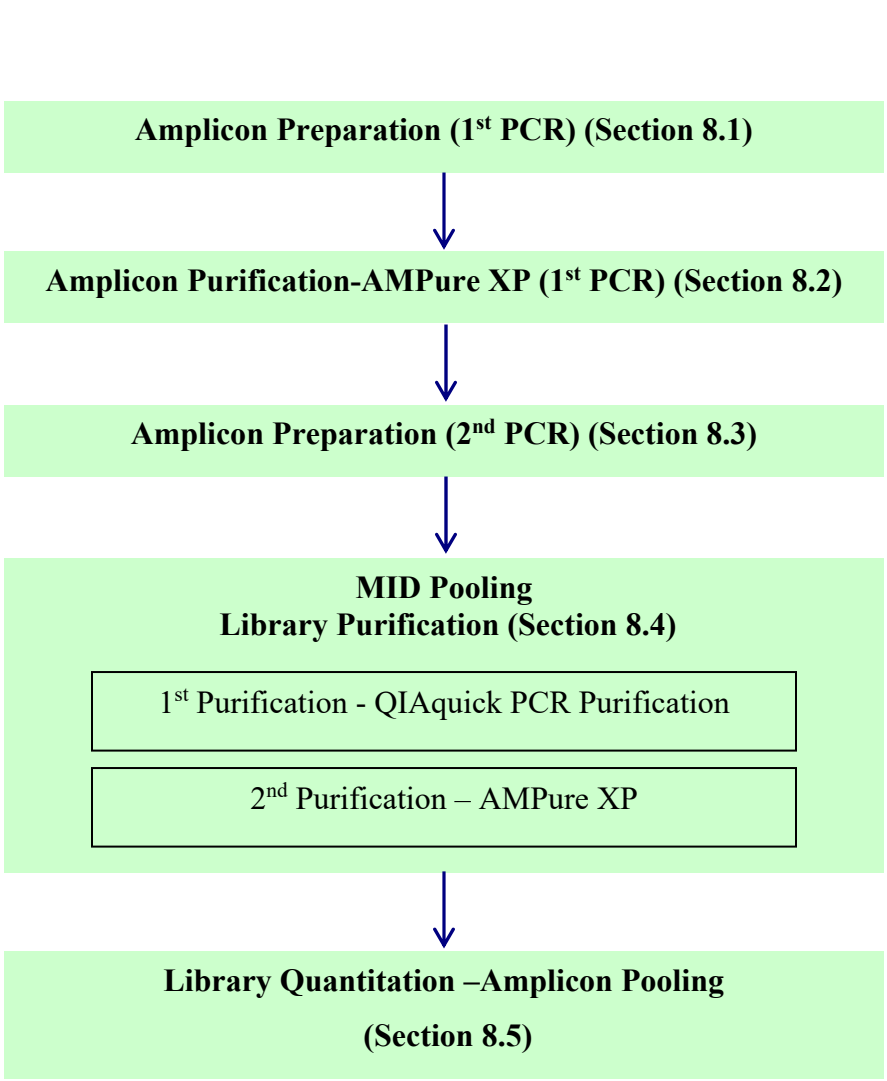
Forward primer (Primer A-Key):

5’-CGTATCGCCTCCCTCGCGCCATCAG-{MID}-{template-specific-sequence}-3’

Reverse primer (Primer B-Key):

5’-CTATGCGCCTTGCCAGCCCGCTCAG-{MID}-{template-specific-sequence}-3’

WORK FLOW OF AMPLICON LIBRARY PREPARATION



Section	Description	Person(s) Responsible

8.1. 1st PCR

Assigned Laboratory Staff

PCR mix preparation

Component	Working Conc	Vol/reaction (µl)
dNTPs (10mM)	10mM	0.8
DMSO	5%	1
10X Buffer (No MgCl)		2
MgCl	25mM	2
RNase-free water		11.08
FastStart Hi Fidelity Taq	250U	0.12
Forward primer	10uM	0.5
Reverse primer	10uM	0.5
cDNA		2

PCR thermalcycling Program

Temperature (°C)	Time	Cycle #
94	2 mins	1 cycle
94	30 sec	40 cycles
*Anneal Temp	1 min	
72	1 min	
72	5 mins	1 cycle
4	Hold	

Anneal Temp: DV1 – 57^{0}C

DV2 – 60^{0}C

DV3 – 56^{0}C

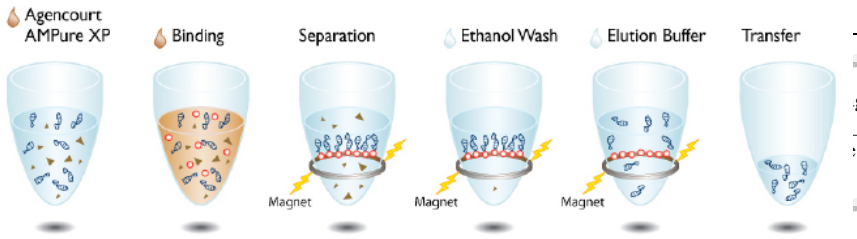
DV4 – 55^{0}C

After thermalcycling program has completed remove plate from thermalcycler. Reactions may be frozen at -20°C or immediately run on a 1% agarose gel to confirm PCR success.

8.2.Purify 1st PCRs

Assigned Laboratory

Process overview



product	8.2.1 Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled. The volume of Agencourt AMPure XP for a given reaction can be derived from the following equation: **Vol of Agencourt AMPure XP per reaction = 1.8 x Reaction Volume** 8.2.2 Mix reagent and PCR reaction thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery. 8.2.3 Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step. 8.2.4 Aspirate the cleared solution from the reaction plate and discard. This step **must be performed** while the reaction plate is situated on the Agencourt SPRIPlate 96 Super Magnet Plate. Do not disturb the ring of separated magnetic beads. If beads are drawn out, leave a few microliters of supernatant behind. 8.2.5 Dispense 200µl of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. **Do not disturb the separated magnetic beads**. **Be sure to remove all of the ethanol from the bottom of the well** 8.2.6 Off the magnet plate, add 20uL 1x TE buffer to each well of the reaction plate and pipette mix 10 times. Incubate at RT for 10	Staff

<table>
<tr><td></td><td>mins.
8.2.7 Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for 3minute to separate beads from the solution.
8.2.8 Transfer the eluant to a new plate.</td><td></td></tr>
<tr><td>8.3. 2nd PCR</td><td>PCR mix preparation
<table>
<tr><th>Component</th><th>Working Conc</th><th>Vol/reaction (µl)</th></tr>
<tr><td>dNTPs (10mM)</td><td>10mM</td><td>0.2</td></tr>
<tr><td>10X Buffer (No MgCl)</td><td></td><td>1</td></tr>
<tr><td>MgCl</td><td>25mM</td><td>1</td></tr>
<tr><td>FastStart Hi Fidelity Taq</td><td>250U</td><td>0.04</td></tr>
<tr><td>RNase-free water</td><td></td><td>4.96</td></tr>
<tr><td>Forward primer</td><td>10uM</td><td>0.4</td></tr>
<tr><td>Reverse primer</td><td>10uM</td><td>0.4</td></tr>
<tr><td>1st PCR product</td><td></td><td>2</td></tr>
</table>
PCR thermalcycling Program
<table>
<tr><th>Temperature (°C)</th><th>Time</th><th>Cycle #</th></tr>
<tr><td>94</td><td>2 mins</td><td>1 cycle</td></tr>
<tr><td>94</td><td>30 sec</td><td rowspan="3">35 cycles</td></tr>
<tr><td>57</td><td>30 sec</td></tr>
<tr><td>72</td><td>50sec</td></tr>
<tr><td>72</td><td>5 mins</td><td>1 cycle</td></tr>
<tr><td>4</td><td>hold</td><td></td></tr>
</table>
After thermalcycling program has completed remove plate from thermalcycler. Reactions may be frozen at -20°C or immediately run on a 1% agarose gel to confirm PCR success.</td><td>Assigned Laboratory Staff</td></tr>
</table>

8.4. MID Pooling and Library Purification	8.4.1 **MID Pooling**: pool MID (at least 3µl) of 1 fragment from 96 samples together. For example: DV1 has totally 16 PCR plates for 16 different fragments. Pool 96 amplicons (96 MIDs) from 1 plate (same fragment) together. Thus, there are totally 16 amplicons tubes of 16 different fragments. *** DO NOT POOL POS AND NEG CONTROLS.** **MID pooling is purified 2 times by column and AMPure bead.** 8.4.2 **Purify pool amplicon by QIAquick PCR Purification kit**: 8.4.2.1 Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. 8.4.2.2 Place a QIAquick spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column and centrifuge at 13,000rpm/ 30–60 s. 8.4.2.3 Discard flow-through. Place the QIAquick column back into the same tube. To wash, add 0.6 ml Buffer PE to the QIAquick column and centrifuge 13,000rpm/ 30–60 s. 8.4.2.4 Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min. 8.4.2.5 Place QIAquick column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane, let the column stand for 20 mins and centrifuge the column for 1 min. 8.4.3 **Purify pool amplicon by AMPure XP: refer to section 8.2** **Vol of Agencourt AMPure XP per reaction = 1.0 x Reaction Volume** 8.4.4 Run purified 16 amplimers on gel to check the purification step.	Assigned Laboratory Staff
8.5 Library Quantization	8.5.1 Thaw DNA standard (100ng/ul = 100ug/mL) 8.5.2 Prepare TE 1X. Add TE 1X as follows:	Assigned Laboratory

and Pool Bar-coded Amplicon	a. Tube 1: 594ul TE 1X b. Tube 2-8: 300ul TE 1X 8.5.3 Transfer 5ul DNA standard to tube 1 (100X dilution: 1ng/ul), vortex 10 sec. 8.5.4 Transfer 300ul from tube 1 to tube 2, vortex 10 sec (dilute ½ times). (0.5ng/ul). 8.5.5 Continue until tube 7. Tube 8 contain no DNA control. 8.5.6 Transfer 100ul of each DNA standard dilution to 8 wells. The amounts of DNA per standard well are listed as following table (duplicate 2 rows). 8.5.7 Add 100ul of 1:200 dilution of Picogreen reagent (protected from light) to each well. Mix carefully. (1:1) 8.5.8 Measure FAM fluorescent (emission 520nm)	Staff

Tube #	Well (100+100ul)	DNA concentration
Tube 1 (1ng/ul)	A1-2	100ng/well
Tube 2 (0.5ng/ul)	B1-2	50ng/well
Tube 3 (0.25ng/ul)	C1-2	25ng/well
Tube 4 (0.125ng/ul)	D1-2	12.5ng/well
Tube 5 (0.0625ng/ul)	E1-2	6.25ng/well
Tube 6 (0.0313ng/ul)	F1-2	3.13ng/well
Tube 7 (0.0156ng/ul)	G1-2	1.56ng/well
Tube 8 (blank)	H1-2	0.00ng/well

8.5.8 Calculate the concentration of each Amplicon in molecules/µl, using this page

http://cels.uri.edu/gsc/cndna.html

8.5.9 Preparing the Amplicon Library by adjusting each MID Pooling to the same value of molecules/µl and then mix an equal volume (e.g. 10µl) of each diluted MID Pooling. Store the stock of MID Pooling and the amplicons library at -15 to -25°C.

9. Quality Control

9.1 PCR step

+ Run 6 samples of 1st round PCR from each 1st amplimer plate (4 samples-1 Negative- 1 Positive control).

+ Run 6 samples of 2nd round PCR from each 2nd round amplimer plate (4 samples-1 Negative-1 Positive control) and 1 positive control for each amplimer from 1st amplimer PCR plate.

2. Vol of Agencourt AMPure XP per reaction can be used = **0.7uL** x reaction volume if there are unspecific bands in 2nd round PCR.

9.2 Library Quantitation

1. Verify that the R^2 value of the standard curve is at least 0.98.

a. If it is not, check if the top point of the curve is below the curve; your fluorometer may not have a sufficient dynamic range for the top point. If that is the case, eliminate the top point and recalculate.

b. If the R^2 value is below 0.98 and it is not due to a dynamic range issue, repeat the assay, pipetting carefully.

2. Verify that the sample readings fall within the range of the standard curve.

a. If any sample readings exceed the highest standard curve value, dilute and re-measure these samples, and take the additional dilution factor into account when calculating final concentration.

b. If any sample readings fall below 5ng/µl, it is recommended to verify the size and purity of the Amplicon before proceeding. If necessary, repeat the purification or preparation of these Amplicons.

10. Calculations

Calculate the concentration of each Amplicon in molecules/µl, using this page

http://cels.uri.edu/gsc/cndna.html

11. Interpretation of results and report

N/A

12. Reporting of results

N/A

13. Reference range and critical value:

N/A

14. Limitations:

N/A

15. Safety

Universal precautions should be used when working with clinical specimens.

When working with chemicals, always ware a suitable lab coat, disposal gloves, and protective goggles.

Place any waste in the clinical/ infectious waste bins provided.

16. Supplementary notes

Change gloves frequently.

Soak tube crack in bleach after using, clean pipette after each step

Clean working bench with bleach-water-Ethanol.

17. Risk assessment and risk management:

N/A

18. Related Documents:

Document name	Document code

19. Document History:

Revision no.	Effective date	Details of amendment
001	18-10-2013	New issue
002	07-02-2013	Add more details in Quality control section. Change PCR components in 1st and 2nd round PCR Using higher range of DNA standard for DNA library quantization

APPENDIX -1st round DENV PCR primers

Primer Name	Primer sequence (5' to 3')	Product size (1st round	Product size (2nd round)
DV1-F1-454-Fw	GACACTATAGTCTAGCCATACCCCCAACAG	725	817
DV1-F1-454-Rv	CACTATAGGGAAGTCYCTGYTGCCTATTCC		
DV1-F2-454-Fw	GACACTATAGATYAAAGTGYTACGGGGTTTC	661	753
DV1-F2-454-Rv	CACTATAGGGAAGTCYCTGYTGCCTATTCC		
DV1-F3-454-Fw	GACACTATAGATYAAAGTGYTACGGGGTTTC	841	933
DV1-F3-454-Rv	CACTATAGGGTCGGTGGTGGTGTTTGATAT		
DV1-F4-454-Fw	GACACTATAGGCRATGGATTTGGGAGAGTTA	694	786
DV1-F4-454-Rv	CACTATAGGGAACGTTCGTCGACACACAAA		
DV1-F5-454-Fw	GACACTATAGCGRATCACTGAGGCGGAACC	861	953
DV1-F5-454-Rv	CACTATAGGGGGAGCTTGAGGTGTTATRGTT		
DV1-F6-454-Fw	GACACTATAGAGAACCGAAACRTGGATGTC	709	801
DV1-F6-454-Rv	CACTATAGGGGGAGCTTGAGGTGTTATRGTT		
DV1-F7-454-Fw	GACACTATAGGCACATGCCATAGGAACATC	838	930
DV1-F7-454-Rv	CACTATAGGGTTCTTTGCATGAGCTGTCTT		
DV1-F8-454-Fw	GACACTATAGGAAGGACTGTCAGGAGCAAC	769	861
DV1-F8-454-Rv	CACTATAGGGGTGTGCATTGCTCCTTCTTGT		
DV1-F9-454-Fw	GACACTATAGTCAAGATGTCCAACACAAGG	547	639
DV1-F9-454-Rv	CACTATAGGGTTCTTTGCATGAGCTGTCTT		
DV1-F10-454-Fw	GACACTATAGCGTGTGCTAAGTTYAAGTGT	815	907
DV1-F10-454-Rv	CACTATAGGGCCACGATGTARCTCTCACCA		
DV1-F11-454-Fw	GACACTATAGCAACYATAACACCTCAAGC	674	766
DV1-F11-454-Rv	CACTATAGGGCCACGATGTARCTCTCACCA		
DV1-F12-454-Fw	GACACTATAGCAGCTCATGCAAAGAAGCAG	715	807
DV1-F12-454-Rv	CACTATAGGGCGTGCTCCTTGARTTTAATCC		
DV1-F13-454-Fw	GACACTATAGTGACYGGRGCGACAGAAATC	649	741
DV1-F13-454-Rv	CACTATAGGGCGTGCTCCTTGARTTTAATCC		
DV1-F14-454-Fw	GACACTATAGACGTCWGGAACGACAACAAT	740	832
DV1-F14-454-Rv	CACTATAGGGTCCACATTTGAGTTCTCTRCC		
DV1-F15-454-Fw	GACACTATAGTGGGAGATTGATAACAGCCAA	510	602
DV1-F15-454-Rv	CACTATAGGGTCCACATTTGAGTTCTCTRCC		
DV1-F16-454-Fw	GACACTATAGGAGACACCGCATGGGACTT	788	880
DV1-F16-454-Rv	CACTATAGGGGGGTGTAGGAGTCACGCAAT		
DV2-F1-454-Fw	GACACTATAGGGSTTCAGGAAAGAGATTGGAA	682	774
DV2-F1-454-Rv	CACTATAGGGATGTCAACCCARCTYCCTCCTGA		

DV2-F2-454-Fw	GACACTATAGGAGATTGGAAGGATGCTGAAYATC	697	789
DV2-F2-454-Rv	CACTATAGGGGTCACACARCTTCCATGTTCTAA		
DV2-F3-454-Fw	GACACTATAGGGAGAACCACACATGATCGT	736	828
DV2-F3-454-Rv	CACTATAGGGGGTTCYCCTTGTGTTGGGCA		
DV2-F4-454-Fw	GACACTATAGGGAGAACCACACATGATCGT	814	906
DV2-F4-454-Rv	CACTATAGGGCCRCATCCATTTCCCCATCCTC		
DV2-F5-454-Fw	GACACTATAGACYGAAACATGGATGTCATCA	776	868
DV2-F5-454-Rv	CACTATAGGGGTCGAGGCCYGTTCTYGGAGA		
DV2-F6-454-Fw	GACACTATAGATGGATGTCATCAGAAGGGGC	845	937
DV2-F6-454-Rv	CACTATAGGGAAYGGCAGGTCTARGAACCA		
DV2-F7-454-Fw	GACACTATAGCTCCTTCAATGACAATGCGYTG	902	994
DV2-F7-454-Rv	CACTATAGGGCCATTCTCAGCCTGCACTTRAG		
DV2-F8-454-Fw	GACACTATAGAAGYTGTGTGACGACGATGG	805	897
DV2-F8-454-Rv	CACTATAGGGCCATTCTCAGCCTGCACTTRAG		
DV2-F9-454-Fw	GACACTATAGAGAACRGGCCTCGACTTCAATGA	610	702
DV2-F9-454-Rv	CACTATAGGGGGYTCTACTCCTATRATGATGTAG		
DV2-F10-454-Fw	GACACTATAGCAATGGTTCYTAGACCTRCC	661	753
DV2-F10-454-Rv	CACTATAGGGGATCCRAAATCCCARGCTGTGTC		
DV2-F11-454-Fw	GACACTATAGTCYCAAGAAGGGGCCATGCA	863	955
DV2-F11-454-Rv	CACTATAGGGTGAAGGGGATTCTGGTTGGA		
DV2-F12-454-Fw	GACACTATAGGTCAACATAGAAGCAGAACCTCC	712	804
DV2-F12-454-Rv	CACTATAGGGCCTRCCTGCATGATTCCTTTRATG		
DV2-F13-454-Fw	GACACTATAGGGAGACAGCTACATCATYATAGG	838	930
DV2-F13-454-Rv	CACTATAGGGTTCCAAGCTCTRTTTGTGTTGGG		
DV2-F14-454-Fw	GACACTATAGGGCCAAATGTTTGAGACAACAA	763	855
DV2-F14-454-Rv	CACTATAGGGTTCCAAGCTCTRTTTGTGTTGGG		
DV2-F15-454-Fw	GACACTATAGGGAGGAGTGTTYACATCWATAGG	793	885
DV2-F15-454-Rv	CACTATAGGGATGGCYGCTGACATGAGTTTTG		
DV2-F16-454-Fw	GACACTATAGTCACTGTCTGTGTCRCTRGTA	652	744
DV2-F16-454-Rv	CACTATAGGGTTWATGGCYGCTGACATGAGT		
DV3-F2-454-Fw	GACACTATAGTGGCGTTCATAGCTTTCCTCA	712	804
DV3-F2-454-Rv	CACTATAGGGGGATGGRGTGACCAGCATTA		
DV3-F3-454-Fw	GACACTATAGCTTGGCTAGATGGGGAACCT	660	752
DV3-F3-454-Rv	CACTATAGGGGGATGGRGTGACCAGCATTA		
DV3-F4-454-Fw	GACACTATAGTGGTGCAACCTYACATCGAC	819	911
DV3-F4-454-Rv	CACTATAGGGATGCCTGGGGTGTTATCTCA		
DV3-F5-454-Fw	GACACTATAGATGTCGGCTGAAGGAGCTTG	749	841
DV3-F5-454-Rv	CACTATAGGGTGTCCGTGGTGARCATTCTA		
DV3-F6-454-Fw	GACACTATAGGAGAAGGTAGAGACATGGGC	657	749

DV3-F6-454-Rv	CACTATAGGGATGCCTGGGGTGTTATCTCA		
DV3-F7-454-Fw	GACACTATAGCTGGTCACYCCATCCATGAC	671	763
DV3-F7-454-Rv	CACTATAGGGCCATTGYCTATGTACCATCC		
DV3-F8-454-Fw	GACACTATAGCTGGTCACYCCATCCATGAC	817	909
DV3-F8-454-Rv	CACTATAGGGTGCATTGCTCCCTCTTGCGA		
DV3-F9-454-Fw	GACACTATAGACGTGGGTTGAYGTGGTGCT	837	929
DV3-F9-454-Rv	CACTATAGGGCCAATTTGTCCATCTTRAGTC		
DV3-F10-454-Fw	GACACTATAGTGAGGAGCAGGACCAGAACTA	653	745
DV3-F10-454-Rv	CACTATAGGGCCCTTGAGTTCCAATTTGTC		
DV3-F11-454-Fw	GACACTATAGTGAGGAGCAGGACCAGAACTA	671	763
DV3-F11-454-Rv	CACTATAGGGCACATTGCATAGCTCATCCC		
DV3-F12-454-Fw	GACACTATAGAGGYTGGGGAAACGGTTGTG	755	847
DV3-F12-454-Rv	CACTATAGGGGCTTTCCCTTGTCCATCCTC		
DV3-F13-454-Fw	GACACTATAGCAGTGCACACAGGAGAYCAA	627	719
DV3-F13-454-Rv	CACTATAGGGGCTTTCCCTTGTCCATCCTC		
DV3-F14-454-Fw	GACACTATAGTGGGCTAGAATGYTCACCAC	515	607
DV3-F14-454-Rv	CACTATAGGGGCTTTCCCTTGTCCATCCTC		
DV3-F15-454-Fw	GACACTATAGGGATGAGCTATGCAATGTGCT	714	806
DV3-F15-454-Rv	CACTATAGGGTATTGCTCTGTCCAGGTGTG		
DV3-F16-454-Fw	GACACTATAGGAGGATGGACAAGGGAAAGC	541	633
DV3-F16-454-Rv	CACTATAGGGCCACTTCCACATTTGAGTTC		
DV3-F17-454-Fw	GACACTATAGGAGGATGGACAAGGGAAAGC	583	675
DV3-F17-454-Rv	CACTATAGGGTATTGCTCTGTCCAGGTGTG		
DV4-F1-454-Fw	GACACTATAGCAATATGCTGAAACGCGAGA	654	746
DV4-F1-454-Rv	CACTATAGGGCCATGTYTCAGCYCTTGTTT		
DV4-F2-454-Fw	GACACTATAGCAATATGCTGAAACGCGAGA	811	903
DV4-F2-454-Rv	CACTATAGGGGRGCGACYAGCATCATTAG		
DV4-F3-454-Fw	GACACTATAGGYTTGATTCCCACCGTAATG	638	730
DV4-F3-454-Rv	CACTATAGGGTTGTGACRCATCCTCCATGT		
DV4-F4-454-Fw	GACACTATAGACACYGTCACGTAYAAATGC	621	713
DV4-F4-454-Rv	CACTATAGGGTAAGGYTCTCCTTGCGTTGG		
DV4-F5-454-Fw	GACACTATAGTTGGAAACAAGRGCTGARAC	705	797
DV4-F5-454-Rv	CACTATAGGGTTATCGTRGCTGTYACTCCA		
DV4-F6-454-Fw	GACACTATAGAACAGGAATYCAGCGAACWG	736	828
DV4-F6-454-Rv	CACTATAGGGGTAGAGGYAGRTCCAAAAAC		
DV4-F7-454-Fw	GACACTATAGGCCCAGGGAAAACCAACCTT	763	855
DV4-F7-454-Rv	CACTATAGGGTGTCCTGCAAACATGTGWTT		
DV4-F8-454-Fw	GACACTATAGATGTCCAACGCAAGGAGARC	752	844
DV4-F8-454-Rv	CACTATAGGGTGCTGTGTTTCTGCCATCTC		

DV4-F9-454-Fw	GACACTATAGTGCCGGAGAGAYGTGGTAGA	697	789
DV4-F9-454-Rv	CACTATAGGGTGCTGTGTTTCTGCCATCTC		
DV4-F10-454-Fw	GACACTATAGRACAGTCCACAATGGAGACA	806	898
DV4-F10-454-Rv	CACTATAGGGTCAAACATCTTGCCAATGGA		
DV4-F11-454-Fw	GACACTATAGTGGAGTKACAGCCACGATAA	755	847
DV4-F11-454-Rv	CACTATAGGGTCAAACATCTTGCCAATGGA		
DV4-F12-454-Fw	GACACTATAGTCATGCCAAGAGACAGGATG	560	652
DV4-F12-454-Rv	CACTATAGGGCCAAAATCCCARGCTGTTTC		
DV4-F13-454-Fw	GACACTATAGTCATGCCAAGAGACAGGATG	770	862
DV4-F13-454-Rv	CACTATAGGGACTGTGAARCCYAGAAACAG		
DV4-F14-454-Fw	GACACTATAGGAGATGGCAGAAACACAGCA	830	922
DV4-F14-454-Rv	CACTATAGGGGTCATGTCCTCCTTCCCAGA		
DV4-F15-454-Fw	GACACTATAGGTTGGGCGYATCATCTCATC	719	811
DV4-F15-454-Rv	CACTATAGGGGTCATGTCCTCCTTCCCAGA		
DV4-F16-454-Fw	GACACTATAGTCCATTGGCAAGATGTTTGA	770	862
DV4-F16-454-Rv	CACTATAGGGGTTCCATGCTCTYCGTTCAT		

APPENDIX 2 – MID primers

Fusion primer	Primer sequence (5' to 3')	MID code in 454 machine
PrimA-RL013-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGACTCGACGTGACACTATAG	1
PrimB-RL013-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGACTCGACGTCACTATAGGG	1
PrimA-RL014-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTACGAGAGTGACACTATAG	2
PrimB-RL014-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTACGAGAGTCACTATAGGG	2
PrimA-RL015-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTACTACTATGACACTATAG	3
PrimB-RL015-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTACTACTATCACTATAGGG	3
PrimA-RL016-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTAGACGTCTGACACTATAG	4
PrimB-RL016-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTAGACGTCTCACTATAGGG	4
PrimA-RL017-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTCGTACACTGACACTATAG	5
PrimB-RL017-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTCGTACACTCACTATAGGG	5
PrimA-RL018-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTGTAGTAGTGACACTATAG	6
PrimB-RL018-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTGTAGTAGTCACTATAGGG	6
PrimA-RL019-Fw	CGTATCGCCTCCCTCGCGCCATCAGATAGTATACGTGACACTATAG	7
PrimB-RL019-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATAGTATACGTCACTATAGGG	7
PrimA-RL020-Fw	CGTATCGCCTCCCTCGCGCCATCAGCAGTACGTACTGACACTATAG	8
PrimB-RL020-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCAGTACGTACTCACTATAGGG	8
PrimA-RL021-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGACGACGCGTGACACTATAG	9
PrimB-RL021-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGACGACGCGTCACTATAGGG	9
PrimA-RL022-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGACGAGTACTGACACTATAG	10
PrimB-RL022-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGACGAGTACTCACTATAGGG	10
PrimA-RL023-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGATACTACGTGACACTATAG	11
PrimB-RL023-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGATACTACGTCACTATAGGG	11
PrimA-RL024-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTACGTCGATGACACTATAG	12
PrimB-RL024-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTACGTCGATCACTATAGGG	12
PrimA-RL025-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTACTCGTAGTGACACTATAG	13
PrimB-RL025-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTACTCGTAGTCACTATAGGG	13

PrimA-RL026-Fw	CGTATCGCCTCCCTCGCGCCATCAGGTACAGTACGTGACACTATAG	14
PrimB-RL026-Rv	CTATGCGCCTTGCCAGCCCGCTCAGGTACAGTACGTCACTATAGGG	14
PrimA-RL027-Fw	CGTATCGCCTCCCTCGCGCCATCAGGTCGTACGTATGACACTATAG	15
PrimB-RL027-Rv	CTATGCGCCTTGCCAGCCCGCTCAGGTCGTACGTATCACTATAGGG	15
PrimA-RL028-Fw	CGTATCGCCTCCCTCGCGCCATCAGGTGTACGACGTGACACTATAG	16
PrimB-RL028-Rv	CTATGCGCCTTGCCAGCCCGCTCAGGTGTACGACGTCACTATAGGG	16
PrimA-RL029-Fw	CGTATCGCCTCCCTCGCGCCATCAGACACAGTGAGTGACACTATAG	17
PrimB-RL029-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACACAGTGAGTCACTATAGGG	17
PrimA-RL030-Fw	CGTATCGCCTCCCTCGCGCCATCAGACACTCATACTGACACTATAG	18
PrimB-RL030-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACACTCATACTCACTATAGGG	18
PrimA-RL031-Fw	CGTATCGCCTCCCTCGCGCCATCAGACAGACAGCGTGACACTATAG	19
PrimB-RL031-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACAGACAGCGTCACTATAGGG	19
PrimA-RL032-Fw	CGTATCGCCTCCCTCGCGCCATCAGACAGACTATATGACACTATAG	20
PrimB-RL032-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACAGACTATATCACTATAGGG	20
PrimA-RL033-Fw	CGTATCGCCTCCCTCGCGCCATCAGACAGAGACTCTGACACTATAG	21
PrimB-RL033-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACAGAGACTCTCACTATAGGG	21
PrimA-RL034-Fw	CGTATCGCCTCCCTCGCGCCATCAGACAGCTCGTGTGACACTATAG	22
PrimB-RL034-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACAGCTCGTGTCACTATAGGG	22
PrimA-RL035-Fw	CGTATCGCCTCCCTCGCGCCATCAGACAGTGTCGATGACACTATAG	23
PrimB-RL035-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACAGTGTCGATCACTATAGGG	23
PrimA-RL036-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGAGCGCGCTGACACTATAG	24
PrimB-RL036-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGAGCGCGCTCACTATAGGG	24
PrimA-RL037-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGATGAGTGTGACACTATAG	25
PrimB-RL037-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGATGAGTGTCACTATAGGG	25
PrimA-RL038-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGCGAGAGATGACACTATAG	26
PrimB-RL038-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGCGAGAGATCACTATAGGG	26
PrimA-RL039-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGCTCTCTCTGACACTATAG	27
PrimB-RL039-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGCTCTCTCTCACTATAGGG	27
PrimA-RL040-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGTCGCTGATGACACTATAG	28

PrimB-RL040-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGTCGCTGATCACTATAGGG	28
PrimA-RL041-Fw	CGTATCGCCTCCCTCGCGCCATCAGACGTCTAGCATGACACTATAG	29
PrimB-RL041-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACGTCTAGCATCACTATAGGG	29
PrimA-RL042-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTAGTGATATGACACTATAG	30
PrimB-RL042-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTAGTGATATCACTATAGGG	30
PrimA-RL043-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTCACACTGTGACACTATAG	31
PrimB-RL043-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTCACACTGTCACTATAGGG	31
PrimA-RL044-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTCACTAGCTGACACTATAG	32
PrimB-RL044-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTCACTAGCTCACTATAGGG	32
PrimA-RL045-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTCTATATATGACACTATAG	33
PrimB-RL045-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTCTATATATCACTATAGGG	33
PrimA-RL046-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTGATCTCGTGACACTATAG	34
PrimB-RL046-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTGATCTCGTCACTATAGGG	34
PrimA-RL047-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTGCTGTACTGACACTATAG	35
PrimB-RL047-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTGCTGTACTCACTATAGGG	35
PrimA-RL048-Fw	CGTATCGCCTCCCTCGCGCCATCAGACTGTAGCGCTGACACTATAG	36
PrimB-RL048-Rv	CTATGCGCCTTGCCAGCCCGCTCAGACTGTAGCGCTCACTATAGGG	36
PrimA-RL049-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGACACTCACTGACACTATAG	37
PrimB-RL049-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGACACTCACTCACTATAGGG	37
PrimA-RL050-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGACATATAGTGACACTATAG	38
PrimB-RL050-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGACATATAGTCACTATAGGG	38
PrimA-RL051-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGACGTGATCTGACACTATAG	39
PrimB-RL051-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGACGTGATCTCACTATAGGG	39
PrimA-RL052-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGAGTACAGATGACACTATAG	40
PrimB-RL052-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGAGTACAGATCACTATAGGG	40
PrimA-RL053-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGAGTATCTCTGACACTATAG	41
PrimB-RL053-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGAGTATCTCTCACTATAGGG	41
PrimA-RL054-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGATACGCTGTGACACTATAG	42
PrimB-RL054-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGATACGCTGTCACTATAGGG	42

PrimA-RL055-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGATCTAGTCTGACACTATAG	43
PrimB-RL055-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGATCTAGTCTCACTATAGGG	43
PrimA-RL056-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGCAGCGTAGTGACACTATAG	44
PrimB-RL056-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGCAGCGTAGTCACTATAGGG	44
PrimA-RL057-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGCGCACGAGTGACACTATAG	45
PrimB-RL057-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGCGCACGAGTCACTATAGGG	45
PrimA-RL058-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGCGTGTGCGTGACACTATAG	46
PrimB-RL058-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGCGTGTGCGTCACTATAGGG	46
PrimA-RL059-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGCTAGATACTGACACTATAG	47
PrimB-RL059-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGCTAGATACTCACTATAGGG	47
PrimA-RL060-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGCTGTCGACTGACACTATAG	48
PrimB-RL060-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGCTGTCGACTCACTATAGGG	48
PrimA-RL061-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTATGCACGTGACACTATAG	49
PrimB-RL061-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTATGCACGTCACTATAGGG	49
PrimA-RL062-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTCGCGCTATGACACTATAG	50
PrimB-RL062-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTCGCGCTATCACTATAGGG	50
PrimA-RL063-Fw	CGTATCGCCTCCCTCGCGCCATCAGAGTCTGTCTGTGACACTATAG	51
PrimB-RL063-Rv	CTATGCGCCTTGCCAGCCCGCTCAGAGTCTGTCTGTCACTATAGGG	51
PrimA-RL064-Fw	CGTATCGCCTCCCTCGCGCCATCAGATACACACGATGACACTATAG	52
PrimB-RL064-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATACACACGATCACTATAGGG	52
PrimA-RL065-Fw	CGTATCGCCTCCCTCGCGCCATCAGATACGCGTGCTGACACTATAG	53
PrimB-RL065-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATACGCGTGCTCACTATAGGG	53
PrimA-RL066-Fw	CGTATCGCCTCCCTCGCGCCATCAGATACTAGCACTGACACTATAG	54
PrimB-RL066-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATACTAGCACTCACTATAGGG	54
PrimA-RL067-Fw	CGTATCGCCTCCCTCGCGCCATCAGATAGAGCTAGTGACACTATAG	55
PrimB-RL067-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATAGAGCTAGTCACTATAGGG	55
PrimA-RL068-Fw	CGTATCGCCTCCCTCGCGCCATCAGATATAGAGTATGACACTATAG	56
PrimB-RL068-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATATAGAGTATCACTATAGGG	56
PrimA-RL069-Fw	CGTATCGCCTCCCTCGCGCCATCAGATCGCTCACGTGACACTATAG	57

PrimB-RL069-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATCGCTCACGTCACTATAGGG	57
PrimA-RL070-Fw	CGTATCGCCTCCCTCGCGCCATCAGATCGTCAGTCTGACACTATAG	58
PrimB-RL070-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATCGTCAGTCTCACTATAGGG	58
PrimA-RL071-Fw	CGTATCGCCTCCCTCGCGCCATCAGATCTCTCGTATGACACTATAG	59
PrimB-RL071-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATCTCTCGTATCACTATAGGG	59
PrimA-RL072-Fw	CGTATCGCCTCCCTCGCGCCATCAGATCTGAGACGTGACACTATAG	60
PrimB-RL072-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATCTGAGACGTCACTATAGGG	60
PrimA-RL073-Fw	CGTATCGCCTCCCTCGCGCCATCAGATGCTACGTCTGACACTATAG	61
PrimB-RL073-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATGCTACGTCTCACTATAGGG	61
PrimA-RL074-Fw	CGTATCGCCTCCCTCGCGCCATCAGATGTGACTACTGACACTATAG	62
PrimB-RL074-Rv	CTATGCGCCTTGCCAGCCCGCTCAGATGTGACTACTCACTATAGGG	62
PrimA-RL075-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACGAGACAGTGACACTATAG	63
PrimB-RL075-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACGAGACAGTCACTATAGGG	63
PrimA-RL076-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACGCGAGTCTGACACTATAG	64
PrimB-RL076-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACGCGAGTCTCACTATAGGG	64
PrimA-RL077-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACGCTACGATGACACTATAG	65
PrimB-RL077-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACGCTACGATCACTATAGGG	65
PrimA-RL078-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACGTGTATATGACACTATAG	66
PrimB-RL078-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACGTGTATATCACTATAGGG	66
PrimA-RL079-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACTACGATGTGACACTATAG	67
PrimB-RL079-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACTACGATGTCACTATAGGG	67
PrimA-RL080-Fw	CGTATCGCCTCCCTCGCGCCATCAGCACTATACTCTGACACTATAG	68
PrimB-RL080-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCACTATACTCTCACTATAGGG	68
PrimA-RL081-Fw	CGTATCGCCTCCCTCGCGCCATCAGCAGCGTACTGTGACACTATAG	69
PrimB-RL081-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCAGCGTACTGTCACTATAGGG	69
PrimA-RL082-Fw	CGTATCGCCTCCCTCGCGCCATCAGCAGTCTCTAGTGACACTATAG	70
PrimB-RL082-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCAGTCTCTAGTCACTATAGGG	70
PrimA-RL083-Fw	CGTATCGCCTCCCTCGCGCCATCAGCATAGTCGCGTGACACTATAG	71
PrimB-RL083-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCATAGTCGCGTCACTATAGGG	71

PrimA-RL084-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGAGACACTATGACACTATAG	72
PrimB-RL084-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGAGACACTATCACTATAGGG	72
PrimA-RL085-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGAGAGTGTGTGACACTATAG	73
PrimB-RL085-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGAGAGTGTGTCACTATAGGG	73
PrimA-RL086-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGAGTCATCGTGACACTATAG	74
PrimB-RL086-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGAGTCATCGTCACTATAGGG	74
PrimA-RL087-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGATCGTATATGACACTATAG	75
PrimB-RL087-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGATCGTATATCACTATAGGG	75
PrimA-RL088-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGCAGTACGCTGACACTATAG	76
PrimB-RL088-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGCAGTACGCTCACTATAGGG	76
PrimA-RL089-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGCGATCGTATGACACTATAG	77
PrimB-RL089-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGCGATCGTATCACTATAGGG	77
PrimA-RL090-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGCGCTATACTGACACTATAG	78
PrimB-RL090-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGCGCTATACTCACTATAGGG	78
PrimA-RL091-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTACAGATATGACACTATAG	79
PrimB-RL091-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTACAGATATCACTATAGGG	79
PrimA-RL092-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTAGCTCTCTGACACTATAG	80
PrimB-RL092-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTAGCTCTCTCACTATAGGG	80
PrimA-RL093-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTATAGTGCTGACACTATAG	81
PrimB-RL093-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTATAGTGCTCACTATAGGG	81
PrimA-RL094-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTCAGCGACTGACACTATAG	82
PrimB-RL094-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTCAGCGACTCACTATAGGG	82
PrimA-RL095-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTCGCAGTGTGACACTATAG	83
PrimB-RL095-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTCGCAGTGTCACTATAGGG	83
PrimA-RL096-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTCTCACGATGACACTATAG	84
PrimB-RL096-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTCTCACGATCACTATAGGG	84
PrimA-RL097-Fw	CGTATCGCCTCCCTCGCGCCATCAGCGTGACTCAGTGACACTATAG	85
PrimB-RL097-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCGTGACTCAGTCACTATAGGG	85
PrimA-RL098-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTACACGCTCTGACACTATAG	86

PrimB-RL098-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTACACGCTCTCACTATAGGG	86
PrimA-RL099-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTACGATATGTGACACTATAG	87
PrimB-RL099-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTACGATATGTCACTATAGGG	87
PrimA-RL100-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTAGACAGACTGACACTATAG	88
PrimB-RL100-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTAGACAGACTCACTATAGGG	88
PrimA-RL101-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTAGTACTCATGACACTATAG	89
PrimB-RL101-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTAGTACTCATCACTATAGGG	89
PrimA-RL102-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTATATGTCGTGACACTATAG	90
PrimB-RL102-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTATATGTCGTCACTATAGGG	90
PrimA-RL103-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTATCGACACTGACACTATAG	91
PrimB-RL103-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTATCGACACTCACTATAGGG	91
PrimA-RL104-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTATGTAGAGTGACACTATAG	92
PrimB-RL104-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTATGTAGAGTCACTATAGGG	92
PrimA-RL105-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTCACGTACATGACACTATAG	93
PrimB-RL105-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTCACGTACATCACTATAGGG	93
PrimA-RL106-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTCGAGTCTCTGACACTATAG	94
PrimB-RL106-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTCGAGTCTCTCACTATAGGG	94
PrimA-RL107-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTCGTCGAGATGACACTATAG	95
PrimB-RL107-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTCGTCGAGATCACTATAGGG	95
PrimA-RL108-Fw	CGTATCGCCTCCCTCGCGCCATCAGCTCTACAGCGTGACACTATAG	96
PrimB-RL108-Rv	CTATGCGCCTTGCCAGCCCGCTCAGCTCTACAGCGTCACTATAGGG	96

1. Purpose

To provide a qualitative procedure for performing emPCR Amplification for 454 sequencing.

2. Principle:

N/A

3. Scope and responsibilities:

Lab technicians have responsibility for performing testing following defined procedures

4. Reference

N/A.

5. Definition

N/A

6. Specimen requirement

cDNA and PCR product

7. Materials/Equipments used

7.1	GS Junior Titanium emPCR kit (Lib-A)	Roche – 05996520001
7.2	GS Junior Titanium PicoTiter Plate Kit	Roche – 05996619001
7.3	Centrifuge rotor for 50 ml tubes	Eppendorf – 5430
7.4	Microcentrifuge	Eppendorf – 5424
7.5	Dry-Block Heater	
7.6	96-well, 0.2 ml block for Thermocycler	
7.7	Vortex	
7.8	LabQuake Shaker	
7.9	Particle Counter	Roche

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7.10 Pipette Tips

7.11 Ethanol

7.12 96-well plates, semi skirted

7.13 Cap strips for 96-well plates

7.14 Molecular Biology Grade Water

7.15 2-Propanol (Isopropanol)

7.16 NaOH

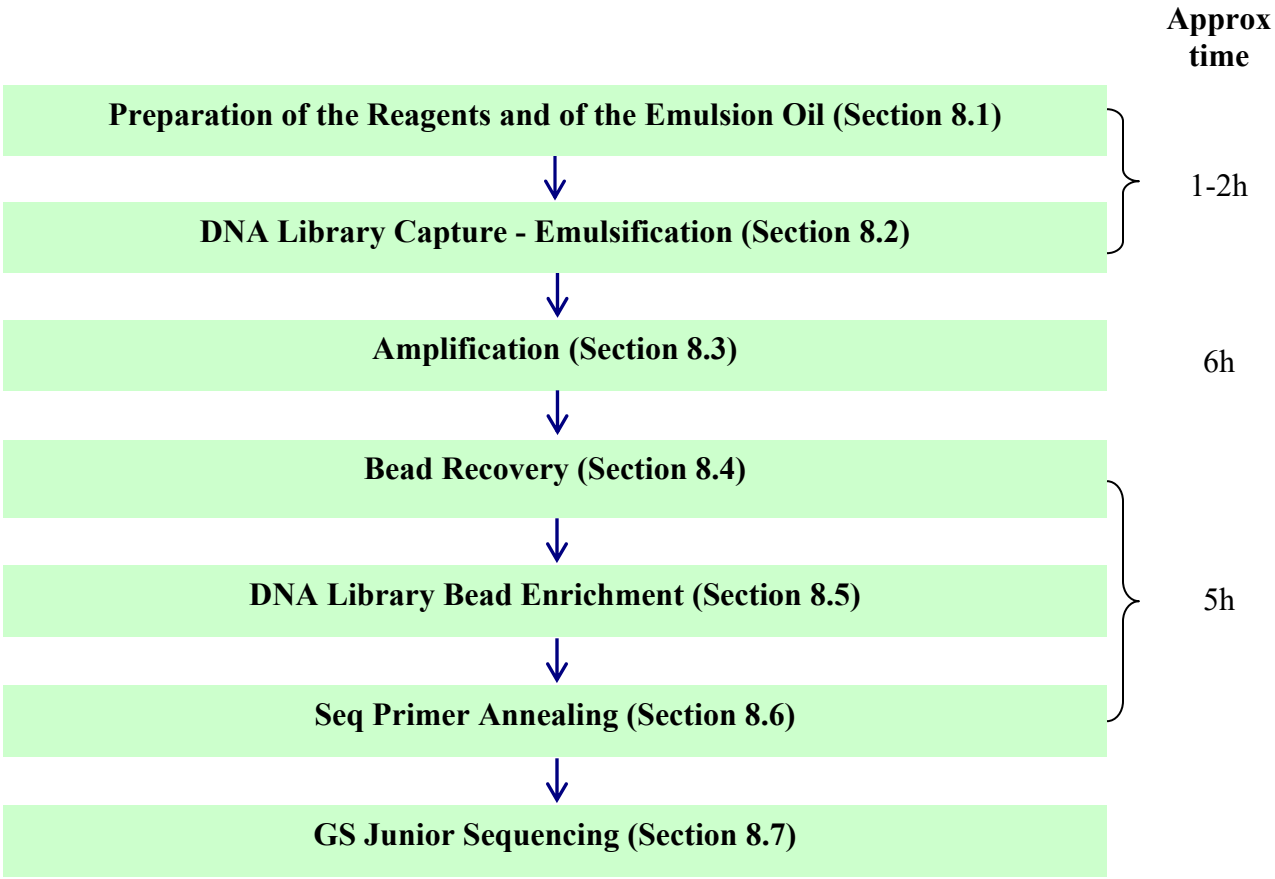
7.17 Magnetic Particle Concentrator

7.18 Falcon tube 50mL

7.19 Petri dish/ chemical container 50mL

8. Procedures

The emulsion-based clonal amplification (emPCR amplification) of a DNA library sample involves 6 major steps, described below. The method described in this manual is for the GS Junior Titanium Series of PCR products with bi-directional fusion primers (Lib-A).



Section	Description	Person(s) Responsible
8.1 Preparation of the Reagents and of the Emulsion Oil	**8.1.1 Determining the Amount of Library to Use in emPCR Amplification:** Calculate the volume of DNA library needed by using the following equation: $$\text{µl of DNA library per tube} = \frac{\text{Desired molecules per bead x } 5.10^6 \text{ beads}}{\text{Library concentration (in molecules/µl)}}$$ Take the vol of DNA library needed in the clean tube and put it in wet ice. **8.1.2 Preparation of the Mock Mix and Pre-Emulsion:** 8.1.2.1 Open the emPCR Reagents box and thaw the kit components at room temperature, except the Enzyme Mix and the PPiase tubes, which should be kept at -15°C to -25°C. Once thawed, vortex for 5 seconds. 8.1.2.2 Vortex and heat the tube of Additive at 55ºC for 5 minutes to aid dissolving. If a precipitate persists, centrifuge the tube and use the supernatant. 8.1.2.3 Spin all the kit components (including enzymes) in a bench top mini centrifuge for 10 seconds. 8.1.2.4 Return the enzymes to -15 to -25°C. Leave the other reagents at room temperature. 8.1.2.5 Vortex vigorously the tube of emulsion oil for 10 seconds at maximum vortex speeds, and pours the entire content (4 ml) into the Turrax stirring tube. 8.1.2.6 Prepare 1x Mock Mix by adding 430 µl of Mock Mix to 1.72 ml of Molecular Biology Grade Water. Vortex to mix. 8.1.2.7 Add 2.0 ml of 1x Mock Mix to the Turrax stirring tube	Assigned Laboratory Staff

containing the emulsion oil. Set the Ultra Turrax Tube Drive (UTTD) to 4000 rpm for 5 minutes. Place the stirring tube in the UTTD and start the UTTD to mix the emulsion.

8.1.2.8 When finished, remove the stirring tube from the UTTD.

8.1.3 Preparation of the Live Amp Mixes A and B

8.1.3.1 Prepare the Live Amp Mix A and the Live Amp Mix B in two separate tubes, according to Table 1. Add the reagents in the order they are listed in the table.

8.1.3.2 Vortex the Live Amp Mixes A and B for 5 seconds, and store them on ice.

A: Live Amp Mix A

Reagent	Volume (µl)
Mol. Bio. Grade Water	179.5
Additive	260
Amp Mix	148.5
Amp Primer A	52
Enzyme Mix	35
PPiase	1
Total	**676**

B: Live Amp Mix B

Reagent	Volume (µl)
Mol. Bio. Grade Water	179.5
Additive	260
Amp Mix	148.5
Amp Primer B	52
Enzyme Mix	35
PPiase	1
Total	**676**

8.2 DNA Library Capture - Emulsification	**8.2.1** Prepare 1x Wash Buffer by mixing 0.5 ml of Wash Buffer with 4.5 ml of Molecular Biology Grade Water. **8.2.2** Add 1 ml of 1x Wash Buffer to both tubes of Capture Beads and vortex. **8.2.3** Pellet the Capture Beads in a bench top minifuge, by spinning for 10 seconds, rotating the tube 180°, and spinning again for 10 seconds (spin-rotate-spin). **8.2.4** Carefully remove and discard the supernatants without disturbing the bead pellets. **8.2.5** Wash the Capture Beads once more with 1 ml of 1x Wash Buffer. Vortex to resuspend the beads, spin-rotate-spin, and discard the supernatant. **8.2.6** Add the calculated volume of the Amplicon DNA library to the tube of washed Capture Beads A. **8.2.7** Add the same calculated volume of Amplicon DNA library to the tube of Capture Beads B. **8.2.8** Vortex the tubes for 5 seconds to mix. **8.2.9** Add 600 µl of Live Amp Mix B to the tube of captured library B. Vortex, and transfer the entire content into the Turrax stirring tube (from Section 8.1.2). **8.2.10** Set the UTTD to 2000 rpm for 5 minutes and start the UTTD to mix the emulsion. **8.2.11** Add 600µl of Live Amp Mix A to the tube of captured library A. Vortex, and transfer the entire content into the same Turrax stirring tube as in Step 3.11	Assigned Laboratory Staff
8.3 Amplification	**8.3.1** Aliquot 100µl of emulsion into nine 8-strip cap tubes or one 96-well plate (~70 wells), by slowly aspirating, taking care not to draw air. **8.3.2** Cap the wells and make sure that all the wells are properly	Assigned Laboratory Staff

<table>
<tr>
<td></td>
<td>sealed.
8.3.3 Place the tube strip/plate in a thermocycler and start the amplification program with the heated lid turned on. The program takes ~6 h to complete.
<table>
<tr><th>Temp</th><th>Time</th><th>Cycle</th></tr>
<tr><td>94°C</td><td>4 minutes</td><td>1x</td></tr>
<tr><td>94°C</td><td>30 seconds</td><td rowspan="2">50x</td></tr>
<tr><td>60°C</td><td>10 minutes</td></tr>
<tr><td>10°C</td><td colspan="2">hold</td></tr>
</table>
</td>
<td></td>
</tr>
<tr>
<td>8.4 Bead Recovery</td>
<td>8.4.1 Emulsion Collection and Initial Washes
8.4.1.1 Pipette the emulsions (A and B) from all the wells and collect them in the chemical container.
8.4.1.2 Pour 6mL isopropanol into chemical container or petri dish. Rinse the wells with 200µl of isopropanol per well up to spend up 6mL isopropanol.
8.4.1.3 Transfer all the emulsions to Falcon 50mL.
8.4.2 Bead Washes and Recovery
8.4.2.1 Vortex the 50 ml tube of collected emulsions.
8.4.2.2 Add isopropanol to a final volume of 35 ml and vortex to resuspend the pellet.
8.4.2.3 Pellet the beads in a centrifuge at 930 x g for 5 min (2813 RPM for the Eppendorf 5430 centrifuge, rotor F-35-6-30) and carefully pour out the supernatant.
8.4.2.4 Add 10 ml of Enhancing Buffer and thoroughly vortex to resuspend the pellet (it is important to properly rinse the beads. Use glass rod or a spatula to break the aggregates, if necessary).
8.4.2.5 Add isopropanol to a final volume of 40 ml and vortex well.</td>
<td>Assigned Laboratory Staff</td>
</tr>
</table>

	8.4.2.6 Pellet the beads in a centrifuge at 930 x g for 5 min and carefully remove the supernatant. 8.4.2.7 Add isopropanol to a final volume of 35 ml of and vortex well. 8.4.2.8 Pellet the beads in a centrifuge at 930 x g for 5 min and carefully remove the supernatant. 8.4.2.9 Add ethanol to a final volume of 35 ml of and vortex well. 8.4.2.10 Pellet the beads in a centrifuge at 930 x g for 5 min and carefully remove the supernatant. 8.4.2.11 Add Enhancing Buffer to a final volume of 35 ml of and vortex well. 8.4.2.12 Pellet the beads in a centrifuge at 930 x g for 5 min and carefully remove the supernatant, leaving approximately 2 ml of Enhancing Buffer. 8.4.2.13 Transfer the DNA bead suspension using a 1000µl pipette into a provided eppendorf tube. 8.4.2.14 Spin-rotate-spin and discard the supernatant. 8.4.2.15 Rinse the 50ml Falcon tube with 1 ml of Enhancing Buffer, and add this rinse to the eppendorf tube. Spin-rotate-spin and discard the supernatant. 8.4.2.16 Thoroughly rinse the bead pellet twice with 1ml of Enhancing Buffer. Spin-rotate- spin and discard the supernatant	
8.5 DNA Library Bead Enrichment	**8.5.1 Preparation for Enrichment** 8.5.1.1 Turn on the heating dry-block and set it to 65°C. 8.5.2 Prepare the Melt Solution by mixing 125 µl of NaOH (10 N) in 9.875 ml of Molecular Biology Grade Water. 8.5.3 Add 1 ml of Melt Solution to the 1.7 ml tube of beads	Assigned Laboratory Staff

and vortex. Incubate for 2 minutes at room temperature. Spin-rotate-spin and discard the supernatant.

8.5.4 Repeat Step 3 once.

8.5.5 Add 1 ml of Annealing Buffer to the 1.7 ml tube of beads and vortex. Spin-rotate-spin and discard the supernatant.

8.5.6 Repeat Step 5 twice.

8.5.7 Add 45µl of Annealing Buffer, 15µl of Enrich Primer A and 15µl of Enrich Primer B to the 1.7 ml tube of beads, and vortex.

8.5.8 Place the tube in a heat block at 65°C for 5 minutes, and then promptly cool on ice for 2 minutes.

8.5.9 Add 1 ml of Enhancing Buffer to the 1.7 ml tube of beads and vortex. Spin-rotate-spin and discard the supernatant.

8.5.10 Repeat Step 9 two more times.

8.5.11 Add 1 ml of Enhancing Buffer to the 1.7 ml tube of beads and vortex.

8.5.12 Set the tube aside at room temperature until Section 8.5.3

8.5.2 Preparation of the Enrichment Beads

8.5.2.1 Vortex the tube of brown Enrichment Beads for 1 minute to resuspend its contents completely.

8.5.2.2 Place the tube in a Magnetic Particle Concentrator (MPC) and wait ~3 minutes to pellet the Enrichment Beads.

8.5.2.3 Discard the supernatant, taking care not to draw off any Enrichment Beads.

8.5.2.4 Add 500µl of Enhancing Buffer and vortex.

8.5.2.5 Pellet the Enrichment Beads using an MPC.

8.5.2.6 Discard the supernatant, taking care not to draw off any Enrichment Beads.

8.5.2.7 Repeat Steps 2.4 to 2.6 once. After discarding the supernatant, remove the tube from the MPC

8.5.2.8 Add 80µl of Enhancing Buffer and vortex.

8.5.3 Enrichment of the DNA-Carrying Beads

8.5.3.1 Add 80µl of washed Enrichment Beads to the tube of beads (from Section 8.5.1) and vortex to mix completely.

8.5.3.2 Rotate the tube on the LabQuake, at room temperature for 5 minutes.

8.5.3.3 Place the tube in the MPC, and wait 3-5 minutes to pellet the Enrichment Beads.

8.5.3.4 Invert the MPC several times and wait for the beads to pellet.

8.5.3.5 Carefully discard the supernatant using a 1000µl pipette, taking care not to draw off any brown Enrichment Beads.

8.5.3.6 Wash the beads with Enhancing Buffer until there are no visible white beads remaining in the supernatant, as follows:

a. Add 1 ml of Enhancing Buffer to the tube.

b. Remove the tube from the MPC and vortex well.

c. Place the tube back into the MPC to pellet the beads on the wall of the tube with the magnet. Invert the MPC and wait for the beads to pellet.

d. Carefully discard the supernatant with a 1000µl pipette, taking care not to draw off any Enrichment Beads.

e. Repeat 6 to 10 times until white DNA beads are no longer being aspirated.

8.5.4. Collection of the Enriched DNA Beads

8.5.4.1 Remove the tube of enriched beads (enrichment tube)

	from the MPC and resuspend the bead pellet in 700µl of Melt Solution. 8.5.4.2 Vortex for 5 seconds, and place the enrichment tube in the MPC until the Enrichment Beads have pelleted. 8.5.4.3 Transfer the supernatant containing the enriched DNA beads to a new eppendorf tube. 8.5.4.4 Add once again 700µl of Melt Solution to the enrichment tube. 8.5.4.5 Vortex for 5 seconds, and place the enrichment tube in the MPC until the Enrichment Beads have pelleted. 8.5.4.6 Transfer the supernatant containing enriched DNA beads into the same 1.7 ml tube, from Step 3. 8.5.4.7 Discard the enrichment tube. 8.5.4.8 Spin-rotate-spin the 1.7 ml tube and discard the supernatant. 8.5.4.9 Add 1 ml of Annealing Buffer and vortex for 5 seconds. 8.5.4.10 Spin-rotate-spin and discard the supernatant. 8.5.4.11 Repeat Steps 4.9 and 4.10 two times. 8.5.4.12 Add 100µl of Annealing Buffer and vortex to resuspend the beads.	
8.6 Seq Primer Annealing	**8.6.1** Add 15µl of Seq Primer A and 15µl of Seq Primer B, and vortex. **8.6.2** Place the 1eppendorf tube in a heat block at 65°C for 5 min, and then promptly cool on ice for 2 min. **8.6.3** Add 1ml of Annealing Buffer and vortex for 5 seconds. Spin-rotate-spin, and discard the supernatant. **8.6.4** Repeat Step3 two times. 8.6.5 Add 1 ml of Annealing Buffer to the bead pellet and	Assigned Laboratory Staff

	vortex. 8.6.6 Spin-rotate-spin to pellet the beads. 8.6.7 A GS Junior sequencing Run requires an input of 500,000 enriched beads (around 5%).	
8.7 GS Junior Sequencing	Refer to GS Junior Sequencing Manual	Assigned Laboratory Staff

9. Quality Control

9.1 emPCR

The recommended input bead number for a GS Junior sequencing Run is 500,000 enriched beads. To evaluate the amount of enriched beads, use the GS Junior Bead Counter v2, which is an accessory to the GS Junior Instrument.

Hold the Bead Counter at eye level with the side reading Single Prep facing you. Look in the window to evaluate the amount of enriched beads. The top of the bead pellet should be within the window.

a. If the top of the bead pellet is within the window and above the bottom edge of the window (500,000 beads) (Figure 3A), remove the excess beads and store them into a clean 1.7 ml micro centrifuge tube for later use, as they are valuable. Load the remaining 500,000 beads on the GS Junior Instrument.

b. If the top of the bead pellet is below the bottom edge of the Bead Counter window (i.e. not visible), the preparation contains less than 500,000 beads. However, the beads are valuable and the amount could be sufficient for the experimental design.

c. If the top of the bead pellet is above the top edge of the Bead Counter window, the preparation has failed. Discard the beads and repeat the emulsion process with 3 times less library sample.

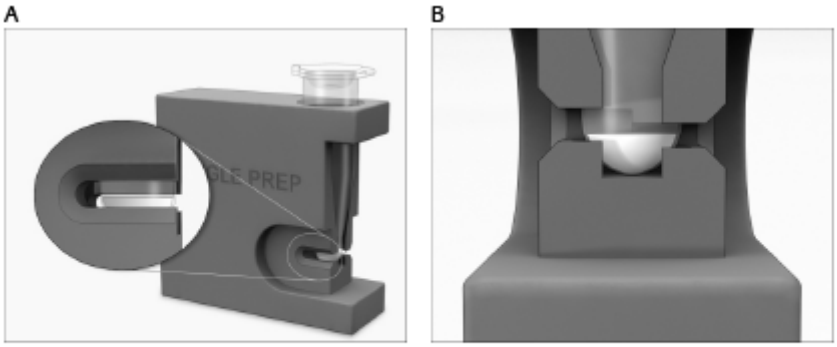


Figure 3: (A) The Single Prep side view of the GS Junior Bead Counter v2, (B) a front view of the GS Junior Bead Counter v2, with the Single Prep side on the left and the Multiple Prep side on the right

9.2 GS Junior sequencing:

Depth of coverage (5000x Amplicon coverage, 14 Amplicon Libraries) needs for one amplicon with variant to 1% frequency,

Criteria	Requirement
Average read length	> 300bp
Total Passed filter bases	19 - 27 million
Total Reads or Total Passed Filtering	> 70,000

10. Calculations

N/A

11. Interpretation of results and report

N/A

12. Reporting of results

N/A

13. Reference range and critical value:

N/A

14. Limitations:

N/A

15. Safety

Universal precautions should be used when working with clinical specimens.

When working with chemicals, always ware a suitable lab coat, disposal gloves, and protective goggles.

Place any waste in the clinical/ infectious waste bins provided.

16. Supplementary notes

Change gloves frequently.

Soak tube crack in bleach after using, clean pipette after each step

Clean working bench with bleach-water-Ethanol.

17. Risk assessment and risk management:

N/A

18. Related Documents:

Document name	Document code
GS Junior Sequencing Manual	N/A

19. Document History:

Revision no.	Effective date	Details of amendment
001	01-06-2013	New issue
002	14-12-2013	Edit emPCR thermalcycling

1. Purpose

This procedure describes the process used to proliferate culture cells, particularly with BHK-21 and C6/36 cell lines.

2. Principle:

Cell Culture is the process by which cells are grown under controlled conditions, outside their natural environment. These conditions include but is not limited to a suitable vessel with medium that supplies essential nutrients and an incubator with appropriate temperatures.

Cells are allow to grow on a surface of the vessel until they are confluent. Then they are detached from the surface by enzymatic activities of trypsin, and diluted with GM. GM, with high levels of nutrients, is used for high rate of cell proliferation. GM is prepared with the SOP code: TP-TO-DNG-019. Different cell lines require different conditions. For example, BHK-21 cells need vented flasks and an 37°C incubator with CO_2, while C6/36 cell culture need plug-seal-cap flasks. The incubator for C6/36 is exceptionally kept at 28°C, and CO_2 is absent.

This procedure is applied by the dengue group to culture BHK-21 and C6/36 cells as the two cell lines are susceptible to DENV infection. BHK-21 cells are also a host cell line for EAV, used as controls for dengue PCR. This procedure is also used to have cells for storage in, or thaw from, liquid nitrogens.

3. Scope and responsibilities:

This procedure must take place in a clean area of level-2 laboratories, designed for cell culture. Aseptic facilities are also required to avoid microbial contamination, and aseptic technique must be used at all times to avoid contamination of the cell lines. The procedure is only performed by trained personels. The training log will be signed by the trainee, and co-signed by the supervisor beforehands. Appropriate PPE is required to be worn by staff members at all times when undertaking this procedure.

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4. Reference

Do Quang Ha. Procedures used in the virology laboratory OUCRU-HTD HCMC (an OUCRU guideline)

5. Definition

- BHK-21 cells — Kidney cells originating from newborn Syrian hamsters (Clone-13)
- C6/36 cells — Cells derived from *Aedes albopictus*
- Confluency — A term used estimate of the number of adherent cells in a culture dish or a flask, referring to the proportion of the surface which is covered by cells. For example: 90% confluency means about 90% of the flask surface is covered with cells.
- GM — Growth Media
- PBS — Phosphate Buffer Saline
- DENV — Dengue viruses
- EAV — Equine arteritis virus

6. Specimen requirement

C6/36 (CRL1660) *Aedes albopictus* cell line Supplier: ATCC Cat number: CRL-1660

BHK-21 Kidney cells from hamsters Supplier: ATCC Cat number: CCL-10

7. Materials/Equipments used

- PBS Supplier: Sigma Cat number: P5368
- Trypsin Supplier: Gibco Cat number: 25200056
- Sterile pipettes (10ml, 5ml, transfer pipettes)
- Timer
- Flask T-25 plug seal cap (for C6/36) Supplier: Sigma Cat number: C7046
- Flask T-25 vented cap (for BHK-21) Supplier: Sigma Cat number: CLS430639

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8. Procedures

The volume following is used for cells in T25-flask. With other size of flasks, the volume would be different.

1) Check the confluence of cells under an inverted microscope. If the cells are confluent, the following steps are proceed.
2) Use the 5-mL pipettes to remove the medium inside the cell flask
3) Rinse cell briefly with 5ml PBS
4) Coat the cell layer with 0.5ml Trypsin 0.25% for 3 minutes at 37°C. Visually check detachment of the cells. The cells detach when they are round. If the cells are not entirely removed from the surface of the flask, incubate for another 2 minutes. Complete detachment may take:
   a. up to 3 minutes for C6/36; and
   b. up to 5 minutes for BHK-21.
5) Add 5ml GM to inactivate Trypsin.
6) Calculate the number of viable cells according to the protocol TP-TO-DNG-001
7) Dilute cell suspension with GM to the desired concentration
   a. 10^6 cells/ml for C6/36
   b. 10^5 cells/ml for BHK-21.
8) Transfer 5ml of diluted cell suspension after dilution into a new T-25 flask labelled with cell line name and passage number.
9) Keep BHK-21 flasks at 37°C with CO_2, C6/36 flasks are at 28°C without CO_2.

9. Quality Control

- All reagents should be stored at appropriate temperatures.
- Trypsin should be aliquoted for single use, and stored at -20°C.
- Cell morphology should be monitored on a daily basis for all cultures, using inverted microscopes.

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10. Safety

- Wear laboratory coat, gloves.
- Place waste in appropriate waste bins provided.
- All aseptic procedure must be performed in the class II safety cabinet.

11. Risk assessment and risk management:

There are no hazard identified with this procedure. However, the laboratory staff must obtain proper training to understand emergency procedures and waste disposal procedures, indicated below.

<table>
<tr><td>Department
Dengue group
Location of work
Clean room, lab 115</td><td>Persons involved
Dengue staff</td></tr>
<tr><td>Description of procedure
The procedure describe how culture cells</td><td>Substances used
N/A</td></tr>
<tr><td>Quantities used
N/A</td><td>Frequency of use
N/A</td></tr>
<tr><td>Hazards identified
N/A</td><td>Could a less hazardous substance be used
instead?
N/A</td></tr>
<tr><td colspan="2">What measures have you taken to control risk?
PPE: i. Designated gown for BSCII work ii. Gloves iii. Cover shoe</td></tr>
<tr><td colspan="2">Checks on control measures
Supervision and observation by senior staff</td></tr>
<tr><td>Is health surveillance required?
No</td><td>Training requirements:
Good Laboratory practice and PPE.
Specific training in this procedure, aseptic techniques especially
(with associated documentation of training by staff and supervisor)</td></tr>
<tr><td>Emergency procedures:
Spills: follow “biological and chemical clean up procedure” PR-HS-ALL-005.
Staff must report incidents to supervisors immediately.</td><td>Waste disposal procedures:
OUCRU BSK2 manual of waste management SD-HS-ALL-009</td></tr>
</table>

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18. Related Documents:

Document name	Document code
Amplification of Equine artieritis virus	*TP-TC-DNG-006*
Preparation of DENV in C6-36 Cells	*TP-TO-DNG-007*
Preparation of cell culture media	*TP-TC-DNG-079*
Cell counting and determing cell viability	*TP-TO-DNG-001*
Isolation of dengue viruses	*TP-TC-DNG-060*
Cryopreservation and thawing of cell lines	*TP-TO-DNG-062*

19. Document History:

Revision #	Effective date	Details of amendment
000	30 Dec 2013	New issue
001	06 Apr 2018	Converted to new OUCRU template for SOPs. Merged the SOPs relating to the culture of BHK-21 with C6/36. Added sections: purpose, principle, scope and responsibilities, reference, definition, safety, risk assessment and risk management, related documents. Clarified sections: specimen requirement, materials/equipments used, procedures, quality control – Trung Vu

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1. Purpose

This procedure aims to provide the instruction on isolating of dengue viruses (DENV) from plasma of dengue patients.

2. Principle:

C6/36 mosquito cells, which retains high susceptibility to dengue virus, are often used to isolate DENV from clinical specimens such as plama of dengue patients. The plasma sample should be diluted 10-fold before incubating with C6/36 cells to avoid cytotoxicity.C6/36 should be confluent at the time of DENV isolation, and the cell growth is kept minimum by the use of MM. The result of DENV isolation must be confirmed by a virological method that detect the presence of DENV. For example, PCR with the DENV-containing isolates.

DENV-postitive isolates can be used for various analysis purposes. For example: genome sequencing, positive control for PCR, and reference DENV strains.

3. Scope and responsibilities:

This procedure must take place in level-2 laboratories with aseptic facilities, and only be performed by trained personels. The training log will be signed by the trainee, and co-signed by the supervisor beforehands. Appropriate PPE is required to be worn by staff members at all times when undertaking this procedure.

4. Reference

Do Quang Ha. Procedures used in the virology laboratory OUCRU-HTD HCMC (an OUCRU guideline)

5. Definition

- PBS: Phosphate Buffer Saline
- GM: Growth Media
- MM: Maintenance Media
- DENV: Dengue viruses

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- PPE: Personal protective equipment
- PCR: Polymerase chain reaction
- BSA: Bovine Serum Albumin

6. Specimen requirement

- Viremic plasma specimens from dengue patients

7. Materials/Equipments used

• Sterile pipettes	(10ml, 5ml, transfer pipettes)	
• Flask T-25 plug seal cap	Corning	Cat number: 430168
• Filter	Merck	Cat number: SLHV013SL
• Bovine serum albumin	Sigma	Cat number: A7409-50ml
• PBS	Sigma	Cat number: P5368
• 2 mL screw cap tube	Scientific Specialities	Cat number: 2341-S0

8. Procedures

The volume following is used for cells in T25-flask. With other size of flasks, the volume would be different.

1) C6/36 cells are prepared according to the SOP: *TP-TO-DNG*-061
2) After incubation for 1–3 days, when C3/36 cells are confluent in a flask, prepare plasma specimens for isolation. Each plasma sample is assigned an identical number; for example, a study code, given to a patient from whom the plasma sample is originated.
3) Keep a sterile effendorf on ice
4) Add 450 µl of MM to the effendorf. The preparation of MM can be found in the SOP: *TP-TO-DNG-019*
5) Add 50 µl of plasma to the MM to avoid cytotoxicity to the cells, caused by components in plasma. This creates a 1/10 dilution.
6) Label the flasks with the sample numbers
7) Use 5 ml pipettes to remove as much medium as possible from the flasks

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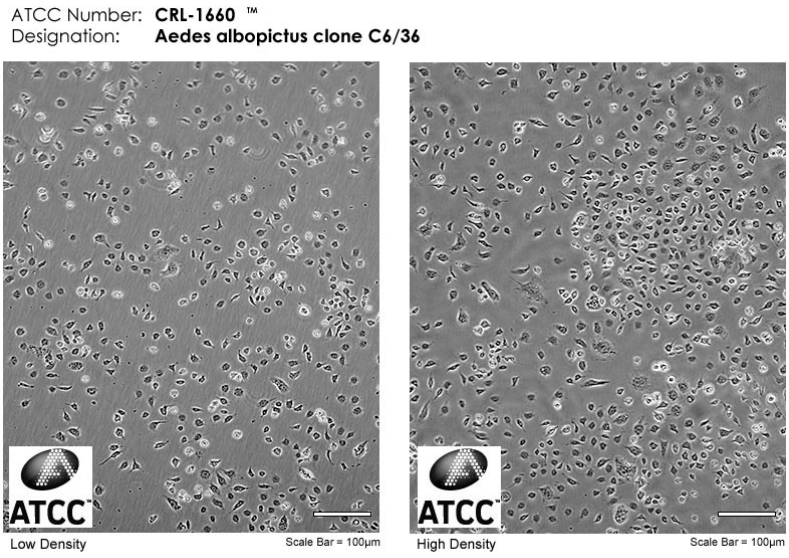
8) Add 4.5 ml of MM into the flasks.
9) Inoculate 500µl of diluted plasma samples into flasks. This is now a 1/100 dilution.
10) Incubate these flasks at 28°C without CO_2 for 7 days
11) Check the morphology and vialbility of C6/36 cells by inverted microscopes everyday.
12) At 7 days after DENV infection of C6/36 cells, check the presence of viable C6/36 cells under an inverted microscope. The cells are viable if they maintain their morphology, as in Figure 1. Only proceed to following steps if cells are viable.
13) Use 5 ml sterile pipette to transfer 5 ml of supernatant into a falcon tubes
14) Centrifuge at 1500rpm for 5 minutes to separate remmant of cells
15) Collect 4.5 ml supernatant without cell remnant
16) Dilute BSA (35%) with PBS to obtain BSA (7.5%),
17) Add 0.5 ml of BSA 7.5% into the 4.5 ml supernatant.
18) Aliquot into 5 × 2-mL screw cap tubes, each tube contain 1 mL of supernant and store the tubes in -80°C.

Figure 1: examples of C6/36 confluency: low (left) and high (right) density cells

(Source: https://www.atcc.org/Products/All/CRL-1660.aspx)



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9. Quality Control

- MM should be stored at 4°C. The plasma samples, before and after diluting with MM, should be kept on ice to avoid viral death.
- Allocate one flask for negative control (incubate with saline) and one positive control flask (incubate with supernatant containing DENV). The same steps above are applied for these 2 flasks.
- Check cell morphology and vialbility by inverted microscopes every day.
  - If there are abnormal cell growth or death, proceed to the step 12 immediately and look for the presence of DENV in infected C6/36 or in isolates. If DENV are not detected, attempt another isolation. Note the number of infectious DENV in plasma will decline if using the same plasma tube.
  - If there is microbial contamination, filtered the cell supernatant, then inoculate the supernant onto new C6/36 cells.

10. Safety

- Safety issues including PPE
- Use appropriate lab coat, gloves, and PPE
- Only trained person could be allowed to work in virology .
- All sterile processes are performed in the bio safety cabinet level 2
- Place waste in appropriate waste bins provided.

11. Risk assessment and risk management

Department Dengue group **Location of work** Lab 115	**Persons involved** Dengue staff
Description of procedure The procedure describe how to isolate DENV in plasma of dengue patients.	**Substances used** DENV
Quantities used 50 µl at any one sample at any one time	**Frequency of use** Frequent
Hazards identified Potential DENV infection, and other blood-	**Could a less hazardous substance be used instead?**

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borne infections.	No
What measures have you taken to control risk? This procedure involves the risk of DENV infection and other blood-borne infections, and must therefore only be performed by trained staff members, when they are feeling alert and well. Appropriate PPE is provided and must be worn at all times when performing the procedure (i.e.: lab coat, gloves, eye protection); SOP of *Universal precaution and Personnel Protective Equipment* guidelines for working in the laboratories, *PR-HS-ALL-006* is to be followed.	
Checks on control measures Supervision and observation by senior staff	
Is health surveillance required? Yes, Hepatitis B serology	**Training requirements:** Good Laboratory practice and PPE. Specific training in this procedure, aseptic techniques especially. (with associated documentation of training by staff and supervisor)
Emergency procedures: Spills: follow "biological and chemical clean up procedure" PR-HS-ALL-005. Staff must report incidents to supervisors immediately.	**Waste disposal procedures**: OUCRU BSK2 manual of waste management SD-HS-ALL-009

18. Related Documents:

Document name	Document code
Preparation of cell culture media	*TP-TC-DNG-079*
Collection and identification of isolates using IFA	*TP-TC-DNG-059*
Cell propagaion	*TP-TC-DNG-061*
Serotyping and quantification of Dengue viruses by multiplex PCR	*TP-TC-DNG-010*

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19. Document History:

Revision #	Effective date	Details of amendment
000	30 Dec 2013	New issue
001	09 Apr 2018	Converted to new OUCRU template for SOPs. Rewrited sections: principle, scope and responsibility, reference, quality control, risk assessment and risk management. Clarified sections: purpose, definition, specimen, material and equipments, procedures and safety – Trung Vu & Lauren Carrington

Note: This is a CONTROLLED document. Any documents in paper form not stamped "Controlled copy" are not controlled and should be checked against the master document.

1. Purpose

To describe the procedure for preparation of DENV stocks, by amplification of the virus in C6/36 cells.

2. Principle:

Stock virus (and working dilutions of stock virus) are needed for use in experiments as positive controls, among other things. DENV can be amplified in the cultured Aedes albopictus C6/36 cell line. After inoculating the virus onto a confluent monolayer of cells, the virus replicates in the cells, before infectious virus particles are released into the supernatant, which can be harvested. Virus stocks are first prepared, before diluted working stocks are made, and all aliquots are frozen ready for use. Infection of cells can be confirmed after the fact, using techniques such as DENV PCR.

3. Scope and responsibilities:

This procedure must take place in level-2 laboratories with aseptic facilities, and only be performed by trained personels. The training log will be signed by the trainee, and co-signed by the supervisor beforehands. Appropriate PPE is required to be worn by staff members at all times when undertaking this procedure

Each DENV culture control batch must be recorded into DENV culture control batch number index. Each sample tube must be labelled with the DENV culture control batch number as defined by the:

- DENV-1 culture control batch number index *FM-TO-DNG-019*
- DENV-2 culture control batch number index *FM-TO-DNG-020*
- DENV-3 culture control batch number index *FM-TO-DNG-021*
- DENV-4 culture control batch number index *FM-TO-DNG-022*

4. Reference

Do Quang Ha. Procedures used in the virology laboratory OUCRU-HTD HCMC (an OUCRU guideline)

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5. Definition

- C6/36 = Aedes albopictus cells, originally derived from larvae.
- GM = Growth Media
- MM = Maintenance Media
- BSA = Bovine Serum Albumin
- PBS = Phosphate Buffered Saline
- PCR = Polymerase Chain Reaction
- T-75 = T-75 cm2 flask

6. Specimen requirement

- C6/36 cells
- Supernatants of each DENV (1-4)

7. Materials/Equipments used

• BSA	Supplier: Sigma	Cat number: A7409-50ml
• PBS	Supplier: Sigma	Cat number: P5368
• 2 mL screw cap tube	Supplier: Scientific Specialities	Cat number: 2341-S0
• T-75 cm2 flask	Supplier: Sigma	Cat number: CLS430641

- Falcon tube (50ml)
- Sterile Pasteur pipette
- Label

8. Procedures

The volume following is used for cells in T75-flask. With other size of flasks, the volume would be different.

Virus inoculation

1) The procedure begins with a confluent flask of C6/36 cells.
2) Wash the cell monolayer in each flask with 15ml of warm PBS.

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3) Inoculate 1.5ml of virus supernatants of DENV (1-4) into each of four T-75 flasks.
4) Add 13.5ml of MM into each of the T-75 flask
5) Incubate flasks at 28°C without CO_2 for 7 days.
6) Pour the cell supernatant in a Falcon tube (50ml) and centrifuge at 1500rpm for 5min.
7) Separate the supernatant from the cell pellet. The supernatant will be stored down, as described below.
8) In a 50ml Falcon tube, add 1.5ml BSA solution and transfer 13.5ml of supernatant. The final volume of BSA will be equal to 10% of the supernatant volume.
9) Aliquot supernatant into sterile tubes (1000µl/tube), label, and store at -80°C. These aliquots are DENV culture stocks. The following is an example of the label placed on the aliquots.

Culture stock
DENV___/YY/NNNNN
Harvesting date: ___/___/_____
Aliquot Vol./No.: _____/_____

where **YY/ NNNNN:** show as year/number of batches in year
Date of harvesting: show as dd/mm/yy
Aliquot. No: Aliquot "2 of 6" for example
***Be sure to fill in DENV serotype also.**

Optimizing virus stock dilutions for use in DENV RT-PCR

10) After referring to "Optimizing internal control and external control for Dengue PCR" (*TP-TO-DNG-008*) to calculate the optimal dilution of viruses for RT-PCR, dilute viruses with MM media to the required dilution factor.

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11) Aliquot diluted virus into 1ml volumes, and label as the 'working stock'. Attach the following label to tubes and store at -80°C until ready for use in PCR.

Working Stock
DENV___/YY/NNNNN
Aliquot Vol./No.: _____/_____
Dilution: _____

9. Quality Control

N/A

10. Safety

Wear laboratory coat, gloves. Place any waste in the clinical waste bins provided. All procedure must be performed in Class II biosafety cabinet at Virology Lab. Use Universal precautions at all times when working with tissue culture. Only trained person could be allowed to work in virology. Place waste in appropriate waste bins provided.

11. Risk assessment and risk management:

Department Dengue group **Location of work** Lab 115	**Persons involved** Dengue staff
Description of procedure The procedure describes how to prepare DENV external controls in C6/36 cells.	**Substances used** Dengue virus
Quantities used <5 ml	**Frequency of use** Frequent
Hazards identified Infection with DENV	**Could a less hazardous substance be used instead?** NO.
What measures have you taken to control risk? **PPE:** i. Designated gown for BSCII work ii. Gloves iii. Cover shoes All staff are trained in working with infectious virus, especially that at high concentration.	
Checks on control measures	

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Supervision and observation by senior staff	
Is health surveillance required? NO.	**Training requirements:** Good Laboratory practice and PPE. Specific training in this procedure, aseptic techniques especially. (with associated documentation of training by staff and supervisor)
Emergency procedures: Spills: follow "biological and chemical clean up procedure" PR-HS-ALL-005. Staff must report incidents to supervisors immediately.	**Waste disposal procedures**: OUCRU BSK2 manual of waste management SD-HS-ALL-009

12. Related Documents:

Document name	Document code
Optimizing External Control And Internal Control For RT-PCR	*TP-TO-DNG-008*
Preparation of cell culture media	*TP-TC-DNG-079*
Cell propagation	*TP-TC-DNG-061*
DENV1 Culture control batch number index	*FM-TO-DNG-019*
DENV2 Culture control batch number index	*FM-TO-DNG-020*
DENV3 Culture control batch number index	*FM-TO-DNG-021*
DENV4 Culture control batch number index	*FM-TO-DNG-022*

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13. Document History:

Revision #	Effective date	Details of amendment
00	18-DEC-2009	New issue
001	04-AUG-2014	Converted to new OUCRU SOP template. More detailed information added for procedural preparations, and removal of section on standard cell culturing description due to overlap between SOPs – Lauren Carrington.
002	01-OCT-2015	Added Principle – Lauren Carrington
003	06-APR-2018	Changed the title. Clarified sections: Procedures, Risk assessment and risk management. Added reference – Trung Vu

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1. Purpose

This procedure describes the process used to inject the saliva from infected mosquitoes into clean mosquitoes, via intrathoracic inoculation.

2. Principle:

As a natural vector of a number of arboviruses, *Aedes aegypti* can act as a vessel to amplify virus for experimental purposes. Inoculation of virus into the thorax of a mosquito bypasses the midgut infection barrier and therefore allows the virus to replicate more easily.

This method can be used to amplify small concentrations of virus that are undetectable in a PCR assay, to higher, detectable concentrations. For example, a sample of saliva from an index mosquito may contain only a small number of viral particles. This number might be lower than limit of detection of a PCR assay, even though the sample is still infectious. With inoculation of the saliva sample into the body of naïve mosquitoes, any infectious viral particles present in the saliva will be able replicate to a higher concentration. After a period of incubation (often 5-7 days), inoculated mosquitoes are collected and tested by RT-PCR for virus infection. If the pool of inoculated mosquitoes is positive for virus by RT-PCR, the initial, index mosquito is to be considered infectious for analysis purposes.

Accordingly, this method can be applied to measure virus transmission potential by mosquitoes. The technique may also be used to amplify virus from patient isolates, from filtered mosquito homogenates, or for serial passaging of virus through mosquitoes.

3. Scope and responsibilities:

This produce must take place within the insectary (Room 115A) at OUCRU, and only be performed by trained personnel. This procedure requires a high level of concentration and so research assistants are responsible for carrying out this procedure only when they are feeling alert and focused. All staff are to be trained, and deemed competent at this task by senior insectary staff members, before the training log will be signed by the trainee, and co-signed by the supervisor. All staff members must follow the directives listed here, and senior staff members are responsible for monitoring adherence to this procedure by junior staff members. Appropriate PPE is to be worn by staff members at all times when undertaking this procedure. **If any changes to**

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this SOP are requested, these must be discussed with the supervisor, updated in the revised SOP and approved before being implemented.

4. Reference

Phillips, A., Mossel, E. Sanchez-Vargas, I., Foy, B. Olsen, K. (2010). Alpha virus transducing system: Tools for visualizing infection in mosquito vectors. *Journal of Visualized Experiments*, 45: 2363.

5. Definition

- PBS: Phosphate Buffer Saline
- DENV: Dengue virus
- Index mosquito: The mosquito from which a saliva sample was collected, that was inoculated into the recipient mosquito
- Naïve mosquito: A mosquito that has never been exposed to virus before
- Recipient mosquito: The mosquito that received the virus inoculation into the thorax
- Inoculum: The fluid that is used to inoculate of the mosquitoes

6. Specimen requirement

- Naïve mosquitoes (the species/strain will be dependent upon the experiment)
- Inoculum (which can be any of the following
  - ✓ Mosquito Saliva samples (see Collecting mosquito saliva, TP-TC-DG-052)
  - ✓ Virus culture

7. Materials/ Equipment used

- 15% Sucrose
- Light Microscope fitted with 10x objective
- Filter tip (20µl)
- Pipette (2-20µl)
- Fine tipped forceps

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- Petri dish
- Marker
- Fine glass capillary needle
- Needle puller
- Glass slides
- Mosquito cups and secondary containment
- Adult mosquito cages (30cm x 60 cm x 30cm)

8. Procedures

Making a glass microinjection needle:

1) Using the needle-puller machine, adjust the temperature to level 61.2°C and the number of weights as desired (usually 2 light weights and 2 heavy weights). Use one-step pulling.
2) Insert the glass capillary into position in the machine.
3) Press start and wait until the weights drop down.
4) Take the needles out and keep in the 15ml falcon tube and the fine end of the needles are up
5) When you take the needle out of the falcon tube to perform the inoculation, you have to do gently and slowly. Put the needle on soft paper and make sure the tip of the needle is remains intact.
6) Create a very fine hole in the tip of the needle. The hole is needed so that the inoculum can be taken into the needle, and then injected into the mosquito. The diameter of hole needs to be as small as possible, and without jagged edges, to reduce injury caused to mosquitoes in the inoculation procedure. The hole is made in the needle using a fine pair of forceps.

   Note: *This technique requires on-the-job training and experience for the staff member to know the appropriate size of the needle tip for inoculation.*

Preparing the inoculum:

7) For inoculation of saliva into naïve mosquitoes, refer to protocol Collecting Mosquito Saliva (TP-TC-DG-052) for directions for collecting the required sample ready for inoculation into the naïve mosquitoes.

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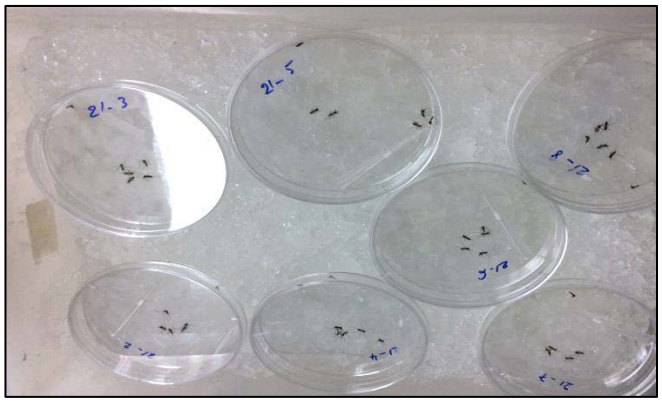
8) For inoculation of virus culture into naïve mosquitoes, virus should be provided to staff in a labelled tube, ready for inoculation.
9) It is important to note that the inoculum samples waiting to be inoculated should be maintained on ice. The ice may need to be replaced mid-way through the experiment to ensure the inoculum stays in contact with the cold surface, but the petri dish is not submerged in the ice.

Anaesthetisation and preparation of clean mosquitoes for inoculation:

10) Naïve mosquitoes for inoculation are to be anesthetized in the freezer for 1-1.5 minutes. The mosquitoes cup is then taken out of the freezer and put on ice to keep the mosquitoes completely immobile. The mosquitoes should be confirmed immobile before opening the mesh cover.
11) Count the number of inoculum samples that need to be inoculated, and then calculate the required number of mosquitoes that will be inoculated in advance. Prepare the required number of mosquitoes[1] for each sample in individual petri dishes (1 sample of inoculum = 1 petri dish).
12) Cover all petri dishes immediately once prepared, and place on ice, ensuring mosquitoes have contact with the cold surface to keep them anaesthetised. Label all petri dishes carefully and clearly, as shown below.



[1] The number of mosquitoes to be inoculated with a single sample will be dependent upon the experiment, and should be confirmed in the experimental study design before commencement.

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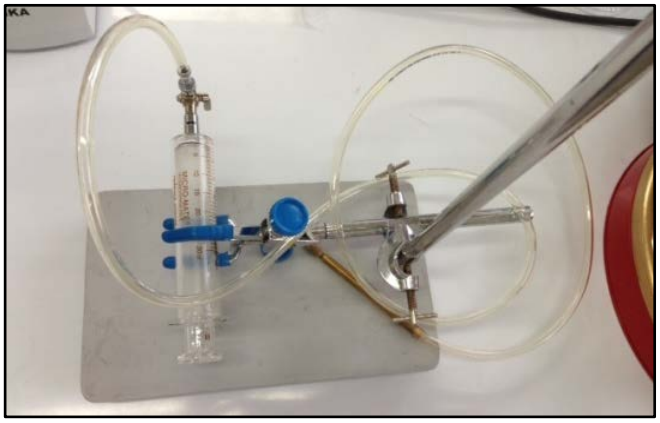
Injection system:

13) Fix a 20ml, 30ml or 50ml syringe to a cinder block.



14) Attach an adjustable 1-way valve to the end of the syringe.



15) Attach an airtight plastic hose to this outlet. The hose should be long enough to be easily moved without pulling tight.

16) Insert a micro-capillary adapter onto the free end of the hose. Ensure the adapter has all parts, including a small rubber o-ring.



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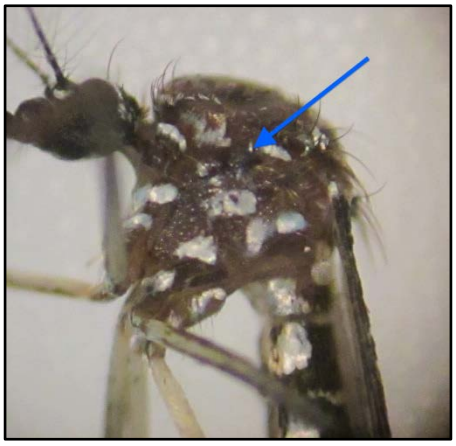
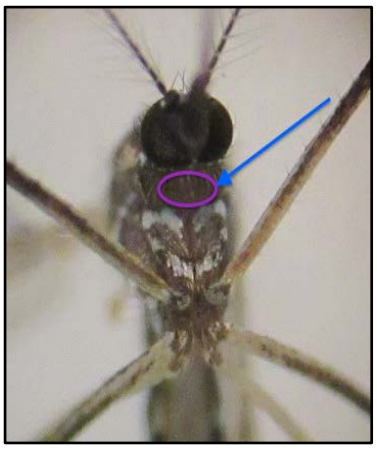
Inoculations:

17) Put a single glass needle into the adapter of injection system.
18) Open the air valve, compress the plunger to expel of any air in the syringe.
19) Adjust the air valve to the "resting position".
20) Pick up the sample that is to be inoculated into the naïve mosquitoes, and pipette the solution onto a clean glass slide[2].
21) Put the needle into the inoculum drop at 45° angle; and then pull the plunger to suck the fluid into the needle. Try not to get any air in the tip of the needle.
22) Adjust the air valve to "injection position".
23) Look for the injecting site on the mosquito. There are two locations on the mosquito that induce the least damage mosquitoes during inoculation[3]. The first position is a area which is between head and thorax, the second area is in the metathoracic spiracle. These are indicated in the following figures, both photographically, and schematically:





[2] A clean glass slide should be used for each samples of inoculum to avoid any risk of cross contamination.
[3] Either of these two locations is acceptable for inoculation, however technicians should be regularly monitoring the survival of the mosquitoes they inoculated to ensure their technique remains effective.

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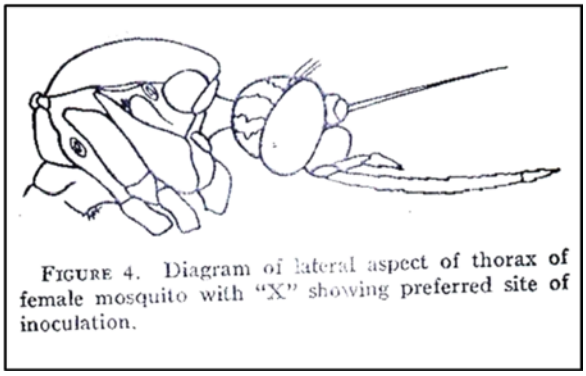

FIGURE 4. Diagram of lateral aspect of thorax of female mosquito with "X" showing preferred site of inoculation.

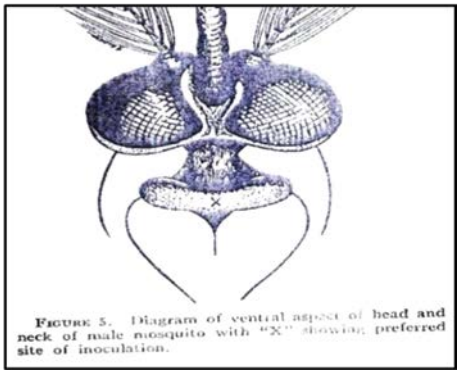

FIGURE 5. Diagram of ventral aspect of head and neck of male mosquito with "X" showing preferred site of inoculation.

24) Insert gently the tip of the needle into the injection site to just below the exoskeleton (not too deep, or else the mosquito becomes injured). Compress the plunger of the syringe to inject 1µl of inoculum into the mosquitoes. Look to see when the mosquito's abdomen becomes distended, when this happens, the mosquito has been inoculated with a sufficient volume.



25) Create a small opening in the mosquito post-injection cup with the mesh, and place the inoculated mosquito inside the cup. Immediately pull down the net to make sure the mosquitoes cannot escape. Place the cup back onto the ice to keep the mosquitoes sedated, until all mosquitoes in that cohort have been inoculated.

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26) Labels must be written clearly and accurately on all mosquito cups, with the following information:
    - Study code
    - Patient code (and day of patient sample, if applicable)
    - Mosquito strain (and whether or not they are 'INO' mosquitoes)
    - Number of mosquitoes added to each cup / total number that cohort
    - Cup number / total number of cups for that cohort
    - Inoculation date
    - Type of mosquitoes being inoculated into

Study code *44DX-250* Experiment name *Ev / MS*
Mosquito strain *15* Mosquito# *3* #mosquitoes in pool *5* DPI *14*
Cup #/total cups *1/3* # mosquitoes/total in cohort *6/20* .
Inoculation date *25 / 10 / 2018* ***Ino**: Type of ino - 07*

27) When each cup of mosquitoes contains all the required mosquitoes, both the contents of the cup and the label on the side, should be double-checked by the person inoculating, and then triple-checked again by an independent technician.
28) Both the staff performing the inoculation, and the staff member that re-checks the container must sign in the appropriate (study-specific) worksheet that the contents and labels are correct. In signing their names, they take responsibility for the contents.
29) Before starting with the next cohort of mosquitoes, discard the needle into the biohazard sharps container, and replace with a fresh needle. This is to make sure there is no cross-contamination between mosquito cohorts.
30) Repeat the process of inoculation for the required number of recipient mosquitoes, and the number of cohorts for the experiment in question.
31) All staff should note well, and be reminded that there should NEVER be a cup of mosquitoes open at ANY time (for any experiment, for any reason) unless that mosquito cup has been on ice, and all mosquitoes in that cup are CONFIRMED ASLEEP. If the cup needs to be re-opened, to add more mosquitoes (or for any other reason), all mosquitoes must be RE-

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CONFIRMED asleep before opening the mesh cover. Any negligence in this step will not be tolerated.

Mosquito maintenance and monitoring survival/mortality post-inoculation:

32) Maintain inoculated mosquitoes daily with 10% sucrose and water until ready for harvesting, generally 7-14 days post-inoculation.

33) Monitoring survival/mortality of the inoculated mosquitoes is often a part of the prescribed experimental procedures. However, even if it is not, all staff should note that every time a mosquito cup is picked up, the staff member has the responsibility to check and confirm that the cup of mosquitoes physically contains precisely the number of mosquitoes that is listed on the side of the container – no more, and no less. All containers must have the number of mosquitoes inside written on the label. Any negligence in checking the contents of cups will not be tolerated.

9. Quality control

- All reagents should be kept in 2-8°C.
- Using appropriate negative controls (the saliva of a mosquito never exposed to virus, that is inoculated into naïve mosquitoes) to ensure that the tools and process exclude sample contamination.
- Saliva samples prior to inoculation should be maintained on ice to ensure that any virus collected in the samples remains viable.
- A fresh inoculation needle is to be used with every sample of saliva/inoculum.

10. Safety

- Each microinjection needle is single use only.
- After use, needles are to be discarded in the sharps/glass waste container for appropriate disposal. The sharps container is to be located on the benchtop of the work area to eliminate the need to transport the needles any distance away from the work area.
- Appropriate PPE (a minimum of lab coats, mask, fully-enclosed shoes, long trousers, and double gloves) should be worn at all times while performing this procedure.

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- This inoculation procedure requires alertness and focus – it should not be performed when staff are tired or unwell, as this will increase the risk of manual error and needle stick injury. All staff are responsible for self-assessment of their capacity to perform the procedure each day, and coordinate work requirements with colleagues accordingly.
- Should a needle-stick injury occur, the wound must be washed thoroughly under running water and the immediate supervisor be informed ASAP. The injured person should go to the Emergency Department at the HTD for treatment.

11. Supplementary notes

Special mosquito handling precautions

- All mosquitoes must be accounted for at all times. To manage the risk of a mosquito (or mosquito cup) going missing, detailed labelling of cups, and detailed recording of information (surviving mosquitoes, dead mosquitoes, discarded mosquitoes) must be performed.
- While preparing the mosquitoes for inoculation in petri dishes, it is important to make sure all mosquitoes are kept on ice, and remain covered by the lid of the petri dish, and actively anaesthetised by the cold. The petri dish should not contain any more mosquitoes than the staff member can safely observe through the microscope objective, at any given time when the lid of the petri dish is uncovered.
- While inoculating a cohort of mosquitoes with a sample of inoculum, the staff member must make sure that the cup containing the freshly-inoculated mosquitoes sits on ice, and is completely covered with mesh. Each time the technician opens the cup, they must reconfirm that all mosquitoes already in the container are still immobile before opening the mesh.
- When a staff member finishes inoculating all the required mosquitoes for a cohort, they should secure the cup with the mesh, and re-count its contents to make sure it is correct. The labelling of the cup should be accurate, and must indicate the numbers of mosquitoes inside that cup. Their signature should be recorded in a worksheet, certifying these points.
- The cup will then be double-checked by a colleague, to double check that the labelling is correct, and the contents of the cup is also correct. This second staff member will co-sign the worksheet to confirm this is correct. This is to avoid any possible cases of cups being identified that contain the incorrect number of mosquitoes at any time.

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- All staff are to be systematic in their approach to setting up mosquito cohorts, monitoring mosquitoes for maintenance (and any other purposes). If a mosquito cohort (of a given strain, or group of mosquitoes have been inoculated with the same inoculum) is divided across a number of cups, then all of those cups should be checked as a single unit, to ensure the contents of each cup, and the number of mosquitoes as a whole, are complete and accounted for.
- Each time a staff member picks up any cup of mosquitoes while it is being maintained (for experimental purposes or otherwise), that staff member has the explicit responsibility to check the contents of the cup against that which is indicated on the label. This applies to all mosquitoes, of all strains, of all experiments, without exception.
- All cups that contain mosquitoes that have been exposed to any form of infectious agent MUST be contained in a primary container (the cup itself) and a SECONDARY container (multiple cups inside a secured cage).
- Any negligence displayed in regards to any of the above mosquito handling procedures will not be tolerated.

12. Risk assessment and risk management:

Mosquito escape:

- Staff must ensure all mosquitoes in any cup or container are completely immobile before opening the container. In this procedure, a cup is opened several times when individual mosquitoes are placed into the cup after inoculation. Each time the cup is opened, there is an increased risk for mosquito escape. To reduce this risk, the staff member must keep the cup on ice until its contents is complete, and re-confirm the status of the mosquitoes each time they need to open the container.
- Once containers are filled with enough mosquitoes for that cohort, they should then be housed in secondary containment. This provides an additional layer of security to maintain the mosquitoes should they happen to escape from the first container.
- If one or more mosquitoes are released, it is essential that all personnel in the insectary are alerted immediately to the situation. As a priority, all available personnel must:

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- Temporarily stop their work at the soonest possible moment, secure any mosquitoes they are working with themselves; and
- Assist other staff in the room to find, and kill the escaped mosquito(es).

Needle-stick injury:

- The use of micro-capillary needles containing infectious substances increases the risk of staff infection. All stuff must self-examine their capacity to perform this technique each time they are needed to perform the task. If they feel fatigued they are to coordinate the workload with colleagues.

Accident reporting:

- All incidents MUST be reported to the supervisor immediately

13. Risk assessment and risk management

University of Oxford COSHH Assessment Form	
Description of procedure Intrathoracic inoculations of mosquitoes	**Hazardous substances used** DENV
Quantities used <10 µl of any one sample at any one time	**Frequency of use** Frequent
Hazards identified - Potential DENV infection - Needle stick injury - Mosquito escape	**Could a less hazardous substance be used instead?** No
What measures have you taken to control risk? - This procedure may only be performed during weekdays, when there is at least one other staff member in the room, and never on weekends. This is to ensure that if an incident does occur, there is always people nearby to assist if needed. - This procedure involves the risk of needle stick injury and infection with dengue virus, and must therefore only be performed by trained staff members, when they are feeling alert and well. The decision about an individual's ability to work safely is the responsibility of the staff member, being fully informed of the risks. The staff member must be diligent in their assessment of their capacity to perform the technique safely on a daily basis. - Should a needle stick injury occur, standard first aid measures will be put into place, i.e.; the cut will be placed under running water and contact will be made with the supervisor and Lab	

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<table>
<tr><td colspan="2">Management team immediately. The staff member might need to go to the Hospital for treatment by a medical professional. Follow SOP of Accidental Occupation Exposure PR-HS-ALL-001.
- Appropriate PPE is provided and must be worn at all times when performing the procedure (i.e.: lab coat, gloves, eye protection); SOP of Universal precaution and Personnel Protective Equipment guidelines for working in the laboratories, PR-HS-ALL-006 is to be followed.
- The Disposable Sharps Containers must be easily accessible to staff at the work bench. Sharps are to be disposed of immediately after use, and not placed into an intermediate container before disposal.
- Other measures as described in Section 11: “Special Mosquito Handling Precautions”.</td></tr>
<tr><td colspan="2">Checks on control measures
Supervision and observation by senior staff.</td></tr>
<tr><td>Is health surveillance required?
Yes, Hepatitis B serology.</td><td>Training requirements:
Good Laboratory practice and PPE
Specific training in this procedure
(with associated documentation of training by staff and supervisor).</td></tr>
<tr><td>Emergency procedures:
Spills: follow “biological and chemical spill clean up procedure” PR-HS-ALL-005.
Staff must report incidents to supervisors immediately.</td><td>Waste disposal procedures:
OUCRU BSL2 manual of waste management SD-HS-ALL-009.</td></tr>
</table>

14. Related Documents:

Document name	Document code
Collecting mosquito saliva	TP-TC-DNG-052
Mosquito container labelling	TP-SM-DNG-003

15. Document History:

Revision #	Effective date	Details of amendment
00	01-11-2012	New issue
01	18-08-2013	Revision to Quality Control and Procedures, Scope and Responsibilities, Safety and Risk assessment and Risk management. Altered title. – Lauren Carrington
02	11-09-2014	Revised Principles, and improved description of procedures. – Lauren Carrington

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03	16-09-2014	Removed unnecessary headings, reformatted, edited Principles. – Lauren Carrington
04	27-12-2014	Added formal Risk Assessment table and pictures showing the location for inoculating mosquitoes. – Lauren Carrington
05	27-03-2018	Improved description of mosquito monitoring and recording requirements, labelling for cups, and mosquito handling for safety. Updated the Risk Assessment (description of procedure, control measures in place to minimise risk, and emergency procedures). Added “Special mosquito handling precautions” section, outlining requirements for mosquito handling procedures and a double checking “buddy” system to ensure cup contents and labelling is accurate. Indicated level of “no tolerance” for negligence in this aspect. Generalised the description of the procedure so it is adaptable to multiple experimental designs. – Lauren Carrington

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COLLECTING MOSQUITO SALIVA

1. Purpose

This procedure describes the process used to collect saliva from mosquitoes exposed to dengue virus.

2. Principle

Saliva is collected from mosquitoes exposed to arboviruses to test whether the saliva contains infectious virus particles after an appropriate extrinsic incubation period. Mosquitoes are de-winged and de-legged to induce salivation, and the proboscis is inserted into the end of a small pipette tip containing medium. The saliva/medium mix is then inoculated into the thorax of an uninfected mosquito (inoculation into the thorax bypasses any midgut infection/escape barriers for the virus), virus replication occurs rapidly in the inoculated mosquito. Inoculated mosquitoes are then tested after 7 days by RT-PCR for DENV infection; the development of infection in the inoculated mosquitoes is evidence of the initial mosquito being able to transmit infectious DENV virus.

3. Scope and responsibilities

This procedure will take place in the “dirty” room of the OUCRU insectary (Room 115A). Only trained people are allowed to implement the procedure, and are responsible for following these directives. All staff are to be trained, and deemed competent at this task by senior insectary staff members, before the training log will be signed by the trainee, and co-signed by the supervisor. All staff are responsible for adhering to this procedure precisely. Senior staff members will help monitor junior staff members. Appropriate PPE is to be worn by staff members at all times when undertaking this procedure.

4. Reference

World Health Organization, Geneva. Part II Tutor’s Guide: Entomological laboratory techniques for malaria control.

Website: http://whqlibdoc.who.int/hq/1994/a71209_%28part2%29.pdf

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5. Definition

- IFCS: Inactivated Fetal Calf Serum
- PBS: Phosphate Buffer Saline
- Complete Medium: R10: RPMI, gentamycin, pen/strep Fetal Bovine Serum
- PPE: Personal Protective Equipment
- DENV: Dengue virus
- Inoculum: The fluid that is used to inoculate of the mosquitoes
- Naïve mosquito: A mosquito that has never been exposed to virus before
- The "dirty" room: The "dirty" side of the insectary where **experimental** mosquitoes that have been **exposed** to a blood meal (**infectious or non-infectious**) are maintained and handled. Even if experimental mosquitoes have been fed on non-infectious blood (for example, as a negative control), they MUST be moved to the dirty room.

  Once an 'exposed' mosquito enters this room, it is not to leave again, unless it has been disposed of, or has been harvested, killed, and is in a sample tube. This specialized room has an additional white mesh curtain prior to entry, is specifically designed to contain the area in which infectious material is being used, and to further minimize the risk of mosquito escape.
- The "clean" room: The "clean" side of the insectary is where immature mosquito rearing, adult mosquito maintenance, and preparation for experiments takes place.

  Experimental mosquitoes: Adult mosquitoes that are intended for use in experiments can only be in this room while they are waiting to be used in experiments, this means that they have **NEVER been exposed to ANY blood meal**.

  Colony mosquitoes: The only mosquitoes that are allowed to be blood fed in this room are the colony mosquitoes, and these MUST be in specialised colony cages – never in a cup.

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6. Specimen requirement

- Arbovirus infected mosquitoes (*Aedes aegypti* or *Aedes albopictus*)
- Inoculum (the mosquito saliva samples)

7. Materials/Equipments used

- Pen/marker
- Timer
- IFCS
- 10% Sucrose
- Fine-tipped forceps
- Petri dish
- Filter tip (10µl)
- Pipette (1-10µl)
- Mosquito cups and secondary containment

8. Procedures

Rearing/maintaining infected mosquitoes prior to harvesting

1) Day 0-21: After an infectious blood meal, mosquitoes that are fully engorged with viraemic blood are maintained for (up to) the next 21 days (normally 14, but depends on experimental design in question), with 10% sucrose and water.
2) Labels of each cup must be checked before sampling any mosquitoes. The contents of the cup must also match the label. The labels should indicate:
    - Study code
    - Patient code (and day of patient sample, if applicable)
    - Mosquito strain
    - Number of mosquitoes added to each cup / total number that cohort
    - Cup number / total number of cups for that cohort

    This information will be needed for ensuring the subsequent saliva samples are labelled accurately also, so make sure everything is written on the labels.

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3) In the morning of the day before mosquitoes are harvested, keep water and only remove the sucrose from the mosquitoes, to encourage salivation when they are offered the IFCS:Sucrose 30% solution.

Anaesthetisation

4) 24 hours after removing the sugar source, before anesthetizing mosquitoes, check and confirm that the cups of mosquitoes physically contains precisely the number of mosquitoes that is listed on the side of the containers – no more, and no less. **Any negligence in checking the contents of cups (at any point) will not be tolerated.** All containers must have the number of mosquitoes inside written on the label**.**
   - Counting the number of dead mosquitoes in each cup
   - Counting the number of alive mosquitoes in each cup
   - Counting the number of cups

   Check to make sure all the mosquitoes you expect to be harvesting are there. **All of these numbers should all up to the correct number of mosquitoes and cups as initially recorded for this cohort and is labelled on the side of the container.** Record this information in the appropriate worksheet.

   Note: *The number of mosquitoes to collect saliva with a single sample will be dependent upon the experiment, and should be confirmed in the experimental study design before commencement.*

5) Immobilize mosquitoes by placing them in the fridge for about 1-1.5 minutes. The mosquito cups of a cohort are then taken out of the freezer and put on ice to keep the mosquitoes completely immobile. *The mosquito holding containers will only be opened after all mosquitoes are confirmed completely immobile.* Pour all mosquitoes in cups into a first petri dish. Cover the first petri dish immediately then recount all mosquitoes to make sure none are left inside the cups, and ensuring mosquitoes have contact with the cold surface to keep them anaesthetized.

6) Choose one mosquito randomly from the first petri dish then put into a second petri dish contained filtered pipette tip for collecting saliva. Re-cover the first petri dish. The leg and

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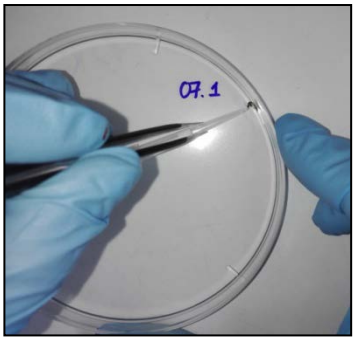
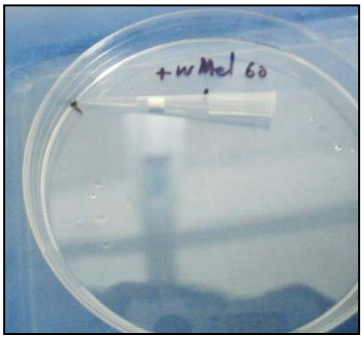
wing of the mosquito are immediately removed to avoid any possibility of the mosquito waking up and flying away.

7) Repeat these steps for each mosquito in the first petri dish but we change a fresh second petri dish to avoid any contamination and mix up of samples. Label all petri dishes carefully and clearly and appropriately to ensure that there is no mix up of samples.
8) Any mosquitoes from the cohort that are not going to be harvested, need to be discarded. For this, they should be frozen -20°C overnight to be killed and discarded in the biohazard container. The number discarded should be recorded, to ensure the fate of every mosquito has been accounted for.
9) Place contaminated petri dishes with legs and wings into a biohazard bag.

Collecting saliva

10) For each mosquito, using **sterile forceps**, insert proboscis of the mosquito into a filtered pipette tip, as shown below. The forceps should be decontaminated with ethanol, between each and every mosquito samples. The filter tip must contain a 1:1 solution of filtered IFCS: Sucrose 30%.





11) Only one mosquito and one tip are to be placed in any petri dish at one time, to ensure that there is no mix up of samples. All dishes are to be labelled appropriately.
12) The volume of solution in the pipette tips will vary depending on the subsequent use (normally, 1µl is required for each mosquito that will subsequently be intrathoracically inoculated with the inoculum). For most experiments, no more than 5µL is added to the tube, because no more than 5 mosquitoes are inoculated with a single saliva sample.

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13) Place the petri dish carefully into a big tray filled with ice. Record the time that the mosquito starts salivating into the tip, and remove the proboscis after 30 minutes. It is important to note that proboscis of the mosquito stays in contact with a filtered pipette tip through 30 minutes. If the proboscis comes out during this time, using sterile forceps to insert proboscis of the mosquito into a filtered pipette tip again.
14) The saliva is collected and processed at this stage in one of two ways.
    - Placed into a 2ml screw tubes and stored at -80°C to preserve any viral particles that have been expectorated.
    - Inoculated into naïve mosquitoes to test for virus in the saliva. For this, refer to protocol *Intrathoracic inoculations* (TP-TC-DG-058) for directions.
15) Subsequent harvesting of specific mosquito tissues might follow this point, depending on experimental requirements. It is after the collection of saliva, that the mosquito will be dissected to harvest these tissues. Refer to the appropriate protocols, listed at the back of this SOP for further instructions.
16) Due to the large number of saliva samples being collected at any one time, there is often a delay in inoculating all of the samples at once. Therefore, until someone is available to perform the inoculation, all inoculum samples are to be maintained on ice (to preserve the virus). The ice may need to be replaced mid-way through the experiment to ensure the inoculum stays in contact with the cold surface, but the petri dish is not submerged in the ice.

9. Quality Control

- All reagents should be kept in 2-8°C.
- Be sure to use appropriate negative control during the experiment. These are mosquitoes that have not been exposed to DENV, and their saliva is collected/stored/processed in the same way as those that have been exposed.
- Saliva samples prior to inoculation should be maintained on ice to ensure that any virus collected in the samples remains viable.
- Use one pair of forceps and a scalpel for only each individual mosquito. Alternatively, rinse pair of forceps sequentially into, working Presep solution, ethanol 70% and distilled water before reusing with another mosquito.

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10. Safety

- PPE must be worn at all times while implementing this procedure. This includes gloves and lab coat.
- All mosquitoes are to be considered infectious and will be treated as such, therefore universal precautions should be employed.
- All surfaces are to be cleaned by ethanol. Place contaminated petri dish with legs and wings into a biohazard bag.

11. Special mosquito handling precautions

- **All mosquitoes must be accounted for at all times**. To manage the risk of a mosquito (or mosquito cup) going missing, detailed labelling of cups, and detailed recording of information (surviving mosquitoes, dead mosquitoes, discarded mosquitoes) must be performed.
- Each time a staff member picks up any cup of mosquitoes while it is being maintained (for experimental purposes or otherwise), that staff member has the explicit responsibility to check the contents of the cup against that which is indicated on the label. This applies to all mosquitoes, of all strains, of all experiments, without exception.
- While preparing the mosquitoes for collecting saliva in petri dishes, it is important to make sure all mosquitoes are kept on ice, and remain covered by the lid of the petri dish, and are anaesthetised by the cold. The petri dish should not contain any more mosquitoes than the staff member can safely observe at any given time when the lid of the petri dish is covered, or uncovered.
- Each time the technician opens the cup for inoculating mosquitoes, they must reconfirm that all mosquitoes already in the container are still immobile.
- All cups that contain mosquitoes that have been exposed to any form of infectious agent MUST be contained in a primary container (the cup itself) and a SECONDARY container (multiple cups inside a secured cage).
- Take extra care when removing cups from cages or mosquitoes waking up on the petri dish or the rubber band slipping of the cup to mitigate the risk of mosquito escape.

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- **Any negligence displayed in regards to any of the above mosquito handling procedures will not be tolerated.**

12. Risk assessment and risk management:

Mosquito escape:

- Staff must ensure all mosquitoes in any cup or container or petri dishes are completely immobile before opening the container and re-confirm the status of the mosquitoes each time they need to open the container.
- Containers should be housed in secondary containment. This provides another secure environment to maintain the mosquitoes should they happen to escape from the first container.
- If one or more mosquitoes are released, it is essential that all personnel in the insectary are alerted immediately to the situation. As a priority, all *available* personnel must: temporarily stop their work at the soonest possible moment, secure any mosquitoes they are working with themselves and assist other staff in the room to find, and kill the escaped mosquito(es).
- Mosquito traps are set up in each of the three rooms of the insectary (the ante-room, the "clean" and the "dirty" rooms) to capture any mosquitoes that escape.

Accident reporting:

All incidents MUST be reported to the supervisor immediately.

University of Oxford COSHH Assessment Form	
Description of procedure Collecting Mosquito Saliva	**Hazardous substances used** Potentially infectious saliva of mosquitoes (containing DENV, or other arboviruses)
Quantities used < 10µl	**Frequency of use** Frequent
Hazards identified - Mosquito escape after waking from anaesthetisation	**Could a less hazardous substance be used instead?** No

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- Potentially pathogenic bacteria and virus in mosquito transferred to human	

What measures have you taken to control risk?

- This procedure may only be performed during weekdays, when there is at least one other staff member in the room, and never on weekends. This is to ensure that if an incident does occur, there is always people nearby to assist if needed.
- Mosquitoes have their wings and legs removed prior to the collection of saliva to prevent their flying away.
- Training of staff in procedures of safe mosquito handling. This procedure of removing wings and legs involves the risk of infected mosquito escape therefore only be performed by trained staff members, when they are feeling alert and well. The staff member must be diligent in their assessment of their capacity to perform the technique safely on a daily basis.
- Staff are trained to limit the number of mosquitoes in a petri dish at any one time to minimisthe risk of mosquito escape.
- Staff must wear appropriate PPE (lab coat, gloves, as per SOP of Universal precaution and Personnel Protective Equipment guidelines for working in the laboratories, *PR-HS-ALL-006*.
- Training of staff to ensure labelling of all containers is clear and accurate, and making sure that all staff understand the importance of adhering to these directives listed within.
- Other measures as described in Section 11: “Special Mosquito Handling Precautions”.

Checks on control measures

Supervision and observation by senior staff.

Is health surveillance required? Yes, Hepatitis B serology.	**Training requirements:** Task specific training for all staff (with associated documentation of training by staff and supervisor), plus training in good laboratory practices.
Emergency procedures: - If exposure to virus occurs, follow SOP of Accidental Occupation Exposure *PR-HS-*	**Waste disposal procedures:** OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

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ALL-001. Staff must report incidents to supervisors immediately.	

13. Related Documents:

Document name	Document code
Administering artificial blood meals	*TP-TC-DNG-051*
Direct blood feeding	*TP-TC-DNG-053*
Dissecting head & thorax and abdomens	*TP-TO-DNG-054*
Intrathoracic inoculations	*TP-TC-DNG-058*

14. Document History:

Revision #	Effective date	Details of amendment
00	01-11-2012	New issue
01	12-06-2013	General editing, revision to Quality Control and Procedures. Altered title. – Lauren Carrington
02	15-09-2014	Improved description of Principles and Procedures. – Lauren Carrington
03	10-10-2014	Added formal risk assessment table, removal of unnecessary section headings. – Lauren Carrington
04	23-03-2018	Added section on Special mosquito handling procedures, and a "no tolerance" policy of any mishandling of mosquitoes. Improved description of Definitions (particularly "clean" and "dirty" rooms, descriptions of Procedures, and appropriate references to other SOPs – Lauren Carrington

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1. Purpose

Describe the procedure for the diagnosis of dengue fever by detection of the NS1 protein in biological specimens. This protocol is adapted the Platelia ^{TM} Dengue NS1 Ag Kit from Bio-Rad.

2. Principle

The Platelia Dengue NS1 Ag kit is a one-step sandwich format microplate enzyme immunoassay for the qualitative or semi-quantitative detection of Dengue virus antigen in human serum or plasma. The test uses murine monoclonal antibodies (mAbs) for capture and revelation.Human samples and controls are directly and simultaneously incubated with the conjugate for 90 minutes at 37°C within the microplate wells sensitised with mAb. If NS1 antigen is present in the sample, an immune-complex mAb-NS1-mAb/peroxidase will be formed. After a washing step, the presence of the immune-complex is demonstrated by colour development reaction. After stopping the colour reaction by adding an acid soultion, the optical density reading obtained with a spectrophotometer set at 450/620 nm is proportional to the amount of NS1 antigen present in the sample. The presence of NS1 antigen in an individual sample is determined by comparing the optical density reading of the sample to the optical density of the calibrator.

3. Responsibility

Technicians/research assistants are responsible for performing this procedure following this protocol. For each ELISA batch, the procedure steps must be documented into the "NS1 qualitative ELISA assay worksheet" *FM-TO-DNG-027*. This worksheet provides a step-by-step recording system for the ELISA set-up and analysis. The assay batch must also be recorded into the "NS1 qualitative ELISA assay batch number index" *FM-TO-DNG-028*.

4. Reference

Platelia™ Dengue NS1 Ag manual instruction (Bio-Rad).

5. Definitions

- Ag = antigen
- NS1 = Non-structural protein 1

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- DENV = dengue virus
- MAb = monoclonal antibodies
- ELISA = Enzyme-Linked Immuno Sorbent Assay
- BSA = Bovine Serum Albumin
- FCS = Fetal Calf Serum
- Neg = negative
- Pos = positive

6. Specimen requirement

Serum, plasma (recommended collection in EDTA, citrate or heparin) from suspected dengue patient. Sample should be clotted completely before centrifugation. The specimen can be used up to 24 hrs after collection, given that it has been stored 2°C – 8°C the whole time. If samples older than 24 hrs are to be used, they should be frozen at -20°C.

7. Materials/Equipments

- Platelia Dengue NS1 Ag Kit (Bio-Rad,Cat.No. 72830). The kit components
  - **R1** = Micro plate, coated with anti-NS1 mAb (12 strips x 8 wells each)
  - **R2** = Concentrated washing solution (20x)
  - **R3** = Negative control (human serum negative for DENV NS1 antigen)
  - **R4** = Calibrator (DENV NS1 antigen, BSA, gycerol…)
  - **R5** = Positive control (DENV NS1 antigen, BSA, glycerol…)
  - **R6** = Conjugate (50x) (Anti-NS1 mAb coupled with horseradish peroxydase)
  - **R7** = Diluent (Phosphate buffer, Tween, FCS…)
  - **R9** = Chromogen TMB (tetramethylbenzidine)
  - **R10** = Stopping solution (1N Sulfuric acid solution)
  - Adhesive films for sealing the plate

- **Material required but not provided**

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- Vortex mixer
- Water bath heater or microplate incubator (set at 37°C)
- Automatic plate washer
- Micro plate reader (equipped with 450nm and 620nm filters)
- Sodium hypochlorite (bleach) and sodium bicarbonate
- Deionized water
- Pipettes and filtered tips (50µl, 100µl, 300µl and 1000µl)
- Graduated cylinders (25ml, 50ml, 100ml and 1000ml)
- Disposable tubes

8. Procedures

1) Before use, allow reagents and assay strips (plate) to reach room temperature.
2) Take the carrier tray and the required number of strips (**R1**) out of the protective pouch. Replace those not required and re-seal the bag immediately and store again 2-8°C.
3) Carefully reconsititute and dilute reagents, avoiding any contamination. Prepare enough solution appropriate for the number of samples that you plan to test.
   - **R2 (20x)**: Dilute 1/20 the Washing solution (**R2**) in distilled water. To make 1L diluted washing buffer, add 50mLof 20x wash buffer into 950mL distilled water.
     - 350ml of diluted Washing solution is needed for one plate of 12 strips if washing manually.
     - If automatic plate washer used, it is necessary to prepare an extra amount of washing buffer because it will be used to prime/rinse washer machine before running sample. To wash whole plate, it needs to prepare 700mL diluted washing buffer.
     - This solution can be stored for 2 weeks at 2°C – 30°C.
   - **R6 + R7**: Conjugate (**R6**) is concentrated 50x, and must be homogenised before use. Dilute 1/50 with Diluent (**R7**).
     - For one strip, dilute **20µl of R6 in 980µl of R7** (1ml total volume). Multiply these volumes by 12 for one microplate.

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- This solution is stable for 8 hrs at 2°C – 30°C.

4) Prepare the plate according to the layout below.
   - Use one negative control (**R3**), two calibrators (**R4**), and one positive control (**R5**) in each run to validate the assay.
   - Add the patient samples in the remaining wells. Neat plasma samples are used for the assay

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	R3	S5	S13									
B	R4	S6	S14									
C	R4	S7	S15									
D	R5	S8	S16									
E	S1	S9	…									
F	S2	S10	…									
G	S3	S11	…									
H	S4	S12	…									

5) Gently vortex all reagent before adding to wells. Add the following reagents to the wells, *in the exact order listed*:
   - 50µl of diluent (**R7**) to all of the wells
   - 50µl of controls, calibrators or patient samples to the appropriate wells
   - 100µl of diluted conjugate (**R6**+**R7**) to all of the wells
6) Seal the plate firmly and incubate for 90 ± 5 minutes at 37 ± 1°. Label numbers for each strip to avoid any confusion if there is any strip fallen out of the carrier tray during washing step
7) After incubation, remove the adhesive plate sealer and aspirate out the contents of all the wells into a biohazard waste container (containing sodium hypochlorite) by pipetting if washing manually. If using automatic plate washer, the program set will perform this step before washing step

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8) Wash the plate six times with 300µL/well of washing solution (**R2**). Invert microplate and gently tap on absorbent paper to remove remaining liquid[1].
9) Quickly add 160µL Chromogen solution[2] (**R9**) (*away from direct light*) into each well. Cover the plate with an alumium paper.
10) Incubate the plate *in the dark* for 30 ± 5 minutes at RT to develop enzyme reaction. Do not use an adhesive plate sealer during this time.
11) Stop the enzymatic reaction by adding 100µL of stop solution (**R10**) to all wells.
12) Carefully wipe the plate bottom. Read the optical density of the wells at 450/620 nm using a plate reader within 30 minutes after stopping the reaction. It is important to keep the plates away from light before reading.
13) The final results are recorded in the "NS1 qualitative ELISA assay worksheet" (*FM-TC-DNG-027*) and the electronic result file is saved in assigned folder.

9. Calculations

- Cut-off (CO) value corresponds to the mean value of the optical densities of the calibrator duplicates (**R4)**.
- Calculation of the sample ratio: Sample result is expressed by ratio of S (which is the optical density of the sample) divided by cut off value.
  - Sample ratio = S / CO

10. Quality control

For validation of the assay, the following criteria must be meet:

- Optical density values

  CO > 0.2
- Negative and positive control ratios:
  - Negative control (R3) ratio < 0.4 (R3 ratio = OD_{R3} / Cut off value)
  - Positive control (R5) ratio > 1.5 (R5 ratio = OD_{R5} / Cut off value)

If those specifications are not met, the test run should be repeated.

[1] Take care not to produce splashes during the aspiration and washing steps. This can lead to cross contamination.
[2] Chromagen is light sensitive

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11. Interpretation of results

Refer to the table below for result interpretation:

Sample ratio	Result	Interpretation and recommendations
Ratio <0.5	Negative	Sample considered non-reactive for DENV NS1 antigen
0.5 ≤ Ratio <1.0	Equivocal	Sample considered equivocal for Dengue NS1 antigen
Ratio ≥ 1.0	Positive	Sample considered reactive for Dengue NS1 antigen

12. Reporting of results

The unit of reporting is Optical density (OD) values

13. Limitations

Diagnosis of recent infection by Dengue virus can only be established on the basis of a combination of clinical and biological datas. The result obtained on a single sample does not constitute a sufficient proof for diagnostic of recent infection.

14. Safety

Use Universal precautions at all times when handling clinical specimens as these may be contaminated with any number of blood-borne infections. Wear laboratory coat, gloves and safety glasses when handing biological specimen. The addition of clinical specimens to the assay plate should be performed in the safety cabinet. Place all waste in the clinical waste bins provided.

Chemicals should be handled and disposed of in accordance with Good Laboratory Pratices. Some of the reagents (R2,3,4,5,6,7,9) containing ProClin™ 300 and acid sulfuric (stoping solution) may cause sensitisation by skin and mucosa contact (risk of toxicity, irritation or burn)

- **ProClin300 is classified as a skin sensitizer. It may cause an allergic skin reaction.**
- Precautionary statements for Proclin300
    - Can cause severe burn
    - Avoid breathing mist/dust/fume/gas/vapors/spray.
    - Contaminated work clothing should not be allowed out of the workplace

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15. Supplementary notes

- It is not permissible to use Diluent (R7) from lots other than provided in the kit.
- It is possible to use the Washing solution (R2), Chromogen (R9) and Stopping solution (R10) from other kits, provided that these reagents are strictly equivalent and the same lot is used within a given test run.
- Chromogen Solution (R9) should be colourless. The appearance of a blue color indicates that the reagent must be replaced.
- Washing the microplate is a critical step in the procedure. Be sure to follow the recommended number of washings, and make sure that all wells are completely filled and then completely emptied. Incorrect washings may lead to inaccurate results.
- Do not allow the microplate to dry between the end of the washing operation and the reagent distribution

16. Risk assessment and risk management

Department Dengue group **Location of work** OUCRU-HCM Laboratory	**Persons involved** Dengue laboratory staff
Description of procedure The procedure describes how to perform the DENV NS1 ELISA assay according to the Bio-Rad Platelia Kit.	**Hazardous Substances used** Human plasma samples ProClin™ 300
Quantities used 5Plasma:50µl/sample	**Frequency of use** 4-5times/years
Hazards identified Potential for blood to be infected with blood-borne pathogens. The preservatives in the kit components can cause an allergic skin reaction. o ProClin300 may cause sensitization by skin contact. Avoid contact with skin and eyes.	**Could a less hazardous substance be used instead?** No

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What measures are in place to control risk? Plasma sample must be distributed into wells for the assay under a safety cabinet class II. Follow usual standard laboratory practices. It is indicated throughout the SOP at various points when extra care is needed. The following personal protection is recommended during performing the procedure: • Wear protective gloves made of latex, nitrile rubber, e.g • Safety glasses • Protective work clothing • Avoid breathing mist, vapors • Ensure adequate ventilation	
Checks on control measures • Supervision and observation by senior staff. • Equipment maintenance and calibration. • Spill kit is checked for content regularly.	
Is health surveillance required? Yes, Hepatitis B serology	**Training requirements:** Task-specific training, waste disposal, spill clean up procedures, signs and symptoms of infection.
Emergency procedures: Biological and chemical spill cleanup procedure *PR-HS-ALL-005*	**Waste disposal procedures**: OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

17. Related documents

Document name	Document code
NS1 qualitative ELISA assay worksheet	*FM-TO-DNG-027*
NS1 qualitative ELISA assay batch number index	*FM-TO-DNG-028*

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18. Document history

Revision no	Effective date	Details of amendment
00	29-03-2010	New issue
001	03-10-2014	Converted to new OUCRU template. General editing, changes and improvement of description in the following sections: Purpose, Principle, Definitions, Procedures, Supplementary notes, Risk assessment and management. – Lauren Carrington
002	14-08-2015	General editing, updated Quality Control. – Lauren Carrington
003	6 April 2018	Convert to new OUCRU template Improvement of description in the following sections: -Principle -Safety -Limitations -Risk assessment and management

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1. Purpose

This protocol describes a serological test targeted to detect human IgM and IgG to dengue virus antigens in plasma/sera samples.

2. Principle

This protocol describes a dengue diagnostic capture IgM and IgG enzyme-linked immunosorbent assay (ELISAs). It is performed with use of paired enrolment and convalescent specimens; differences between measurements in the early and late samples are used to determine the outcome of the assay. Reagents are provided by Venture Technologies (Malaysia).

Briefly, 96-well plates are coated with anti-human IgM or anti-human IgG (ordered separately from Sigma and Dako's companies) overnight at 4°C. The next day, the wells were blocked to remove the possibility of non-specific binding of the detection antibodies using 0.5% BSA-PBS for 2 hours at room temperature (RT). After several washes, 100µl of each of positive, negative controls or samples which were diluted 1:100 were added and incubated for 2 hours at RT. After unbound samples are washed off, 100µl of pooled DENV1-4 antigen (pooled C6/36 cultures of DENV1-4) are distributed into wells and incubated at 4°C overnight. Afterwards, the plate is again washed and incubated with a cocktail of mouse monoclonal anti-DENV1-4 E protein antibodies for 1 hour at RT. The antigen-antibody complex is detected with anti-mouse Ig Horseradish Peroxidase (HRP) for 1 hour in the dark at RT. The color reaction is developed with substrate o-phenylenediamine dihydrochloride (OPD) in the dark for 30 minutes and then stopped by adding 50µl of 10% H_2SO_4. The optical density (OD) is read at 490nm.

3. Scope and responsibilities

Dengue staffs (Research assistant, lab technicians) have responsibilities to perform this procedure for dengue diagnostic tests. This work is to be performed at Immunology lab (Room 111) in OUCRU.

4. Reference

This test is based on the in-house MAC ELISA instruction of Venture Technologies, Sarawak, Malaysia.

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5. Definition

ELISA:	Enzyme Linked Immunosorbent Assay
PBS:	Phosphate buffer Saline
MAC-ELISA:	IgM Antibodies Capture ELISA
DENV:	Dengue virus
OD:	Optical Density
CO:	Cut off

6. Specimen requirement

Serum or plasma samples from dengue patients. Paired acute and convalescent samples are required for each patient to determine the ratio of IgM:IgG, and therefore determine infection history.

7. Materials

- Anti-human μ chain (Anti-IgM) Dako- Cat. No. A0425
- Anti-human γ chain (Fc specific) (Anti-IgG) Sigma-Cat.No.İ2136
- DENV antigen Venture Technologies, Malaysia
- Mouse monoclonal DENV1-4 E protein antibodies Venture Technologies, Malaysia
- Polyclonal Rabit Anti-Mouse immunoglobulins/HRP Dako, P0260
- Sigma Fast™ o-phenylenediamine dihydrocloride template sets Sigma-Cat. No. P9187
- PBS tablets Sigma- Cat.No. P3813
- Albumin bovine serum (BSA) Sigma- Cat.No. A7906
- Tween®20 (Polyoxyethylenesorbitan monolaurate) Sigma, Cat.No. P1379
- Coating buffer (0.05 M carbonate-bicarbonate buffer tablets, pH9.6) Sigma. Cat.No. C3041
- 10% H2SO4 (made from concentrate H_2SO_4) Merck. Cat.No. 100748

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8. Equipment

- Incubator (temperature set 37°C)
- ELISA Plate (Nunc Immuno plate). Nunc. Cat.No. 442404
- Plate washer BioTek, ELx405
- Microplate reader model 5.5 (or any microplate reader) BioRad
- Multi-channel pipettes (50 µl -300 µl)
- Single channel pipettes (10 µl – 1000 µl)

9. Procedures

Reagent preparation

- **Coating buffer (enough for 10 plates)**
  - Dissolve 1 tablet of carbonate bicarbonate buffer into 100ml dH_2O to make coating buffer. Ensure the pH = 9.6.
  - Filter through 0.22µm filter, and store at RT.
- **Anti-human IgM/IgG**
  - Make 1:2000 dilution of the antibodies, by diluting 5µl anti-human IgM/IgG in 10 ml coating buffer. This is enough for **1 plate**, and should be prepared fresh before use.
- **PBS**
  - Dissolve 1 tablet PBS into 100mL distilled water. Store this at RT.
- **1% BSA-PBS solution**
  - Dissolve 1g BSA into 100mL PBS. Filter through 0.22µm filter.
  - This can be stored at 4-8°C for 1 month. Write the date of preparation on the bottle.
- **0.1% or 0.5% BSA-PBS**
  - Dilute 1:10 of 1% BSA-PBS in PBS to make 0.1% BSA-PBS solution
  - Dilute 1:2 of 1% BSA-PBS in PBS to make 0.5% BSA-PBS solution
  - This can be stored at 4-8°C for 1 month. Write the date of preparation on the bottle.
- **Preparing specimen, positive and negative control**
  - Dilute specimen and controls at 1:100 dilution in 0.1% BSA–PBS.
  - This should be made freshly each time.
- **Washing buffer**

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- Washing buffer is 0.05% Tween20 in PBS.
- Add 1ml Tween into 2 litres PBS. This should be freshly prepared each time.

- **DENV antigen (enough for 1 plate)**
  - Combine 6ml of DENV antigen with 4ml PBS. This should be freshly prepared each time.
- **Mouse monoclonal DENV 1-4 E protein antibodies (enough for 1 plate)**
  - Combine 2 ml of monoclonal antibody with 8ml of 0.1% BSA-PBS. This should be freshly prepared each time.
- **Conjugate solution**
  - Prepare a 1:2000 dilution of conjugate in 0.1 % BSA-PBS.
  - Add 5 µl conjugate (Polyclonal Rabbit Anti-Mouse immunoglobulins/HRP) to 10 ml of 0.1 % BSA-PBS. This should be freshly prepared each time.
- **Subtrate solution (enough for 2 plates)**
  - 1 tablet of Buffer with urea (Sigma Fast™)
  - Dissolve the tablet completely with 20mL distilled water. Then add 1 tablet OPD.
  - This should be freshly prepared each time
- **Stopping solution (10% H_2SO_4) (must prepare under a fume hood)**
  - Add 10mL of concentrate H_2SO_4 into 90 mL distilled water to make 10% solution
  - Stored at RT

Testing the samples

- **Plate Coating**
  - Plate is coated with 100µL/well of 1:2000 anti-human IgM/IgG coating buffer
  - Incubated overnight at 4°C.
- **Washing (1)**
  - Wash 3 times with washing buffer using the automatic washer, using 300µL/well to remove the excess coating antibody.
- **Blocking plate**
  - Each well of the plate is added with 200µL of 0.5% BSA-PBS at RT in 2 hrs or at 37°C in 1 hour. The blocking step is applied to eliminate the residual binding capacity of the wells.
- **Washing (2)**

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- Wash 3 times, using 300µL/well

- **Samples incubation**
  - Add 100µL of diluted specimen and controls into well.
  - Incubate at RT for 2 hours
- **Washing (3)**
  - Wash the assay plate 5 times, using 300µL/well
- **Antigen incubation**
  - Add 100µL/well of diluted DENV antigen.
  - Incubate at 4°C overnight
- **Washing (4)**
  - Wash the assay plate 5 times, using 300µL/well
- **Mouse monoclonal antibodies incubation**
  - Add 100µL of diluted anti-mouse Ig HRP in to well.
  - Incubate at RT for 1hr
- **Washing (5)**
  - Wash the assay plate 5 times, using 300µL/well
- **Developing**
  - Add 100µL OPD in to each well.
  - Leave the plate in dark for 30 minutes for the colour to develop
- **Stopping**
  - Stop the assay by adding 50µL/well of 10% H_2SO_4
- **Reading**
  - The optical density (OD) is read at 490nm

10. Quality Control

- The negative control sample was a mixture of plasma from dengue-naïve healthy adult volunteers who had just arrived from regions without known dengue transmission.

  In the template assay must include
  - 2 positive wells: added with positive control plasma 3 negative wells: added with negative control plasma

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- 3 background wells: added with positive control plasma; *PBS is added to background wells instead of DENV antigen*

template	1	2	3	4	5	6	7	8	9	10	11	12
A	pos											
B	pos											
C	neg											
D	neg											
E	neg											
F	Background											
G	Background											
H	Background											

11. Calculations

- OD_B = mean of OD of 3 background samples
- OD_N = mean of OD of 3 negative samples
- Negative control value (NC) = OD_N - OD_B
- Cut-off (CO) = 5x NC
- Sample ratio (RS) = $(OD_S - OD_B)/CO$

12. Interpretation of results and report

Sample ratio (RS)	Result	Interpretation
If RS < 0.8	Negative	No detectable IgM/IgG antibodies
If $0.8 \leq RS \leq 1.2$	Equivocal	Equivocal samples should be repeated.
If RS >1.2	Positive	Presence of detectable IgM/IgG antibodies

Serological tests are based on the principle that anti-dengue IgM and IgG antibodies increase in response to dengue infection. In case of the paired samples, early sample (S1) and late sample (S2), used for dengue lab confirmation, the presence of rising levels of IgM/IgG or IgM/IgG ratio or IgM/IgG seroconversion in S1 and S2 could be applied to make a definitive diagnosis. There

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are different algorithms based on S1 and S2 serological results used to define infectious status and the definition in each study is different.

13. Reporting of results

The unit of reporting is Optical density (OD) values

OD values (intensity) are clearly different between S1 and S2 samples if there is any the presence of increased levels of IgM/IgG or IgM/IgG seroconversion.

14. Reference range and critical value:

For validation of the assay, the following criteria must be met:

- Cut off value (CO): $0.2 < CO < 0.6$
- Negative control value (NC): $NC \leq 0.12$

If the above QC criteria are not met, the test should be repeated.

15. Limitations:

- This assay can give results of positive serology due to cross-reactivity of the DENV antigens with the antibodies of the other Flaviviruses (e.g JEV)
- To define immune status of samples, or discriminate between acute and recent DENV infections, it is necessary to combine these results with the different dengue laboratory confirmation algorithms based on the different studies.

16. Safety

- Sample preparation step should be performed under a biosafety cabinet class II to avoid the direct contact with the infectious pathogen.
- Human plasma samples should be decontaminated in PRESEPT before discarding
- Any material which contact with samples should be treated as clinical waste
- Wear protective gloves, clothing, eye and face protection when working with chemicals and clinical specimens.
- **H_2SO_4 is hazardous.** In case of small spill of 10% H_2SO_4: must dilute with water and mop up, or absorb with an inert dry material and place in an appropriate waste disposal container.

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- If necessary: Neutralize the residue with a dilute solution of sodium carbonate.

17. Supplementary notes

- Acute plasma from Vietnamese confirmed dengue patients which gave strong positive results with the capture IgM/IgG ELISA were pooled to create the positive control.
- To make 10% H_2SO_4 solution: never add water to concentrated product, should do in inverse

18. Risk assessment and risk management:

COSHH Risk Assessment COSHH risk assessment - University of Oxford COSHH Assessment Form	
Description of procedure This protocol is applied to detect human IgM and IgG to dengue antigens in dengue infected plasma/sera samples.	**Hazardous substances used** Dengue plasma specimens 10% H_2SO_4
Quantities used Plasma: 10µl/sample H_2SO_4: 50µ/sample	**Frequency of use** 4-5times/year is minimum. The number of samples tested depends on the studies
Hazards identified • Potentially infectious pathogens in clinical samples • 10% H_2SO_4 solution can cause extremely hazardous in case of skin contact (corrosive, irritant), of eye contact (irritant), of ingestion, of inhalation. Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth and respiratory tract. Skin contact may produce burns.	**Could a less hazardous substance be used instead?** No
What measures have you taken to control risk? Follow usual standard laboratory practices. It is indicated throughout the SOP at various points when extra care is needed. For personal protection in case of preparing 10% $H2SO_4$: use face shield, full suit, vapor respirator. It is recommended that you must use an approved/certified respirator or equivalent, gloves and boots if it is possible in your lab condition to prepare the 10% H_2SO_4 Normally, we prepare 10% $H2SO_4$ under a fume hood.	

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Checks on control measures • Supervision and observation by senior staff. • Equipment maintenance and calibration. • Spill kit is checked for content regularly.	
Is health surveillance required? Yes, Hepatitis B serology.	**Training requirements:** Task specific training for all staff, plus training in good laboratory practices.
Emergency procedures: Biological and chemical spill cleanup procedure *PR-HS-ALL-005*	**Waste disposal procedures:** OUCRU BSL2 manual of waste management *SD-HS-ALL-009*.

18. Related Documents:

Document name	Document code
N/A	

19. Document History:

Revision #	Effective date	Details of amendment
00	01 July 2010	New issue
01	27 Mar 2018	Updated version. Major changes include: improved description of the Principle of the assay, limitations of the assay, interpretation of results, risk assessments and management. The SOP has also been converted to the new OUCRU template. – Lauren Carrington

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SOP for - MILLIPLEX MAP Kit, Human Cytokine/Chemokine Panel 1, cat# HCYTOMAG-60K-PX41

Sample receipt and storage

1. Receive frozen samples from user. Keep them in -80°C until run.
2. Prepare sample layout as outlined below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK										
B	Standard 1	Standard 1										
C	Standard 2	Standard 2										
D	Standard 3	Standard 3										
E	Standard 4	Standard 4										
F	Standard 5	Standard 5										
G	Standard 6	Standard 6										Internal Control
H	QC-1	QC-2										Internal Control

Sample preparation

1. Thaw samples completely.
2. Mix well by vortexing and centrifuging prior to use in the assay to remove particulates.
3. Keep samples at 4°C while preparing other reagents.

Reagent preparation

A. Preparation of Standards

1. Reconstitute the Human Cytokine Standard with 250 µL deionized water.
2. Invert the vial several times to mix and vortex.
3. Allow the vial to sit for 10 minutes.
4. Label 6 polypropylene Eppendorf tubes as S1, S2, S3, S4, S5, S6.
5. Transfer reconstituted Human Cytokine Standard to tube S6.
6. Add 200 µL of Assay Buffer to S1, S2, S3, S4, S5.
7. Transfer 50 µL of S6 to S5, and mix well.
8. Transfer 50 µL of S5 to S4, and mix well.
9. Transfer 50 µL of S4 to S3, and mix well.
10. Transfer 50 µL of S3 to S2, and mix well.
11. Transfer 50 µL of S2 to S1, and mix well.

B. Preparation of Quality Controls

1. Reconstitute the Quality Control 1 and Quality Control 2 with 250 µL deionized water.
2. Invert the vial several times to mix and vortex.
3. Allow the vial to sit for 10 minutes.
4. Label polypropylene Eppendorf tubes as QC-1 and QC-2.
5. Transfer reconstituted Quality Controls 1 and 2 to respective tubes.

D. Preparation of Wash Buffer (PBS + 0.05% Tween + 0.1% BSA)

1. Add 1ml of Tween 20 and 50ml of 2g BSA to 1.95L of Filtered PBS.
2. Mix Well.

D. Preparation of Serum Matrix

1. Reconstitute the bottle containing lyophilized Serum Matrix with 1.0 mL deionized water.
2. Mix well.
3. Allow to stand for at least 10 minutes for complete reconstitution.

Plate set-up

1. Pre-coat the plate by dispensing 200 µL of Assay Buffer into each well of the black Flat bottom 96-well plate.
2. Seal and shake on a plate shaker at 700rpm for 10 minutes at room temperature.
3. Remove Assay Buffer by aspiration. Blot excess Assay Buffer on to absorbent pad or paper towels.
4. Add 25 µL of standards, QC-1 and -2, samples and internal controls into appropriate wells.
5. Add 25 µL of Assay buffer to Blank and samples wells.
6. Add 25 µL of Serum Matrix to Blank, Standards, and QC-1 and -2 wells.
7. Vortex Beads bottle and add 25 µL of bead mix to each well. During addition of beads, vortex Mixing bottle intermittently to avoid settling.
8. Seal the plate with a plate sealer.
9. Incubate with agitation on a plate shaker overnight (16-18 hours) at 4°C.

Addition of Detection antibodies, Streptavidin-PE and sheath fluid

1. Wash plate 2 times with 200 µL of Wash Buffer in each well.
2. Add 25 µL of Detection Antibodies into each well.
3. Seal plate and incubate with agitation on a plate shaker for 1 hour at room temperature.
4. Add 25 µL of Streptavidin-Phycoerythrin (SA-PE) into each well.
5. Seal plate and incubate with agitation on a plate shaker for 30 minutes at room temperature.
6. Wash plate 2 times with 200 µL of Wash Buffer in each well.
7. Add 150 µL of Sheath Fluid into each well.

Data acquisition

1. Read plate on FLEXMAP 3D® machine.
2. Save and analyze the Median Fluorescent Intensity (MFI) data using Bio-plex Manager 6.1 to obtain a weighted 5-parameter logistics or 4-parameter logistics for calculating cytokine/chemokines concentrations in samples.