# CHARACTERIZATION OF HUMAN ANTIBODY RESPONSES TO DENGUE VIRUS INFECTIONS IN A SRI LANKAN PEDIATRIC COHORT

Kizzmekia Shanta Corbett

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology in the School of Medicine.

Chapel Hill
2014

Approved by:

Aravinda de Silva

Ralph Baric

Mark Heise

Ronald Swanstrom

Jennifer Webster-Cyriaque

## ABSTRACT

Kizzmekia Shanta Corbett: Characterization of Human Antibody Responses to Dengue Virus Infections in a Sri Lankan Pediatric Cohort
(Under the direction of Aravinda de Silva)

Dengue virus (DENV) is the most significant mosquito-borne viral infection of humans. People infected with dengue viruses present with subclinical (inapparent) or clinically-apparent infections ranging from undifferentiated fever to dengue hemorrhagic fever or dengue shock syndrome. It is not completely understood why some people get more severely ill from DENV infections than others, but antibody responses have been implicated in playing a role facilitating severe dengue disease. To that end, we analyzed samples from a pediatric dengue cohort study in Sri Lanka to explore if antibody responses differentiated clinically inapparent and apparent infections. 799 children living in a heavily urbanized area of Colombo, Sri Lanka were followed for one year; samples were collected at enrollment, at follow-up, and intermittently from any child who acquired fever.

Using those samples, we first obtained accurate estimates of the incidence of DENV infection and disease in the cohort. At enrollment, dengue sero-prevalence was 53.07% demonstrating high transmission in this population. Over the study year the incidence of DENV infection and disease were 8.39 (95% CI: 6.56-10.53) and 3.38 (95% CI: 2.24-4.88), respectively per 100 children per year. All together, we identified 35 primary (20 inapparent and 15 apparent) and 32 secondary/repeat infections (20 inapparent and 12 apparent) over the 12-month study period. The ratio of inapparent to apparent infections was 1.48:1. Secondly, we defined temporal regulation of the DENV-infected children's antibody responses. Children who experienced primary infections had broad, serotype cross-neutralizing responses that narrowed in breadth to a single serotype over a 12-month period post infection. In children who

experienced repeat infections, IgG antibody levels fluctuated following infection, but neutralization breadth remained steady. Thirdly, we observed baseline antibody responses of children who got repeat infections. Children with pre-existing monotypic neutralizing responses were more likely to develop fever than children with cross-neutralizing responses. Pre-existing DENV neutralizing antibodies were correlated with protection from apparent dengue disease. Our results will be useful for obtaining more accurate estimates of the burden of dengue in the Indian subcontinent, and, most importantly, provide insight on protective antibody responses as they may be important for vaccine development.

To my ProjectSEED bus partner, my friend, and my angel, Yusuf Neville (06/22/85 - 01/29/14)…
your death motivates me to keep living.
I miss you. I love you.

## ACKNOWLEDGEMENTS

**God.**

Above all, I must thank my Lord and Savior Jesus Christ for giving my life a purpose and directing me so that I can fulfill it.

**Yusuf.**

I dedicate this dissertation to you because, in this moment, I cannot think of anyone else who is more deserving. I remember during my second year when research wasn't going how I'd planned… You drove to Durham, sat in your cousin's driveway with me, and reminded me why I couldn't give up. I remember you always telling me to "man up" when I called you in an emotional frenzy (usually about boys who you thought were no good for me, but I guess that's a different topic… lol). I remember you (and my Dad) being the only two people in the world who remembered my presentation schedule off the top of your heads. I could always count on a message from you 5 minutes before I stepped to the podium with words of encouragement. I met you when I was 15 years old; we both were scientists then. Although you didn't take the science route, you always understood "Kizzy the scientist" in ways most people didn't. ProjectSEED connected us for a lifetime, and every single day, I think about how yours was cut a little too short. May your soul be free, friend. R.I.P.

**Family.**

To my grandmothers, Shirley and Margaret, thank you for praying for me and instilling in me a faith that, although sometimes tested, is forever undying. To my mom, Rhonda, thank you for all of your sacrifices. You were my mom, my dad, and my everything during some of the most tender and impressionable years of my life; thanks for leaving an everlasting impression on what a strong woman should be. To my dad, Terrance, you are my rock and my friend.

Thank you for loving me as if your blood runs through my veins and providing the foundation I needed to grow into the person that I am today. To both of my parents, thanks for keeping your expectations of me high, and even when I didn't always meet them, thanks for loving me anyway. Thanks for every time you supported me, whether it was at a cheerleading competition or scientific presentation. You always found a way to remind me that I was important in times when the world tried to convince me otherwise. Your unconditional love and support got me here, to the day before I submit my PhD dissertation, and I will forever be indebted to you. To my siblings, unbeknownst to you, you set goals for me that I didn't even know I had; because you all looked up to me, I could never give up and I promise I never will. Ciera, I was no longer the oldest when you came into the family. Although I'm sure it's burdensome having 6 younger people look up to you, you carry it well. Thanks for teaching me so much and having my back. Tameka, you were born my cousin and became my sister. At your mom's funeral, I told you that if there is anything you ever need, I got you. Thanks for having me too. Dee, you have so many of Dad's characteristics in you which make you the perfect man and the perfect brother. Thanks for being someone that I can forever call on, whether it be to paint my walls, wash my car, or to simply talk. Tireka, thanks for every single random phone call, text message, or tweet. Sometimes you just need someone to remind you that they love you and are proud of you. Destiny, thanks for being such a genuinely humble and caring human being. Those were always characteristics you exhibited that made me look up to you, although you are 11 years younger. Eric, thanks for always screaming "Hey Kizzy" in the background when I call Mom or Dad, for randomly hanging out with me on Saturdays, and for still giving me huge hugs on sight; small things like that brightened my days. Thanks to my niece and nephew, Keziah and Kaiden, for loving your "Aunt Kitty" unconditionally. It has been amazing to watch you grow into a seven year old dancing machine and a laughing bubbly "Baby Terry", respectively. To my Godchildren, Jaden, Chey, and Kailyn, your parents designated this important role for me in your lives, and I promise to fulfill it wholeheartedly. Thanks for being the children I have yet to birth on my own.

To my extended family… Joyce, for being my mom in Maryland and providing me a home away from home…Tonya, for being a big cousin who I could look up to… Aunt Laura, for being so strong and reminding me that biomedical research is so important… and Lamar, for being more like my brother than my cousin. To my entire family, grandmas, parents, siblings, nieces, nephews, godchildren, aunts, uncles, and cousins, thanks for the moments we shared together in the last five years. I will cherish each and every one forever, no matter how many degrees I acquire or how far away I move.

**Friends.**

To my PICs, Fe, Cool, and Prillert, not many people can look back 12 years and find 3 rock-solid loyal people who they can still call friends. Your love, support, and honesty are so appreciated in times when other people have fallen by the wayside. Thanks for being forever "friends", in the truest meaning of the word. Kenyetta, honestly I would have never thought we'd still be friends 12 years after working as grocery store cashiers together, but we are, and I appreciate you for rocking with my craziness for this long. To my undergrad roommates, Rasheeda and Nerg, thanks for accepting and loving me just as I came and staying my friends "4 Life". Meeting you guys was one of the first times I'd come across other black women with similar goals as me; thanks for that inspiration. To Seghen, thanks for being someone I can always call and pick up right where we left off… "Deuce Deuce". Vic, my "Suga", thanks for relieving me from the stresses of science, whether it was a random getaway or simple text conversation after a long day in lab. Brian, your advice has gotten me through many a rough day; thanks for continuing to be my friend through it all. Daaimah, or shall I say "Baby D", we went from working at NIH to roaming the streets of NYC together; thanks for reminding me that a scientist can be cool too. Isa, from working in the same lab at 16 to arguing like sisters in group chat at 28, thanks for being the "Woo" to my "Woo". Stephani, whether it be Pinot Noir served at perfect temperature, indie hip-hip concerts on Friday nights, or science policy advocacy via social media networks, thanks for being a mature friend with whom I share similar

passions. Jasmyn and Richard, thanks for extending our connection beyond the BBSP incoming Class of 2009 to friendships that I'm sure will last forever. To my extended circle of friends, Taneisha, Ashley, Sophia, Janeice, Alex, Jelly, Noah, Lenard, Taa, Harry, Greg, Jared, Randall, and Jean thanks for simply being there whenever I have needed you.

**Science.**

Thanks to Project SEED, the American Chemical Society, UNC Kenan Labs, Mr. Cutler, and Mr. Cherry for fostering my love for science at 15 years old. Thanks to UMBC, Robert and Jane Meyerhoff, Dr. Hrabowski, Mr. Toliver (R.I.P), Mr. Goodwyn, Mr. Harmon, Mrs. Baker, and the entire Meyerhoff Scholarship Program for affording me an unparalleled undergraduate science education. To the staff of NIH's undergraduate scholarship program, I know how much of a risk it must have been to accept a rising college freshman into such a prestigious program; thank you for believing in me when I was only 18 years old. To the mentors I had in my early scientific career, Dr. Russell, Dr. Viboud, and Dr. Graham, thank you for the time you invested in training me when I had no clue.

**UNC.**

Thank you, Aravinda, for accepting me into your laboratory and giving me the creative freedom to work on a project that is atypical for a PhD student. Thank you for creating a peaceful, loving laboratory environment so that we could all work and grow as scientists. Over the years, my laboratory family became more like real family to me, which is a testament to your mentorship and leadership. Rukie, you have no idea how motivating it was to sit beside someone as brilliant as you every day for three years. Thanks for your advice on experiments and manuscript preparation. Thanks also for all of the times you saved me, whether it be with a hug when I was crying or by running shoes across campus when my flip flops broke. Thank you, Leah, for starting preliminary analyses on the PDVI dataset. Thanks also for that random conversation in the bathroom at the Dengue Vaccine Consultation Meeting, during which I somehow convinced you to do cartography analysis with the neutralization data. Thank you Nick

for patiently teaching me everything I know about dengue cell culture and neutralization assays and for not laughing at me that one time when I clumsily dropped eight plates on the floor. Bhumi and Sandra, thanks for giving me mid-day breaks with random conversations about literally anything, from science to boys to fashion. Bhumi, especially for being someone I could call on to fix my plates or stop my gels from overrunning. Emily, thanks for organizing lab outings and teaching me a thing or two about coffee and beer. Thank you, Anne, for every single flower you brought me from your garden; even when your days weren't the brightest, you made it your duty to make sure mine were. Wahala, thanks for being like my big brother with whom I argued one second but whose advice and direction I still took heed to. Yang, thanks for your sparse but meaningful conversations about science. Thanks to Katie and Eileen for putting up with me emptying all of the stack-packs as soon as you refilled them. To Tony and Jennifer, thanks for digging in the cold room for samples and crunching datasets. To all of our undergraduate lab assistants, thanks for keeping the lab in order and everyone somewhat sane in the process. Thanks to Bill and Doug for meaningful scientific discussions and advice, both during my preliminary exam and thesis work. Thanks to the entire Sri Lanka cohort study group for trusting me to work with such a precious set of samples. Thank you to Dharshan and the rest of my Genetech family for embracing me with open arms during my stint in Sri Lanka. Thanks to the Micro/Immuno administrative staff members who have helped to make the "school" portion of this PhD go so smoothly. Thank you to the Virology Training Grant, IMSD, Off-Campus Dissertation Fellowship, and several travel awards for funding a small part of my scientific endeavors. Funding aside, thank you especially to Pat, Ashalla, and everyone in the IMSD program for being like a family outside of my biological and laboratory families. Lastly, thanks to my committee members, Mark, Ralph, Ron, and Jennifer, for all comments, constructive criticism, and advice that helped me to think about my project from different angles…
Happy Reading!

*There are so many people who, in various capacities, have shared this journey with me. By fault of the head and definitely not the heart, I have likely omitted people who have touched me, my science, or my career in their own special way. To those who go unnamed above, thank you!*

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

1°– primary

2° - secondary

3° - tertiary

4° - quaternary

*Ae. aegypti – Aedes aegypti*

*Ae. albopictus – Aedes albopictus*

ADE – antibody dependent enhancement

AG129 -

BALB/c - Bagg Albino

B cell – B lymphocyte

BoB – blockade of binding

C – capsid

C57BL/6 – Black 6

CD14 – cluster of differentiation 14

c-type lection – calcium-dependent lectin

DC – dendritic cell

DC-SIGN - dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DENV – dengue virus

DENV1 – dengue virus serotype 1

DENV2 – dengue virus serotype 2

DENV3 – dengue virus serotype 3

DENV4 – dengue virus serotype 4

DENV5 – dengue virus serotype 5

DF – dengue fever

DHF – dengue hemorrhagic fever

DSS – dengue shock syndrome

E – envelope

EDI – envelope domain I

EDII – envelope domain II

EDIII – envelope domain III

ER – endoplasmic reticulum

FBS – fetal bovine serum

FcγR – fragment crystallizable gamma receptor

FcγRI – fragment crystallizable gamma receptor 1

FcγRII – fragment crystallizable gamma receptor 2

FcγRIIa – fragment crystallizable gamma receptor 2a

FcγRIIb – fragment crystallizable gamma receptor 2b

FL – fusion loop

HAI - hemagglutination inhibition assay

hsp70 – heat shock protein 70

hsp90 – heat shock protein 90

huMAb – human monoclonal antibody

Gln - glutamine

IFN – interferon

IFNα – interferon alpha

IFNβ – interferon beta

IgA – immunoglobulin A

IgE – immunoglobulin E

IgG – immunoglobulin G

IgM – immunoglobulin M

IL – interleukin

IP10 – interferon inducible protein

LAV – live attenuated vaccine

M – membrane

mAb – monoclonal antibody

MCP-1 - monocyte chemotactic protein 1

MIP1β – macrophage inflammatory protein 1 beta

mθ – macrophages

NEAA – non-essential amino acids

$neut_{50}$ – 50% neutralization

NHP – non-human primate

NHS – normal human serum

NK – natural killer

NS – nonstructural protein

NS1 – nonstructural protein 1

NS2A – nonstructural protein 2A

NS2B – nonstructural protein 2B

NS3 – nonstructural protein 3

NS4A – nonstructural protein 4A

NS4B – nonstructural protein 4B

NS5 – nonstructural protein 5

Pen/Strep – penicillin/streptomycin

PDVI – Pediatric Dengue Vaccine Initiative

PCR – polymerase chain reaction

OD – optical density

pH – potential hydrogen

prM – pre-membrane

rE – recombinant envelope

RNA – ribonucleic acid

SD – standard deviation

STAT2 - Signal transducer and activator of transcription 2

T cell – T lymphocyte

TGN – trans-Golgi network

TNFα – tumor necrosis factor alpha

vRNA – viral ribonucleic acid

WHO – World Health Organization

# CHAPTER ONE

# Background and Significance

## 1.1 Dengue: the Virus

Dengue virus (DENV), the causative agent of dengue disease, is an enveloped positive-strand RNA *Flavivirus,* akin to other disease-causing viruses such as West Nile virus, Tick-borne Encephalitis, and Yellow Fever virus. DENVs have been thought to exist as four distinct but antigenically related serotypes, DENV1-4, but data describing a new fifth DENV serotype, DENV5, has recently arisen [1]. Further complicating the dengue virus species, DENV serotypes can be additionally classified into regionally-specific genotypes.

### Infectious Life Cycle of Dengue Virus

DENV is transmitted to humans via the bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes. Upon injection into the skin, DENV primarily targets mononuclear phagocytic cells, such as monocytes, macrophages, and dendritic cells [2]. To date, a specific human cellular receptor for DENV has not been identified. Several molecules have been identified to act as receptors or co-receptors on various mammalian cell types, including heparin sulfate [3, 4], CD14 [5], and heat shock proteins 70 (hsp70) and 90 (hsp90) [6]. Additionally, a few calcium-dependent (c-type) lectins are thought of as attachment factors for DENV; the most well-studied of these c-type lectins is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, DC-SIGN/CD209 [7-9]. Also, antibody-bound DENV can attach via cellular Fcγ-receptors during a process called antibody dependent enhancement (ADE), which is described in detail later in this chapter.

The methods by which DENV is internalized after attachment to appropriate receptors and/or co-receptors (**figure 1.1A**) are still active areas of research (reviewed in [10]). Clathrin-mediated endocytosis is largely accepted as the main method of internalization by DENV (**figure 1.1B**) [11], although a clathrin-independent, dynamin-dependent internalization method used by DENV2 was identified in Vero cells as well [12]. Upon internalization, DENV particles are trafficked in early endosomes (**figure 1.1C**). Early endosomes mature into acidic late endosomes; low pH of late endosomes triggers DENV envelope (E) conformational changes [13, 14]. E homodimers dissociate, exposing the fusion loop (FL), which is then inserted into the cellular membrane. E homotrimers form, and viral and host membranes bend toward each other and fuse together (**figure 1.1D**) [14, 15].

As a result of viral to host membrane fusion, DENV nucleocapsid is ejected into the cell cytoplasm, where genomic viral RNA (vRNA) (**figure 1.2A**) is uncoated (**figure 1.1E**). DENV vRNA then travels to the rough endoplasmic reticulum (ER) where it is translated as a single polyprotein (**figure 1.1F**). Polyprotein processing occurs resulting in three structural proteins, capsid (C), pre-membrane (prM), and E, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (**figure 1.2B**). Once the viral protease cleaved the precursor to generate active protein products, the proteins aggregate to form a replication complex. Subsequently, repeated cycles of vRNA synthesis and replication occur (reviewed in [16]). RNA is translated to make enough viral proteins to form new viruses, and vRNA is packaged by C to form nucleocapsids. Then, immature DENV virions, containing prM/E heterodimers, are assembled in the ER (**figure 1.1G**) and transported through the trans-Golgi network (TGN) (**figure 1.1H**). It is in the Golgi where furin cleaves prM thus resulting in mature DENV particles (**figure 1.1I**). Progeny DENV virions exit cells via host secretory processes (**figure 1.1J**).

### Dengue Virion Structure

Mature dengue virions have icosahedral enveloped structures with a round core, containing multiple copies of C encapsidating the DENV RNA genome. The core is surrounded by a host-cell lipid bilayer containing 180 copies of membrane (M) and E [17]. The structure of E is biologically important in regards to neutralizing antibody epitopes; briefly, E consists of three domains, EDI-III, and a fusion loop at the tip of EDII (**figure 1.3A**). On a mature DENV virion, E is organized in 90 homodimers, which lay in sets of triples along the viral surface, creating a smooth appearance (**figure 1.3C**). Oppositely, immature dengue particles have a rough appearance because trimers of prM/E heterodimers form spikes on the surface of the particle (**figure 1.3B,D**) [18]. Dengue maturation state, as well as maturation of other *flaviviruses*, has been shown to be relevant to pathogenesis and antibody responses [19, 20]. Dengue virion structure will be related to antibody responses later in this chapter.

## 1.2 Dengue: the Disease

### Global Epidemiology of Dengue

Dengue virus infection is a widespread emerging global epidemic, with a projected two-thirds of the world's population at risk of exposure to DENV [21, 22]. *O*f all *Ae. aegypti*-borne viruses, including yellow fever virus and chikungunya, DENV represents the greatest epidemiological and economic burden [23, 24]. In the last 30 years, DENV has spread into over 100 countries, including the United States of America, leading to a four-fold increase of dengue disease [22]. Recent dengue outbreaks in Texas and Florida are true testaments to the continuous spread of DENV into previously unaffected areas and the emergence of dengue as a serious international disease threat [25, 26].

There are several factors that are contributing to the global emergence of dengue virus infections [21]. For one, *Ae. aegypti* and *Ae. albopictus*, are populating new areas thus contributing to the unwavering spread of DENV globally [21-23, 27]. Notably also contributing to the spread of DENV is the increasing prominence of tropical urbanization worldwide [21]. Ten

years ago, estimates of the global dengue disease burden stated that 100 million total DENV infections occurred each year, 500,000 of which resulted in severe disease and over 20,000 that resulted in death [21, 22]. Since then, those estimates drastically increased as surveillance, detection, and records of dengue cases improved. In 2013, it was estimated that about 390 million dengue infections occur around the world each year, which is a staggering 300% increase over previously published estimates [22, 28].

**Dengue in Sri Lanka**

Risk of dengue virus infection is considered highest in tropical regions of Asia. Latest estimates from the global burden of dengue in 2010 state that Asia contributed to 67% of the world's dengue infections, with 37% occurring in India alone [28]. Sri Lanka is an island country on the southern tip of the Indian subcontinent. Dengue virus has been present in Sri Lanka since at least 1966 [29, 30], though epidemiology of DENV infection in Sri Lanka has changed in recent years. There is documentation of a 10-fold increase in dengue fever (DF) and dengue hemorrhagic fever (DHF) cases in the last 10 years [30, 31]. Expansion of dengue across Sri Lanka coupled with introduction of new, possibly more virulent DENV genotypes have contributed to an increase in Sri Lankan dengue cases [30-33]. Crude estimations, based on surveillance cases reported to the WHO and cartographic estimates of global burden, state that there are about 2 million dengue cases that occur in Sri Lanka per year. [28]. Until our studies were conducted, there was no data on the true burden of dengue virus infections in Sri Lanka, or even the entire Indian subcontinent.

**Clinical Presentation of Dengue Disease**

An estimated 1.4 million (70%) of the 2 million dengue infections that occur in Sri Lanka each year are inapparent infections. That percentage is about the same globally, with 75% of all dengue infections occurring sub-clinically [28]. Inapparent (asymptomatic) dengue infection, as characterized by the World Health Organization (WHO), occurs when an individual is infected with DENV but presents no clinical symptoms. Traditionally, apparent (symptomatic) infections

are thought to manifest clinically as dengue fever (DF), dengue hemorrhagic fever (DHF), or potentially-fatal dengue shock syndrome (DSS). DF consists of mild symptoms including, but not limited to, fever, joint pain, headache, and rash. DHF, the more severe form of dengue disease, includes symptoms of DF and is additionally characterized by plasma leakage. DSS, the most severe category of dengue disease, consists of signs of complete circulatory failure, manifesting as hypertension or irregular pulse [34]. In 2009, WHO modified its traditional classification system to broaden the complexity and applicability of apparent DENV case definitions. The newly revised system characterizes apparent dengue disease as dengue without warning signs, dengue with warning signs, and severe dengue, which are respectively similar to DF, DHF, and DSS, in regards to symptom descriptions [35]. Treatment of clinical dengue disease is simply a matter of close observation and symptom maintenance, mostly consisting of fluid replenishment [34]. (Refer to **table 1.1** for a complete outline of WHO dengue case classification.)

### 1.3 Role of Viral Factors in Dengue Disease Severity

It is not fully understood why some individuals become more severely ill after dengue infections than others, although evidence shows viral factors play a role. Studies within the same cohort population have correlated specific DENV serotypes with severe disease outcomes. For example, a study in a Thai population showed DENV2 induced more severe disease than any of the other DENV serotypes [36, 37]. Introduction of new DENV clades into populations often have an effect on dengue disease severity, as well [32, 38-40]. DHF epidemics in Nicaragua have been associated with the introduction of new DENV2 clades [38]. A similar story exists with DENV3 clades in Sri Lanka [32, 39]. Recently even, a new DENV1 genotype was associated with a severe dengue epidemic in Sri Lanka [33]. Subtle genetic differences between DENV strains have been shown to correlate with pathogenesis [31, 38, 41, 42]. Additionally, DENV-encoded immune antagonists enable DENV to circumvent the host

immune system [43, 44]. NS5, for example, has the ability to inhibit type I interferon (IFN) signaling by binding to STAT2, which is a key component of the IFN pathway [43].

## 1.4 Role of Host Factors in Dengue Disease Severity

### Immune Responses to Dengue Virus Infection

Although DENVs encode human immune system antagonists, as a whole, immune responses are typically efficient enough to clear DENV infection. Dendritic cells (DCs) residing in the skin are thought to be the first line of defense against invading DENVs [45]. DCs become activated and produce tumor necrosis factor alpha (TNFα) and IFNα [46]. Generally speaking, IFN responses to DENV infection are robust as evidenced by upregulation of IFN-related genes in DENV-infected cells [47-51]. In several cell types, type I IFNs, IFNα [52] and IFNβ [53], play a role in controlling DENV early in infection. Additionally, natural killer (NK) cells have been shown to induce proinflammatory type II IFN production and lyse DENV-infected cells [54].

Innate immune activation initiates an adaptive immune response to dengue infection. Antigen processing and presentation is an integral part of adaptive immunity. DCs are essential in trafficking DENVs to the lymphatic system, from where the virus then spreads to other cell types. Macrophages (mθ) process and present DENV antigen to T and B lymphocytes (T cells and B cells) [55-57]. Antigens stimulate CD4+ T cells to produce IFNγ [58]. Strong CD8+ T cell responses are stimulated by DENV infection as well [59-61]. Upon seeing antigen, B cells clonally expand to produce DENV-specific antibodies [55, 56], which are discussed later in this chapter.

### Immunopathology of Dengue Virus Infection

Immune responses are tailored to defend against invading pathogens, but in the case of DENV infection, immunopathology can play a key role in facilitating severe disease outcomes. Protection from or progression towards dengue disease is essentially a competition between protective versus pathogenic immune responses, respectively. As aforementioned, cytokines are instrumental components of the initial immune response to DENV infection, but

dysregulation of cytokine responses, namely “cytokine storm”, correlates with severe dengue disease. Comprehensively, analysis of cytokine profiles in dengue patients reveals many cytokines, including IFNγ, TNFα, and several interleukins (ILs), are associated with dengue disease severity (reviewed in [62]). One of the more recent of these studies showed that MIP1β, IP-10, and MCP-1, which are all associated with inflammatory responses, are higher in patients with dengue with warning signs compared to patients with dengue without warning signs [63].

Cross-reactive CD8+ T cells are also correlated with DHF presence [59]. One mechanism of T cell immunopathology is decreased degranulation [64], and another is cytokine induction [64]. The standing theory is that cross-reactive T cells produce elevated levels of cytokines, and subsequently, cytokine signaling contributes to vascular leakage, which is a classic symptom associated with DHF and DSS [64]. Complement, which is typically thought of as a viral clearance mechanism, is insinuated in inducing vascular leakage as well. While a helpful role for complement was shown in one study where DHF was associated with a reduction in complement components [65], other studies suggest an immunopathological role for complement. DENV NS1, an activator of complement signaling, and other key complement components are increased in fluids of DHF and DSS patients compared to DF patients [66, 67]. Also, *in vitro*, complement activation can enhance DENV uptake and viremia in myeloid cells [68].

## 1.5 Human Antibody Response to Dengue Virus

### Role of Antibodies in Enhancement of Dengue Disease

From the host’s perspective, the most significant risk factor for severe disease is previous exposure to DENV. Because DENVs circulate in waves of four serotypes, individuals are susceptible to multiple infections over a lifetime. A first infection is considered primary (1°) infection; repeat DENV infections are considered secondary (2°), tertiary (3°), and quaternary (4°). Secondary infections have long been correlated with more DHF and DSS than 1° infections [36, 69-73]. Studies with human monoclonal antibodies (huMAbs) show that a large proportion

of DENV antibodies induced after 1° infection are weakly neutralizing but cross-reactive [74-77]. Cross-reactive, sub-neutralizing DENV antibodies are thought to induce severe disease through a phenomenon known as antibody dependent enhancement (ADE) ([78] | reviewed in [79] and [80]) (**figure 1.4**).

Serotype cross-reactive antibodies have been mapped to NS1, prM, and E [81-84]. These weakly neutralizing cross-reactive antibodies bind to DENVs (**figure 1.4A**), and antibody-bound DENV particles then bind to Fc-gamma receptors, FcγRI and FcγRII, on appropriate moncocytic cells [85] (**figure 1.4B**). ADE via FcγRII can be tricky because isoforms FcγRIIa and FcγRIIb, which are found on most DENV target cells, have stimulatory and inhibitory effects on DENV ADE, respectively [86, 87]. Entry via ADE is thought to occur in one of two fashions: (1) endocytosis, similar to the classical DENV entry pathway described above, or (2) phagocytosis, which occurs when aggregates of antibody-bound DENV particles sit atop a cell (**figure 1.4C**). In all, DENVs are engulfed by host cells thus increasing viral burden (extrinsic ADE) (**figure 1.4E**) and inducing cellular changes (**figure 1.4D**) that result in increased viral production (intrinsic ADE) (**figure 1.4F**).

*In vitro* studies suggest extrinsic ADE can result in upwards of a 1000-fold increase in virus infection [88]. Increased DENV infection by ADE has been proven repeatedly in multiple cell systems, including human-isolated mθ, monocytes, and DCs, using huMAbs [89], mouse MAbs [90], and serum from DENV-infected individuals [91]. ADE may be a more effective method of infection due to the ability of immature DENV particles, which are noninfectious via classical receptor-mediated pathways, to be bound by prM-specific antibodies and taken up by FcγRs [19, 81]. DENV infection via ADE pathways dramatically changes innate immune responses. Most notably, ADE has been correlated with cytokine storm [92-94] and complement activation, both of which were discussed previously [65-67]. Effects of DENV ADE on IFN regulation are controversial, as some studies cite increases [95] and other studies cite decreases [96, 97] in IFN production following ADE. TLRs are instrumental in sensing

pathogens and inducing cytokine and IFN production. DENV ADE may affect IFN production by reducing toll-like receptor (TLR) expression and signaling. [94]. Overall, the likely effect of intrinsic ADE is increased viral replication and progeny virus output per infected cell.

**Role of Antibodies in Protection of Dengue Disease**

Similar to other DENV immune responses, antibody responses can be either pathogenic (enhancing), as discussed in detail above, or protective (neutralizing). The DENV neutralizing antibody response consists of type-specific antibodies and cross-neutralizing antibodies. Dogma would have it that these antibodies facilitate protection from severe disease upon 2° infection with a homotypic or heterotypic DENV serotype, respectively. Early in the quest to dissect DENV antibody epitopes, mouse MAbs revealed a large portion of the DENV antibody response was directed to EDIII [98]. However, more recent mapping studies, using huMAbs and polyclonal human sera, show human antibody responses to DENV are different and more complex. In fact, human antibodies recognizing EDIII are relatively sparse and furthermore play only a minute role in DENV neutralization [99].

While strongly neutralizing mouse MAbs bind to recombinant E (rE) protein, strongly neutralizing huMAbs only recognize regions on intact DENV virions [77]. That finding was one of first to suggest a role for complex epitopes recognized by DENV neutralizing antibodies; since then more effort has been spent in understanding epitope specificity of DENV neutralizing antibodies. Strongly neutralizing huMAbs exerted antibody pressure on DENV in cell culture to render neutralization escape mutations around DENV EDI/II [77]. In studies of DENV1 vaccine recipients, it was found that amino acid mutations in EDI and EDII together were responsible for a type-specific antibody response [100, 101]. Structural analysis revealed a type-specific DENV1 neutralizing antibody epitope that spans the EDI/II hinge region of E monomers [102]. This region may be relevant to long-term type-specific DENV antibody response, as evidenced in an elegant EDI/II hinge transplant study [103].

Antibodies that confer potent neutralization across multiple DENV serotypes are less understood than type-specific antibodies. A few huMAbs that have potent broad neutralization capacity have been mapped to the FL region of E [104]. A potent broadly neutralizing huMAb that recognizes a region next to FL known as the “bc loop of EDII” was recently identified. However, FL-specific antibodies are typically weakly neutralizing, and, in fact, can confer enhancing activity [105]. The role for FL-specific antibodies in pathogenesis and/or protection is questionable, but a study using samples from a Nicaraguan cohort provides evidence that anti-FL antibodies generated from 1° DENV infections correlate with protection against 2° heterologous infections [106].

**Temporal Regulation of Dengue Antibodies**

One might imagine that the balance between pathogenic and protective DENV antibody responses is largely dependent on temporal regulation of antibodies following DENV infections. The field’s current understanding of antibody timing following DENV infections is summarized in **figure 1.5** [36, 107, 108]. Upon 1° DENV infection, a person may begin to show symptoms 4-10 days after inoculation. In the symptomatic or acute phase of illness, a person can exhibit viremia for up to 7 days; typical viremia lasts 5 days, peaking at day 3 of illness [36]. Length and magnitude of viremia is shortened during 2° infections [36, 107]. NS1 antigen circulates in the blood stream during the viremic period and is often used as a quick and easy method for acute diagnosis of DENV infection [109-111].

IgG antibodies following 1° infection begin to appear after viremia wanes, at about day five post illness onset, and remain for a lifetime. Upon 2° infection, one is thought to experience a boost in IgG antibody levels that remains steady forever. IgM is more robust than IgG immediately following 1° infection and quickly wane to undetectable levels by 3-6 months [112]. IgM levels following 2° infection are not as robust and are even shorter lived than those following 1° infection [113, 114]. IgA and IgE antibodies have been detected following both

primary and secondary DENV infections, but temporal regulation and biological significance of these antibody subtypes remain unclear [114].

More important than the timing of various types of antibodies is the timing of antibodies as they relate to pathogenic and protective disease outcomes. To that end, studies are being conducted to dissect temporal regulation of enhancing and protective antibody responses. Primary DENV infection with one serotype is dogmatically believed to lead to lifelong protective immunity to the infecting (homologous) serotype, but dengue-immune individuals remain susceptible to 2° infection with a different (heterologous) serotype [115]. It is thought that 2° infections induce antibody responses that are broadly-neutralizing and likely protective against post-secondary infections [116]. Even a 2° antibody response may not be completely protective because 3° and 4° infections, although rare, have been documented [70, 116-118].

Some of the more telling research on how long protective antibody responses last was conducted in the 1950s; Sabin infected humans with DENV and challenged them with either a homologous or heterologous serotype. That study pronounced the period of type-specific protection to be at least 18 months while the period of cross-protection was only about 2 months [119]. Sabin's last timepoint was 18 months, but studies have shown type-specific neutralization lasts up to 60 years post-infection [120]. Sequential infections in Thai and Nicaraguan cohorts reveal that 2° inapparent infections are more likely to occur within a shorter time span following 1° infection than 2° apparent infections. Together, these studies determined the period of cross-protection to be about 2 years [118, 121]. Perhaps, it is after 2 years that concentrations of weakly neutralizing cross-reactive antibodies decline and enhancement begins to occur [87].

## 1.6 Advantages of Studying Dengue Virus Infections in Human Cohorts

### Mouse Models for Dengue Virus Pathogenesis

Studying vaccine-induced immunity and dengue pathogenesis in a small animal model has been difficult (reviewed in [122]). Mice infected with mouse-adapted DENV strains do not typically show classical signs of human disease. In the early stages of mouse model

development, neurological effects and paralysis, which are not clinical symptoms of human dengue disease, were seen in DENV-infected animals [123, 124]. BALBc and C57BL/6 mice have shown human-like DHF symptoms, but only after being inoculated with high doses of DENV [125]. In studies of DENV pathogenesis, humanized mice have a stark advantage over other mouse models because their cell tropism is similar to that of an infected human [126], and their disease presentation is similar to that of human DF [126, 127]. ADE has been shown multiple times in IFNI/II receptor-deficient AG129 mice [128, 129]. In the AG129 model, protection from ADE-induced disease is dependent on neutralizing antibodies [128]. In regards to antibody responses, it is important to restate that antibody responses in mice are drastically different than in humans [79, 99]. Furthermore, temporal regulation of DENV antibody responses and sequential DENV infections cannot be studied in mice due to their short life spans.

**Use of Non-human Primates in Vaccine-Related Studies**

Despite aforementioned pitfalls, mice are still a telling model for preliminary vaccine studies, especially because many mouse models exhibit disease symptoms that can be used as suitable protection endpoints [123, 124]. For advanced vaccine-related studies, it is imperative that disease manifestation and antibody responses resemble those seen in humans'. DENV pathogenesis is less well-studied in non-human primates (NHP) because they do not develop human-like clinical symptoms. However, early on DENV-infected Rhesus macaques were shown to have similar viremia and antibody responses as humans [130]. Before introduction into humans, DENV vaccines are often tested in NHPs [122, 131-136], and NHPs have proven rather useful in predicting vaccine-induced immunogenicity.

**Lessons Learned from Human Cohort Studies**

With knowledge that antibody responses in mice [79, 99] and disease pathology in mice and NHPs alike are drastically different than humans', it is ideal to study DENV pathogenesis in humans [137]. Over the years, several prospective dengue cohort studies have been

completed (**Table 1.2**). The lessons that cohort studies have taught the dengue field are immense (reviewed in [138]). Using samples collected from cohort studies, it has been shown that severe dengue disease correlates with several biological factors, such as age, and clinical outcomes, such as platelet count [139-143]. Results from cohort studies [144] were even influential in WHO's reevaluation of clinically apparent dengue symptomologies [145-148].

Additionally, researchers are making progress with understanding DENV antibody responses in human cohorts. Cohorts in Thailand were the first to show previous DENV infection is a risk factor for severe disease [69, 70]. Later, several cohorts followed suit with similar findings [78, 128, 149-152]. Also, maternal antibodies have been correlated with severe dengue disease in Vietnamese infants [153, 154]. Together, these findings justify the theory of ADE in naturally infected people. As far as protective antibody responses are concerned, determining the natural correlate of protection from DENV infections and/or disease is a continuing challenge for the vaccine field. As previously mentioned, cohort studies provided us with the first evidence of clade-induced shifts in DENV immunity [32, 38-40]. In Thai cohorts, pre-existing neutralization titers did not correlate with protection from DENV infection with the same serotype, as evidenced by clinical symptoms and viremia [152, 155], but Thai cluster studies show that neutralization titer does correlate with protection from DENV infection [*Buddhar, Darunee, et al.* | *unpublished data submitted to PLoS Neglected Tropical Diseases*]. In a cohort in Peru, pre-existing neutralization breath correlated with protection from apparent post-secondary DENV infections [116]. Cross-protection following natural primary infections was determined to last about 2 years in Thai and Nicaraguan cohorts [118, 121].

## 1.7 Dengue Vaccine Development

In the wake of dengue emerging as a serious emerging global health problem, development of a DENV vaccine is urgently needed. However, dengue vaccine development has proven difficult. There are several criteria that a successful DENV vaccine must meet. Although young children are thought to be the target vaccine population, adults are still

susceptible to 3° and 4° DENV infections. Therefore, an ideal DENV vaccine should target a spectrum of age groups. Also, given the widespread circulation of DENV within poor nations, a DENV vaccine must be affordable. Most importantly, serotype-unbalanced vaccine responses run the risk of facilitating ADE upon natural challenge so an effective DENV vaccine must induce long-lived cross-protective responses against all DENV serotypes. Together these criteria present a vaccine development feat that, despite intense research efforts, has not been obtainable. To date, there is no DENV vaccine available to humans.

**Prospective Dengue Virus Vaccine Candidates**

Various approaches have been taken to develop a dengue vaccine. Subunit, subviral particles, or nonliving whole virus vaccines represent the safest class of DENV vaccines. There are a couple of nonliving DENV vaccines that stand out as potential vaccine candidates. The first is a tetravalent DNA vaccine that expresses DENV1-4 prM/E constructs [133], and the second is a tetravalent rE subunit vaccine [132]. These types of vaccines typically induce subpar immunogenicity and require combination with adjuvants and viral vectors to provide an immune boost [156, 157]. Live-attenuated or recombinant DENV vaccines have been tested and generally show stronger, longer-lived neutralizing antibody responses than nonliving counterparts. Dengue virus can be attenuated via serial passage in cell culture so the virus no longer induces disease-causing infection but still induces an effective immune response. The efficacy of live attenuated DENV as a vaccine strategy was first tested by administering attenuated DENV2 to naïve individuals. In that small trial, DENV2-specific neutralizing antibody responses lasted for up to 2 years post-vaccination [158]. Live-attenuated DENV vaccines have evolved since then into tetravalent recombinant formulas administered at various prime/boost timepoints [131, 134, 159-177].

There are three DENV live-attenuated vaccines (LAV) that stand out as promising human vaccine candidates. Steve Whitehead's group has developed a tetravalent attenuated vaccine strategy, namely "TDV", which consists of DENV prM/E from DENV1-3 inserted into an

attenuated DENV4 background. In human trial, vaccinees were challenged with DENV1 or DENV3 and were completely protected or partially protected, respectively [178]. DENVax is a LAV that has a DENV2 backbone with prM/E from the other serotypes inserted. In nonhuman primates, DENVax showed complete protection against DENV3 and DENV4 challenge, but only partial protection against DENV1 and DENV2 [135]. Recently completed human trials revealed DENVax induced broadly neutralizing antibody responses 30 days following inoculation [160]. Lastly is Sanofi Pasteur's version of the DENV vaccine, CYD-TDV, which was thought to be the most promising candidate based on pre-phase IIB studies [179]. The chimeric tetravalent formulation consists of each of DENV1-4 prM/E inserted into the yellow fever vaccine backbone. Unfortunately, this vaccine showed only showed ~30-70% protection against natural DENV challenge in clinical trials, depending on serotype [180-187], and was notably deficient in protecting against DENV2 infection in a Thai trial [186].

## 1.8 Outline of this Dissertation Research

### Research Questions

With levels of protection being lower than predicted with the leading DENV vaccine [180-187], the field was reminded that there are fundamental aspects of protective responses that we do not understand. Natural DENV infection protects most people against severe secondary infection so using protective immune responses to natural infection as a gold-standard may prove beneficial to the vaccine field. Three overarching questions exist about human antibody responses to DENV infection:

(1) What are the differences between antibody responses induced by primary versus secondary infections?

(2) What role do enhancing antibodies play in disease severity?

(3) What role do neutralizing antibodies play in protection from dengue disease?

**Research Approach**

To that end, the overall goal of this dissertation was to characterize the role of antibodies in natural inapparent and apparent dengue infections. Based on knowledge that a balance of pathogenic and protective antibody responses can influence the severity of dengue disease, ***we hypothesize that distinct properties of pre-existing antibodies correlate with inapparent and apparent dengue disease outcome upon secondary infection*.** In collaboration with the Pediatric Dengue Vaccine Initiative (PDVI) and investigators from the Ministry of Health in Sri Lanka, our group completed a prospective dengue study of 799 children, ages 0-12 years, who reside in Colombo, Sri Lanka. Using samples collected from the cohort, we addressed the following specific aims:

**Specific Aim 1** (Chapter Two). **Determine the incidence of DENV infection in cohort.** We set out to characterize the true burden of DENV infection, including inapparent cases, on the Indian subcontinent for the first time.

**Specific Aim 2** (Chapter Three). **Investigate temporal regulation of antibodies following DENV infections.**

- **2A.** Compare quantity and neutralization capacity of antibodies acquired from 1° inapparent and apparent DENV infections.
- **2B.** Determine how time post-infection influences antibody neutralization capacity following 1° infections.
- **2C.** Characterize quantity and quality of antibodies following repeat DENV infections.

**Specific Aim 3**. **Investigate qualities of pre-existing antibodies from repeat inapparent and apparent DENV infections.**

- **3A.** (Chapter Four) Compare quantity, neutralization capacity, and enhancement ability of pre-infection antibodies from repeat inapparent and apparent DENV infections.

- **3B.** (Future Directions) Dissect infection history of children who acquired repeat DENV infection.

**Innovation**

Our study is innovative because most previous studies have only detailed differences between mild DF cases and severe DHF/DSS cases because they are hospital-based studies that enroll patients upon acquisition of symptoms [69, 139, 140]. Given the complexity and cost of cohort studies, very few groups have pre- and post-infection blood samples from both inapparent and apparent cases. Even for studies that have been able to characterize inapparent infections, many have not specifically differentiated between 1° and repeat silent infections in their analyses [140, 141]. The findings that I detail in the following chapters will likely change the way the dengue field thinks about DENV antibody responses to natural infections in ways that will impact future dengue vaccine development.

**Table 1.1. World Health Organization Dengue Case Classifications**

| | 1997 World Health Organization Dengue Case Classifications [34] | | | |
|---|---|---|---|---|
| Case Classification | **Asymptomatic (Inapparent)** | **Dengue Fever (DF)** | **Dengue Hemorrhagic Fever (DHF)** | **Dengue Shock Syndrome (DSS)** |
| Clinical Manifestations | • no symptoms | • fever<br>AND ≥2 of the following…<br>• retroorbital or ocular pain<br>• headache<br>• rash<br>• myalgia<br>• arthralgia<br>• leukopenia<br>• hemorrhagic manifestations not meeting DHF criteria | ALL of the following…<br>• fever (2-7 days)<br>• hemorrhagic manifestations (positive tourniquet test or purpura/ecchymosis or epistaxis or gum bleeding or blood in vomit, urine or stool or vaginal bleeding)<br>• thrombocytopenia<br>• plasma leakage or pleural effusion or ascites or hypoproteinemia | • rapid and weak pulse and narrow pulse pressure<br>OR…<br>• hypertension and cold, clammy skin and restlessness |
| Other possible Symptoms | | • anorexia<br>• nausea<br>• abdominal pain<br>• persistent vomiting | | |
| **2009 World Health Organization Dengue Case Classifications [35]** | | | | |
| | **Asymptomatic (Inapparent)** | **Dengue without Warning Signs** | **Dengue with Warning Signs** | **Severe Dengue** |
| Clinical Manifestations | • no symptoms | • fever<br>AND ≥2 of the following…<br>• aches and pain<br>• nausea, vomiting<br>• rash<br>• positive tourniquet test | Dengue without warning signs<br>AND ANY of the following…<br>• abdominal pain<br>• fluid accumulation<br>• mucosal bleeding<br>• restlessness<br>• liver enlargement<br>• decrease in platelet count | Dengue with ≥1 of the following…<br>• plasma leakage leading to DSS or fluid accumulation with respiratory distress<br>• severe bleeding<br>• severe organ involvement (liver \| CNS – impaired consciousness \| heart or organ failure) |

**Table 1.2. List of Prospective Dengue Cohort Studies**[1]

| Study site | Population size[a] | Age range (years) | Study period | Incidence (average) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Dengue infection (%) | Symptomatic dengue | Hospitalized dengue | Severe dengue | Symptomatic: asymptomatic ratio |
| Rayong, Thailand (2) | 1,056 | 4–14 | 1980–1981 | 39.4 | n/a[b] | 0.7% | 0.7% | n/a |
| Bangkok, Thailand (3) | 1,757 | 4–16 | 1980–1981 | 11.8 | 0.7% | 0.4% | 0.4% | 1:8 |
| Yangon, Myanmar (4) | 12,489 | 1–9 | 1984–1988 | 5.1 | n/a | 0.3% | 0.2% | n/a |
| Yogyakarta, Indonesia (5) | 1,837 | 4–9 | 1995–1996 | 29.2 | 0.6% | 0.4% | 0.4% | n/a |
| Kamphaeng Phet I, Thailand (6) | 2,119 | 7–11 | 1998–2002 | 7.3 | 3.9% | 1.0% | 0.6% | 1:0.9 |
| Iquitos, Peru (7) | 2,300 | 5–20 | 1999–2005 | 34.5 | n/a | n/a | n/a | n/a |
| West Java, Indonesia (8) | 2,536 | 18–66 | 2000–2002 | 7.4 | 1.8% | 0.1% | 0.1% | 1:3 |
| Managua, Nicaragua (9, 10) | 1,186 | 4–16 | 2001–2002 | 9.0 | 0.85% | n/a | n/a | 1:13–1:6 |
| Maracay, Venezuela (11) | 981 | 5–13 | 2001–2002 | 16.9 | n/a | n/a | n/a | n/a |
| Kamphaeng Phet II, Thailand (12) | 2,095 | 4–16 | 2004–2006 | 6.7 | 2.2% | 0.5% | 0.1% | 1:3.0 |
| Ratchaburi, Thailand (13, 14) | 3,015 | 3–11 | 2006–2009 | 3.6 | 3.6% | 1.6% | 0.3% | n/a |
| Managua, Nicaragua (10) | 3,800 | 2–9 | 2004–2010 | 9.0 | 0.85% | n/a | n/a | 1:13–1:6 |
| Long Xuyen, Vietnam (15) | 2,190 | 2–15 | 2004–2007 | 3.0 | 3.0% | 1.2% | 1.2% | n/a |
| Southeast Asia (Indonesia, Malaysia, Philippines, Thailand, and Vietnam (16) | 1,500 | 2–14 | 2010–2011 | 11.4 | n/a | n/a | n/a | n/a |

[a] *Number in cohort tested for dengue antibody (incidence denominator).*

[b] *n/a, not available; not provided in the published paper.*

[1]This table was taken from 138. **Endy, Timothy P**, *Human Immune Responses to Dengue Virus Infection: lessons learned from prospective cohort studies.* Front Immunol, **2014**. 5.

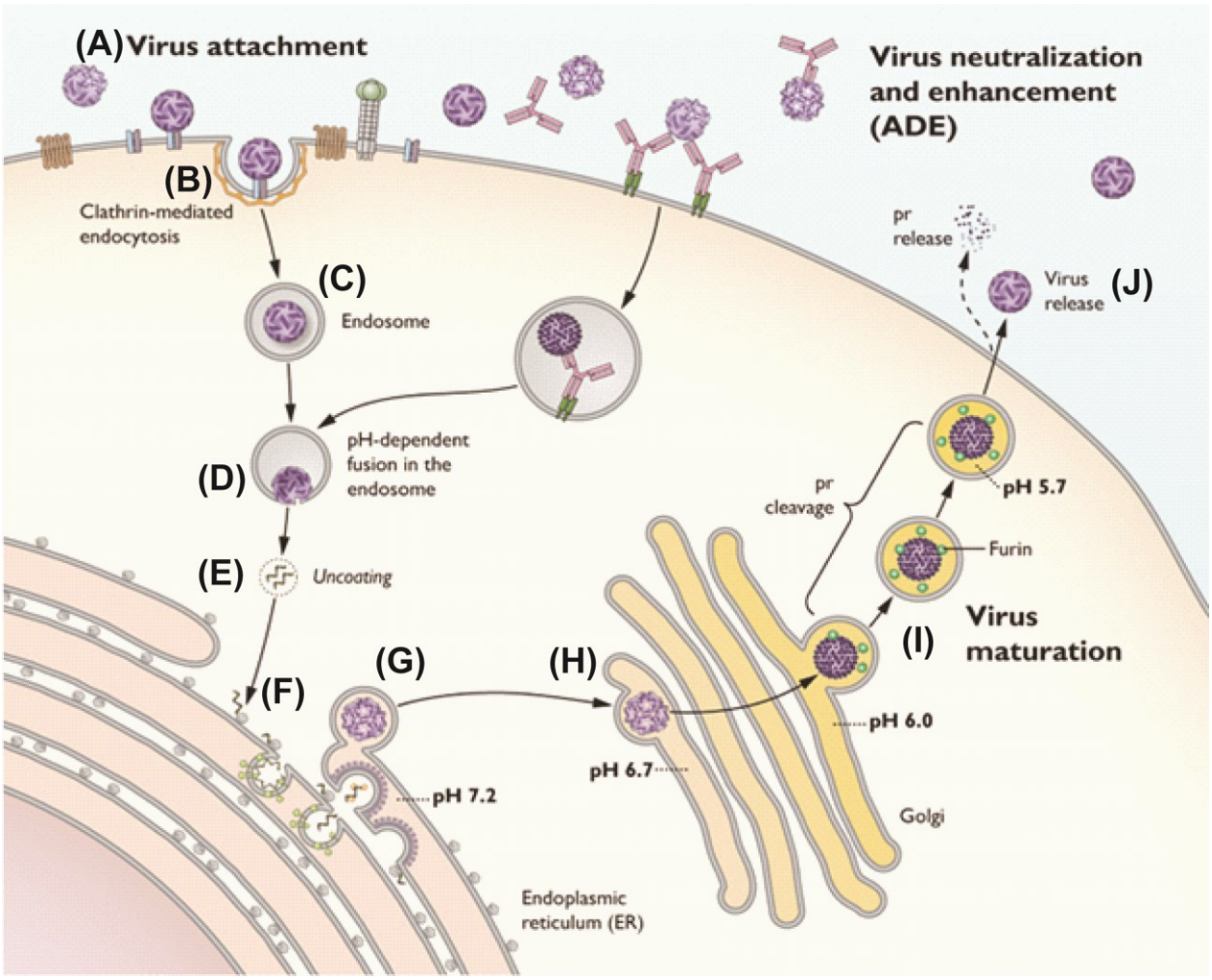


**Figure 1.1. Dengue virus infectious life cycle.** (A) DENV attaches to cells via appropriate receptors and/or co-receptors, and is (B) internalized via clathrin-mediated endocytosis. (C) DENV travels in an early endosome, which matures into a late endosome. (D) pH changes in the late endosome induce DENV envelope conformational changes and the viral and host-cell membranes fuse. (E) DENV nucleocapsid exits into cell cytoplasm, and vRNA is uncoated. (F) vRNA travels to the rough ER where polyprotein processing and RNA synthesis and replication occur. RNA is encapsulated, and (G) an immature DENV particle, with prM/E heterodimers assembles in the ER. (H) Immature DENVs travel to the TGN and undergo (I) viral maturation; prM is cleaved by furin. (J) Lastly, mature virions are released from the host cell. (This figure was adapted from [188].)

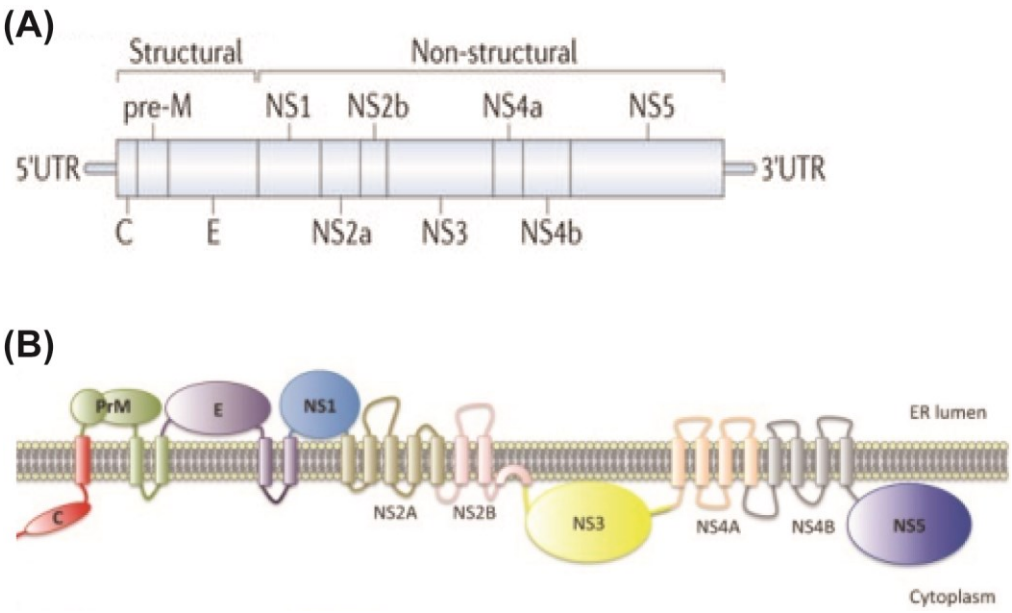


**Figure 1.2. Dengue virus genome and polyprotein.** (A) DENV RNA genome is (B) translated and processed as a polyprotein at the ER. ((A) was adapted from [189]. (B) was adapted from [190].)

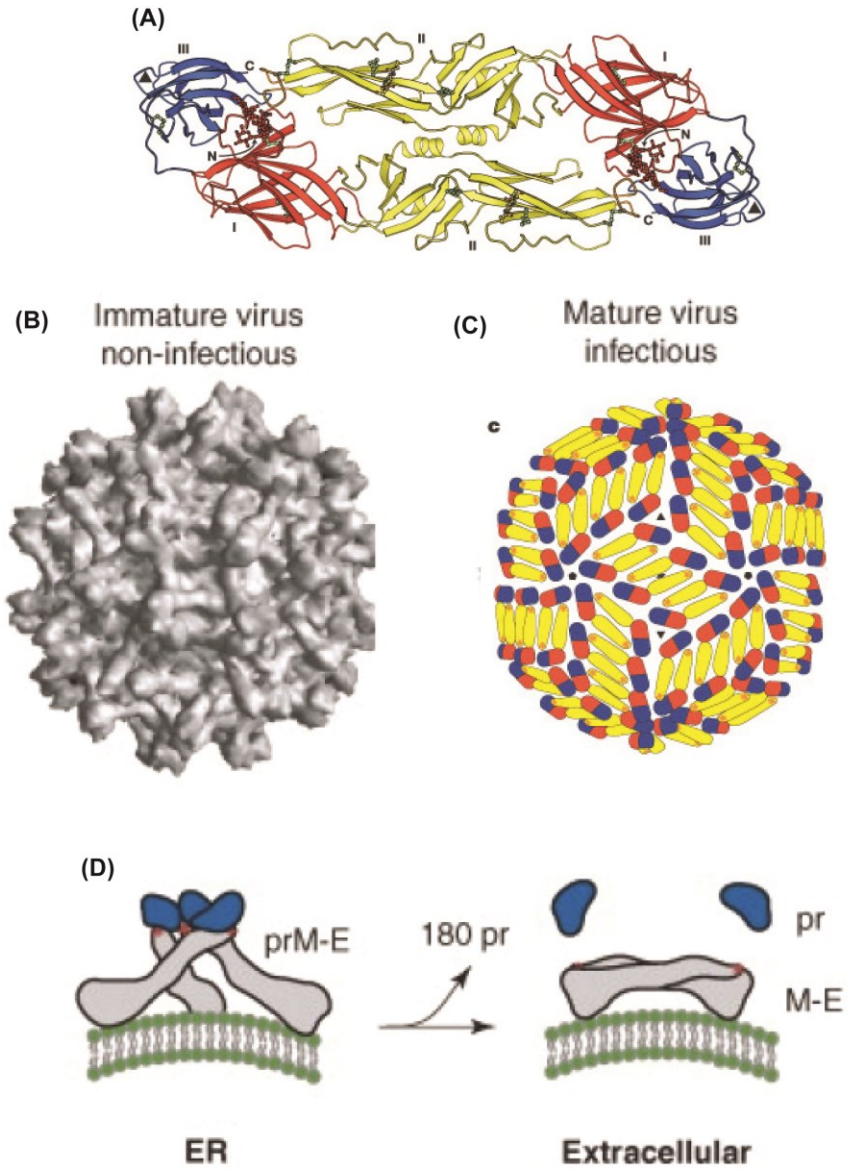


**Figure 1.3. DENV virion structure**. (A) DENV E consists of three domains, EDI (red), EDII (yellow), EDIII (blue) and a fusion peptide. (B) prM/E heterodimers are organized on the surface of an immature DENV particle, creating a jagged appearance. (C) Ninety homodimers of E are organized flatly on the surface of a mature DENV particle, creating a smooth appearance. (D) Immature DENV particles are assembled in the ER and matured in the TGN. Mature DENV particles, and pr peptides, are excreted from the host cell. ((A) and (C) were adapted from [14]. (B) and (D) were adapted from [79].)

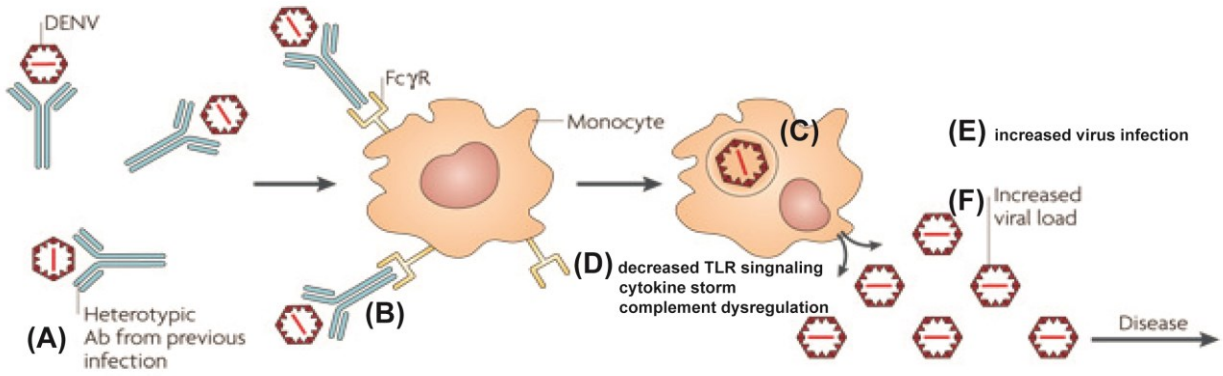


**Figure 1.4. Antibody dependent enhancement of dengue virus.** Upon secondary dengue infection, (A) heterotypic weakly-neutralizing antibodies from previous infection bind to DENV particles. (B) Antibody-bound DENVs are attached to monocytic cells via Fcγ receptors. (C) DENVs are endocytosed or phagocytized. (D) Various internal cellular processes are dysregulated. This entire process results in (E) increased virus infection (extrinsic ADE) and (F) increased viral load (intrinsic ADE). (This figure was adapted from [191].)

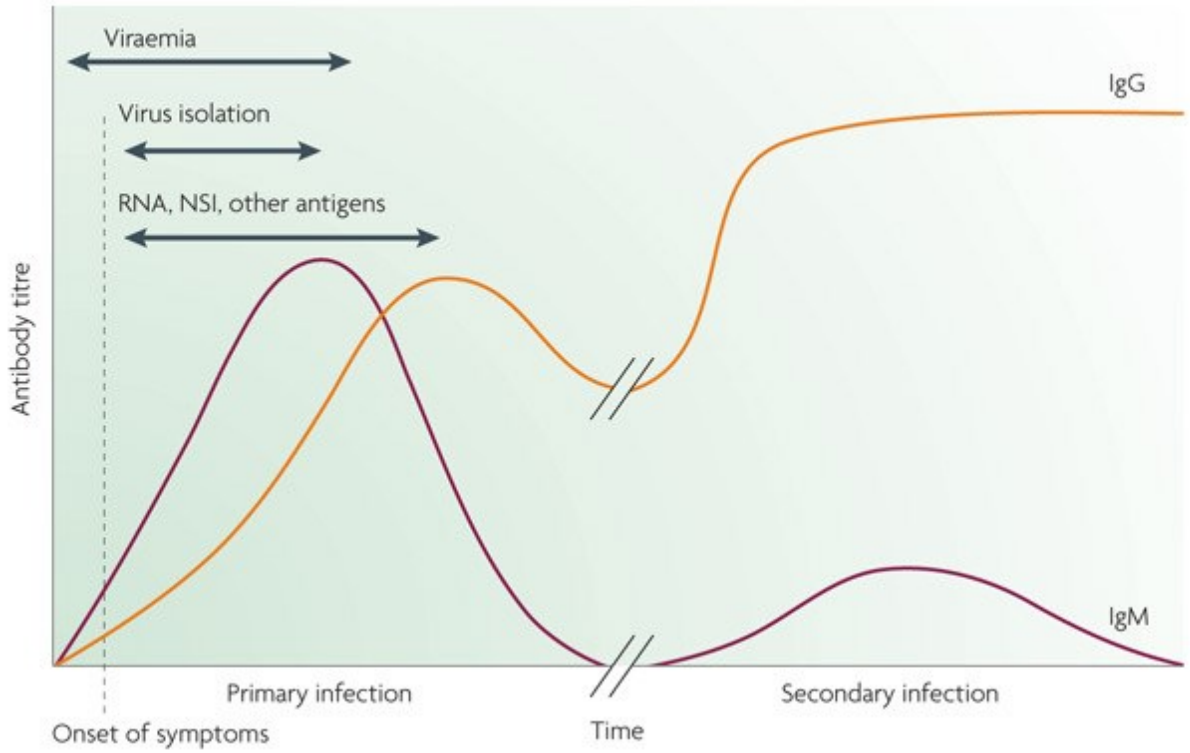


**Figure 1.5. Temporal regulation of antibodies following DENV infection.** (This figure was adapted from [192].) A person can exhibit viremia for up to 7 days [36], although length and magnitude of viremia is shortened during 2° infections [36, 107]. NS1 antigen circulates in the blood stream during the viremic period [109-111]. IgG antibodies (yellow line) following 1° infection begin to appear at about day five post illness onset and remain for a lifetime. Upon 2° infection, one is thought to experience a boost in IgG antibody levels that remains steady forever. IgM (red line) is more robust than IgG immediately following 1° infection and quickly wane to undetectable levels by 3-6 months [112]. IgM levels following 2° infection are not as robust and are even shorter lived than those following 1° infection [113, 114].

# CHAPTER TWO

## Burden of Dengue Infection and Disease in a Pediatric Cohort in Urban Sri Lanka[2 3 4]

### 2.1 Introduction

Dengue is caused by four related mosquito-borne dengue viruses (DENV1-4), which are endemic in many tropical and subtropical regions of the world. Most individuals infected with DENV are asymptomatic or develop a febrile illness known as dengue fever (DF), but in a minority, disease can be more severe and progress to dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). Infection with one DENV serotype confers protective immunity to future infections with that serotype only and these individuals are susceptible to secondary infections with heterologous serotypes [79]. Individuals suffering from secondary DENV infections are at a greater risk of developing DHF/DSS compared to individuals experiencing their first infection [189, 193].

Globally, an estimated 2.5 billion people are at risk for DENV infection, with estimated 390 million annual DENV infections and 96 million dengue cases [28]. From prospective community and school based cohort studies, it has been possible to obtain estimates for the

[2]This chapter was previously published. | **Tissera H, Amarasinghe A, de Silva A.D., Palihawadana P, Kariyawasam P, Gunasena S, Corbett K.S., Katzelnick L, Tam C, Letson G.W., Margolis H.S., and de Silva A.M.**, *Burden of dengue infection in a pediatric cohort in urban Sri Lanka.* **American Journal of Tropical Medicine and Hygiene**, 2014.

[3]Portions of this chapter are in press. | **Corbett K.S., Katzelnick L, Tissera H, Amarasinghe A, de Silva A.D., and de Silva A.M.**, *Pre-existing Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort.* **Journal of Infectious Diseases**.

[4]This study was funded by the Pediatric Dengue Vaccine Initiative (Bill and Melinda Gates Foundation Grant #: 23197). Additional funding was provided by UNC Virology Training Grant (NIH Grant #: T32AI007419) and Initiative for Minority Student Development (NIH Grant #: 5R25GM055336).

incidence of DENV infection and disease for sites in southeast Asia [141, 194-198] and Latin America [199-201]. Although dengue is emerging in the Indian subcontinent and is considered a major health issue, we do not have estimates of the true burden of DENV infection and disease in this region.

Dengue was first reported in Sri Lanka in the 1960s [29]. Studies conducted from 1980-1984 showed a DENV seroprevalence of 50% among school children, with an annual seroconversion rate of 10-15% among 5−7-year-old school children in Colombo, the capital of Sri Lanka [39, 202]. More recently, analysis of age-stratified seroprevalence data indicated that the annual seroconversion rate among children <12 years in Colombo is around 14% [202]. In the past, most dengue cases have been reported from the Colombo district and other neighboring districts in the heavily urbanized southwestern region of the country. However, over the past 10-15 years dengue has been reported from nearly all districts of the island, and over the past two decades, the number of reported DF and DHF cases has increased by over 10-fold [31]. This increase in cases has been attributed to introduction of new genotypes of DENV as well the expansion of the range of the virus on the island [31-33]. In many ways, the changing epidemiology of dengue in Sri Lanka mirrors events in other parts of the subcontinent including India, which has also documented large increases in cases and appearance of new virus strains [203, 204]. We conducted a population-based study to determine the incidence of symptomatic and asymptomatic DENV infection among children living in Colombo, Sri Lanka, an urban setting of the Indian subcontinent.

## 2.2 Materials and Methods

### Ethical Approval for Study

Ethical approval for this research was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka and the Institutional Research Board of the International Vaccine Institute, Seoul, Korea. Only the children whose parents or legal guardians provided written informed consent were enrolled in the study.

**Study Site**

The study was conducted in the city of Colombo, the commercial capital of Sri Lanka, which has a population of 647,100 and is the most densely populated area in the country with 17,353 persons per square kilometer [205]. The city is divided into 47 municipal wards and ward 33 was selected for the study because of its stable population, which reflects the socio-economic status and demographics of the entire municipal area. The ward is endemic for dengue with the catchment population seeking healthcare in the tertiary care institution situated within its boundaries (**Figure 2.1**).

**Study Population**

A prospective cohort study was conducted between November 2008 and January 2010. The protocol for the study is described in detail elsewhere [206]. In brief, a house-to-house census was conducted by research assistants to determine the size, socio-demographic information and health-seeking patterns of the permanent resident childhood population $\leq$12 years of age. A representative sample of 800 children $\leq$12 years of age was recruited for the current study. The sample size calculation was based on the ability to detect an annual incidence of dengue of 10% with an absolute precision of 3%. The estimated sample size was then inflated by 30% to allow for possible loss to follow-up during the study period. All children were enrolled in the study following written informed consent from parent/legal guardian and assent from children > 7 years of age. Each participating household was given a thermometer and each child was given an identification card with a unique study number. A febrile illness was defined as a temperature of ≥38°C lasting ≤7 days in any child in the study cohort documented by the parent, research assistant or health care professional. Following assessment and treatment by a designated physician, the research assistants interviewed the patient and parent using a standard case investigation form.

**Sample Collection**

Blood samples were collected from all children at enrollment (Between November 2008-January 2009) and one year after enrollment (between November 2009 and January 2010) by finger prick and stored as blood spots on protein saver card (Whatman & ID Biological systems, USA) [207, 208]. From children with a documented fever, whole blood was collected by venipuncture into EDTA containing tubes. Some of the whole blood was used to prepare dried blood spots for later serological testing. The remaining blood was centrifuged and the plasma used for molecular diagnostic testing. Ten or more days after recovery from fever, convalescent samples were collected by finger prick and stored as blood spots on protein saver cards.

*DENV Strains Used for Laboratory Assays*

The WHO DENV reference strains, i.e. DENV1 West Pac 74, DENV2 S-16803, DENV3 CH54389 and DENV4 TVP-360 were used for preparing antigen and infectious stocks for serological assays. The WHO reference viruses were initially obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD). Infectious stocks of virus were prepared using the C6/36 mosquito cell line and dengue antigens were harvested from Vero cells as previously described [209].

**Molecular Detection of DENV**

Plasma obtained during the acute phase of the febrile illness was tested by reverse transcriptase polymerase chain reaction (RT-PCR) to detect and serotype DENVs as previously described [31].

**Detection of anti-DENV IgM and IgG Antibodies in Dried Blood Spots**

Antibodies were eluted from dried blood spots by submerging the filter paper in PBS and incubating for 37C for 2.5 hrs. The final volume of the eluted antibody was adjusted to obtain a 1:50 or 1:100 dilution of the original blood volume applied to the filter paper. A recent report validated the use of dried blood spots for dengue serology [210].

We performed dengue IgM capture ELISA as described [211], except we used anti-*flavivirus* monoclonal antibody 4G2 followed by enzyme-conjugated goat anti-mouse IgG to detect captured DENV antigen. In brief, 96-well plates were coated (overnight, 4°C) with 100 µL/well (1 ng/µL) of goat anti-human IgM (Sigma, St. Louis, MO, USA) at a concentration of 0.1 mol/L in carbonate buffer (pH 9.6). Plates were washed 3× in Tris-buffered saline with 0.2% Tween 20 (TBST) and blocked with 200 µL/well of 1× Tris-buffered saline with 0.05% Tween 20 and 3% nonfat dry milk. Paired serum samples were tested on the same plate. Diluted serum (1:50) was loaded in duplicate and incubated (37°C, 1 h) to capture IgM antibody. Unbound antibody was washed, and wells were successively incubated with DENV antigen (mix of serotypes DEN1–4), mouse anti-flavivirus 4G2 mAb, and human-absorbed alkaline phosphatase (AP)–conjugated goat anti-mouse IgG antibody (Sigma). Optical density (OD) was measured at 405 nm after final incubation with AP substrate.

Dengue IgG ELISA was performed as described [212]. Plates were coated overnight (4°C) with 100 µL/well of mouse anti-flavivirus 4G2 mAb at a concentration of 0.1 mol/L in carbonate buffer (pH 9.6) and then washed 3× in TBST. Plates were then blocked with standard diluents and successively incubated (37°C, 1 h) with DEN1–4 antigen, diluted serum (1:100) in duplicate wells, and AP-conjugated goat anti-human IgG (Fc portion), with 3 washings (TBST) between incubations. Plates were read at 405 nm after a final incubation with AP substrate (15 min, room temperature, in the dark).

**Measurement of DENV Neutralizing Antibodies**

The presence of DENV neutralizing antibody was determined using a flow cytometry-based neutralization assay with U937 monocytic cells stably transfected with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) as previously described [213]. This flow cytometry based neutralization test has comparable specificity to the conventional dengue plaque reduction neutralization test and does not detect antibodies against Japanese encephalitis virus (Broadwater and de Silva unpublished data) [213]. Blood samples

were serially diluted (4 fold dilutions starting at 1:40 and ending at 1:10,240) and the serum dilution that neutralized 50% of the viruses was calculated by nonlinear, dose-response regression analysis with Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

**Determining Dengue Serostatus at Study Enrollment**

Dengue virus serostatus (DENV naïve or immune) at enrollment was determined by dengue IgG immunoassay. An OD value $\geq$ 0.3 were considered dengue antibody positive. During the study period, primary infections were defined as dengue naïve children at enrollment who experienced an infection. Secondary infections were defined as dengue immune children at enrollment who experienced an infection.

**Criteria for Laboratory Diagnosis of DENV infections**

A laboratory-confirmed clinically apparent dengue case was a child with a febrile illness who tested positive in at least two out of the three diagnostic assays (PCR, rising levels of IgM and/or IgG antibodies in paired acute and convalescent blood samples). In the few cases where there was only an acute blood sample, the diagnosis was based on RT-PCR testing only. Apparent dengue cases were further classified as primary or secondary based on baseline DENV naïve or immune status, respectively.

Inapparent infections were classified as new DENV infections occurring in children who did not have a laboratory-confirmed apparent dengue fever during the study year. To detect primary inapparent infections, paired baseline and end-of-year blood samples from all children were tested by IgG immunoassay. Dengue-naïve children at enrollment (391 children) who seroconverted over the study year (baseline sample seronegative and end-of-year sample seropositive [$\geq$ 0.3 OD units]) were classified as primary infections. DENV-immune children at enrollment (441 children) who displayed rising levels of anti-DENV IgG antibodies by the end of the year ($\geq$ 0.1 OD units) were initially classified as secondary infections. However, gold-standard neutralization testing of these secondary infections (as classified by IgG increases) indicated no neutralization capacity increase over the study year.

Therefore, all children were screened for increased neutralization capacity over the study year by subjecting paired baseline and 12-month follow up samples from all children (at dilutions of 1:40 and 1:640) to a FACS-based neutralization assay against each of the four dengue serotypes (**Table 2.1**). Those children showing an increase in neutralization capacity by 2-dilution screen were then confirmed by neutralization assay [213, 214] with full serum dilution series (**Figure 2.2**). To establish criteria for defining secondary infections using paired samples collected 12 months apart, we used a test set of 8 symptomatic secondary cases (detected by PCR and serology performed on samples collected within 1 month of acute infection). Only half the cases (4/8) cases displayed a 4-fold or greater increase whereas all cases (8/8) displayed a 2-fold or greater increase in neutralizing antibody levels when paired baseline and end-of-year samples from these children were tested. Therefore, children who had $\geq$2 fold increases in levels of neutralizing antibodies to one or more serotypes by the end of the year were designated as secondary infections. Samples were tested at least twice and only paired samples that displayed a reproducible 2 fold-increase were included as new secondary infections. Although it is possible that some inapparent infections were actually dengue fever cases that were missed, efforts were made to ascertain case detection by educating parents and having a study team visit each house weekly [215]. Loss to follow-up was minimal; of 800 children enrolled, only 1 child dropped out during the 12 months [215, 216].

## 2.3 Results

Notably, all DENV cases (inapparent and apparent) in this study were confirmed by neutralization assay [213, 214] with full serum dilution series (**Figure 2.2**). While the DENV neutralization assay is the gold standard assay for detecting new infections by serology, DENV-specific IgG ELISAs are faster and easier to perform than neutralization assays. Paired samples were tested by both IgG ELISA and neutralization test to assess the performance of the IgG assay for detecting new infections using samples collected 12 months apart (**Table 2.2**). While the IgG assay performed well for detecting primary infections (sensitivity of 94.3% and positive

predictive value (PPV) of 76.7%), the assay performed poorly for secondary infections (sensitivity of 62.5% and a PPV of only 29.9%) These results indicate that IgG ELISA is an unreliable method for identifying true DENV infections, particularly 2° infections, when utilizing paired samples collected 12 months apart.

Between November 2008 and February 2009 a total of 800 children ages 0-12 years were enrolled in the study from ward 33 (**Figure 2.1**), these being representative of the age and demographic distribution of the 2527 children known to be permanent resident of the ward. Only 1 child was lost to follow up during the study period. The dengue seroprevalence at enrollment was determined by testing all children by ELISA for the presence of DENV binding IgG antibodies (**Table 2.3**). To confirm that IgG seropositivity was due to dengue infection and not exposure to related Japanese encephalitis virus (JEV), which is also present in Sri Lanka, all IgG positive samples were tested for the presence of DENV neutralizing antibodies. The vast majority (96%) of IgG positive sera also neutralized DENV indicating that in this population the results of the IgG ELISA reflected dengue seroprevalence. The overall dengue seroprevalence was 53% at enrollment (**Table 2.3**). The age specific seroprevalence steadily increased with advancing age from a low of 22% in the youngest (<1 year) age group to a high of 74.26 % in the oldest (10-12 year) age group (**Table 2.3**).

A total of 681 fever episodes were detected among the 799 children indicating that on average each child reported 0.9 fever episodes over one person-year of follow-up (**Table 2.4**). Dengue was laboratory-confirmed in 3.9% (27/681) of the fever episodes (**Table 2.4**); no child had more than one episode of dengue fever over the course of the study. Therefore, the incidence of clinically apparent dengue in the cohort was 3.38 per 100 children per year (**Table 2.4**). When combined with the clinically inapparent infections, the total incidence of DENV infection was 8.39 per 100 children (**Table 2.4**). The highest incidence of infection and disease was in the 1-3 year group. The overall ratio of inapparent to apparent infections was 1.48.

Out of the 65 new apparent and inapparent DENV infections, 34 were primary infections and 31 were secondary infections (**Table 2.5**). As might be expected, the ratio of primary to secondary infections decreased with age from 3 in the youngest (< 1 year) age group to 0.75 in the oldest (10-12) age group (data not shown). The ratios of inapparent to apparent infections were not statistically significantly different (P>0.1) between primary and secondary infections (**Table 2.5**).

The prevalence of each DENV serotype during the study year was estimated by identifying the serotypes responsible for primary infections (**Table 2.6**). DENV2 was the most common serotype (49%) followed by DENV3 (27%), DENV1 (23%) and DENV4 (3%). The number of people with monospecific neutralizing Abs to each serotype at enrollment into the study was used to estimate the prevalence of each serotype in this population before the start of our study (Table S1). This analysis demonstrated that DENV2 (43%) and DENV3 (29%) were more common than DENV1 (9%) and DENV4 (12%) in the period preceding the study.

## 2.4 Discussion

Over the past two decades dengue has emerged as a major health problem in the Indian subcontinent [203, 204]. Dengue epidemics in the region have been linked to more intense transmission, expansion of the range of the virus and introduction of new strains [31-33, 203, 204]. Most estimates of dengue incidence in the region are based on hospital-based studies and nationally reported cases, which grossly underestimate the true burden of disease and infection [217]. In this study, we followed a cohort of 799 children in Colombo, Sri Lanka to gauge the true burden of infection in this region.

A strength of our study is that infections were detected by testing paired baseline and follow-up blood samples by neutralization test. This approach is not feasible with most cohort studies given the difficulty of testing large sample panels using the DENV neutralization test. We overcame this hurdle by using a 96-well format, flow cytometry based, high throughout neutralization assay [213, 214]. We screened baseline and follow-up samples from all enrolled

children at 2 dilutions for the presence of neutralizing antibody before selecting samples for more comprehensive neutralization testing. Our study demonstrates that while simple to perform, IgG ELISA and related assays are unreliable methods for detection of DENV infections when samples are collected 12 months apart, particularly for repeat infections.

Having fully characterized primary and secondary DENV infections in the cohort using neutralization assay, we estimated the incidence of infection and disease to be 8.39 and 3.38 per 100 children respectively. The ratio of clinically inapparent to apparent infections was 1.48 indicating that for every apparent infection there were approximately 1.5 inapparent infections in children. This high intensity of transmission was also supported by the seroprevalence data that demonstrated a gradually rising prevalence with age that ranged from 22% in the youngest age group to 74% in the oldest age group. The 4 DENV serotypes were circulating in this population both before and during our study, with serotypes 2 and 3 being more prevalent than 1 and 4 (**Table 2.6**). Our study establishes a rigorously measured, accurate burden of dengue infection and disease among children living in an urban area of Sri Lanka.

Recently, we used dengue seroprevalence data from this cohort to model the rate of primary infections in dengue naïve children [202]. Using a catalytic model, we estimated the incidence of primary infection to be 14.1% per year (95% CI: 12.7%– 15.6%), which is higher than the incidence 8.39 % (95% CI: 6.56-10.53) observed here for total infections (primary and secondary infections). A more legitimate comparison would be to compare the model based estimate for primary infections with the incidence of primary infections in our cohort. The incidence of primary infection noted in the current study was 9.8% (95% CI: 6.80-12.80), which is closer to the model-based estimate of 14.1% per year (95% CI: 12.7%– 15.6%). A previous study of school children in Colombo over a 5-year period from 1980-1985 estimated the incidence of new infections to range between 10.78 to 18.54 per 100 children in different years [39]. Collectively, these findings demonstrate intense transmission of dengue among children in Colombo.

Our current estimate for disease (3.38 per 100 under 12 years of age population) is 14-fold higher than the reported national incidence of the disease (0.23 per 100 individuals) among <15 years of age in 2010 in Sri Lanka, which is based on the passive national surveillance system (Epidemiology Unit, MoH). Based on national surveillance for 2010, the disease estimates for children less than 15 years in the Colombo district and the Colombo Municipality (study area) (**See Figure 2.1**) were 0.64 and 1.61 per 100 population respectively. Thus, as expected the passive national program underreports the true burden of disease in Sri Lanka. Recent studies in SE Asia have estimated expansion factors of 8.9 and 9.6 when comparing national data with true disease incidence for Thailand and Cambodia respectively [195, 218]. Moreover, a recent study of the global burden of dengue estimated that there are three times as many infections and dengue cases compared to cases reported to the World Health Organization [28].

For most regions in the world with endemic dengue transmission we do not have accurate estimates of infection and disease because of the cost and infrastructure required to conduct cohort studies. The most comprehensive cohort studies on dengue in South East Asia have been done in Thailand where the incidence of infection has ranged between 2 to 15% depending on the year and location of the study [141, 194, 196, 219, 220]. Studies in Puerto Rico, Nicaragua and Peru have documented incidences that range between 1.34-12% depending on the year and location [199, 200]. Thus, the intensity of dengue transmission among children in Colombo (8.14%) is similar to transmission documented in other cohort studies conducted in South East Asia and Latin America. The ratio of inapparent to apparent infections has also been estimated in different regions of the world. In southeast Asia the ratio has ranged from 3:1 to 1:1, whereas in Latin America the ratio has ranged from 3:1 to 18:1, indicating that infections in Southeast Asia [141, 194, 196] are more likely to lead to clinically apparent cases compared to infections in Latin America [199, 200]. The data we report here

indicates that the situation in Colombo Sri Lanka with a ratio of 1.5:1 is more similar to the situation in Southeast Asia.

The current study was not designed to test hypotheses about risk factors for DHF/DSS because our cohort of 799 children is too small to detect significant numbers of DHF/DSS cases. We did not observe any significant differences in the ratio of inapparent to apparent cases between primary and secondary cases. However, it is premature to conclude from these data that risk of developing a clinically apparent infection is the same in primary and secondary cases as the study was only powered to detect a large difference in risk following primary and secondary infection. We do note that other studies have documented that children exposed to primary and secondary infections have a similar risk of developing clinically apparent infections [194].

It is well documented that the incidence of infection and disease can vary from year to year and our data was collected over a single year only. Our study was confined to a relatively small area and our results may not be generalizable to children in other parts of the city and country. Thus, studies such as this have to be conducted in other parts of the country over a longer period to understand how the incidence of infection and disease vary by location and over time. Dengue vaccines are currently being tested in different regions of the world [162]. It is important that trials also be conducted in the Indian subcontinent given the heavy burden of disease and the distinct strains of DENV circulating in the region. The results of the current study will be useful for designing vaccine trials in the region of South Asia and for making decisions about how best to introduce vaccines.

**Table 2.1. Detection of secondary inapparent dengue cases by 2-dilution neutralization screen[5]**

| | | | Neutralization Capacity (% of virus) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | DENV1 | | | DENV2 | | | DENV3 | | | DENV4 | | | Detected by Neut Screen (N) |
| Serum Dilution | | | 50 | 70 | 90 | 50 | 70 | 90 | 50 | 70 | 90 | 50 | 70 | 90 | |
| Not Infected | Baseline | 1:40 | - | - | - | + | + | + | - | - | - | - | - | - | 421 |
| | | 1:640 | - | - | - | - | - | - | - | - | - | - | - | - | |
| | Follow-up | 1:40 | - | - | - | + | + | + | - | - | - | - | - | - | |
| | | 1:640 | - | - | - | - | - | - | - | - | - | - | - | - | |
| Secondary Inapparent | Baseline | 1:40 | + | - | - | + | - | - | + | + | - | - | - | - | 20 |
| | | 1:640 | - | - | - | - | - | - | - | - | - | - | - | - | |
| | Follow-up | 1:40 | + | + | + | + | + | - | + | + | - | + | - | - | |
| | | 1:640 | + | + | - | + | + | - | + | + | + | - | - | - | |
| | | | | | | | | | | | | | | | 441 |

[5]Children were screened for increased neutralization activity against DENV1-4 over the study period. Paired baseline and follow-up sera at dilutions of 1:40 and 1:640 were subjected to neutralization tests

**Table 2.2. Ability of IgG ELISA to detect true dengue infections based on neutralization assay outcome[6]**

| | | Neutralization Assay Outcome[7] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Primary Cases | | | Secondary Cases | | |
| | | (+) | (-) | Predictive Values (%) | (+) | (-) | Predictive Values (%) |
| **IgG ELISA Outcome[8]** | (+) | 33[9] | 10[10] | 76.7%[11] | 20[9] | 47[10] | 29.9%[11] |
| | (-) | 2[12] | 313[13] | 99.4%[14] | 12[12] | 362[13] | 96.8%[14] |
| | | Sensitivity 94.3%[15] | Specificity 96.9%[16] | | Sensitivity 62.5% | Specificity 88.5% | |

[6]Paired baseline and follow-up samples were subjected to DENV IgG ELISA and DENV neutralization assay.

[7]Neutralization assay outcome criteria: A primary case was DENV seronegative at baseline and DENV seropositive at follow-up. A secondary case was DENV seropositive at baseline and indicated a 2-fold $neut_{50}$ titer increase or seroconversion to at least one DENV serotype at follow-up.

[8]IgG ELISA outcome criteria: A primary case was DENV IgG negative at baseline and DENV IgG positive at follow-up. A secondary case was DENV IgG positive at baseline and showed an increase in DENV IgG OD at follow-up.

[9]True Positive (TP) – correctly identified

[10]False Negative (FN) – incorrectly identified

[11]Positive Predictive Value (PPV) – [TP ÷ (TP+FP) × 100%]

[12]True Negative (TN) – correctly unidentified

[13]False Positive (FP) – incorrectly identified

[14]Negative Predictive Value (NPV) – [TN ÷ (FN+TN) × 100%]

[15]Sensitivity – [TP / (TP + FN) × 100%]

[16]Specificity – [TN / (TN + FP) × 100%]

**Table 2.3. Age-specific dengue sero-prevalence**[17]

| Age group (years) | No. children | No. seropositive by IgG ELISA | No. seropositive confirmed by dengue neutralization test | Seroprevalence (%) |
|---|---|---|---|---|
| 0 | 51 | 12 | 11 | 21.57 |
| 1-3 | 196 | 71 | 67 | 34.18 |
| 4-6 | 191 | 105 | 100 | 52.36 |
| 7-9 | 225 | 150 | 145 | 64.44 |
| 10-12 | 136 | 103 | 101 | 74.26 |
| Total (0-12) | 799 | 441 | 424 | 53.07 |

[17]The dengue seroprevalence at the beginning of study (baseline) was determined by screening all sera by dengue IgG ELISA and then testing the positive samples by dengue neutralization test.

**Table 2.4. Age-specific fever episodes and incidence of dengue infection and illness[18]**

| Age (yrs) | # Children | Fever Episodes | | New dengue infections | | | Incidence of Infection (per 100 children) | Incidence of Disease (per 100 children) |
|---|---|---|---|---|---|---|---|---|
| | | Total | Mean/Child | Total | Inapparent | Apparent | *(95% confidence interval)* | |
| 0 | 51 | 31 | 0.6 | 1 | 0 | 1 | 1.96 *(0.05-10.45)* | 1.96 *(0.05-10.45)* |
| 1-3 | 196 | 252 | 1.3 | 27 | 15 | 12 | 13.78 *(9.28-19.41)* | 6.12 *(3.20-10.45)* |
| 4-6 | 191 | 174 | 0.9 | 13 | 11 | 2 | 6.81 *(3.67-11.36)* | 1.05 *(0.13-3.73)* |
| 7-9 | 225 | 137 | 0.6 | 15 | 6 | 9 | 6.67 *(3.78-10.76)* | 4.00 *(1.85-7.46)* |
| 10-12 | 136 | 87 | 0.6 | 11 | 8 | 3 | 8.09 *(3.25-16.03)* | 2.21 *(0.46-6.31)* |
| 0-12 | 799 | 681 | 0.9 | 67 | 40 | 27 | 8.39 *(6.56-10.53)* | 3.38 *(2.24-4.88)* |



[18]Clinically apparent dengue infections were diagnosed by performing paired serology and PCR on acute and convalescent samples collected from febrile children. Total dengue infections over the study year were detected by testing paired baseline and end of year samples by IgG ELISA and neutralization test. Children who were infected but not identified as apparent cases were designated as inapparent cases.

**Table 2.5. Dengue disease outcome in children exposed to primary versus secondary infections**

| Infection | Total | Disease | | |
|---|---|---|---|---|
| | | Inapparent | Apparent | Inapp./App. ratio |
| Primary | 35 | 20 | 15 | 1.33 |
| Secondary | 32 | 20 | 12 | 1.67 |
| Total | 67 | 40 | 27 | 1.48 |

**Table 2.6. Prevalence of circulating DENV serotypes**

| | DENV Serotype Exposure (before initiation of study)[19] N (%) | DENV Serotype Exposure during study year[20] N (%) |
|---|---|---|
| **DENV1** | 7 (9) | 7 (21) |
| **DENV2** | 32 (43) | 16 (49) |
| **DENV3** | 27 (36) | 9 (27) |
| **DENV4** | 9 (12) | 1 (3) |
| **Total** | 75 (100) | 33 (100) |

[19]A dengue immune individual was considered to have been previously exposed to a particular serotype if the individual had neutralizing antibodies to one serotype only (monotypic) at baseline. Inferences of infecting serotype were not made when more than one serotype was neutralized.

[20]Based on children who were exposed to primary infections during the study year. The serotype was determined by PCR or neutralization test performed with sample collected at the end of the study (follow-up).

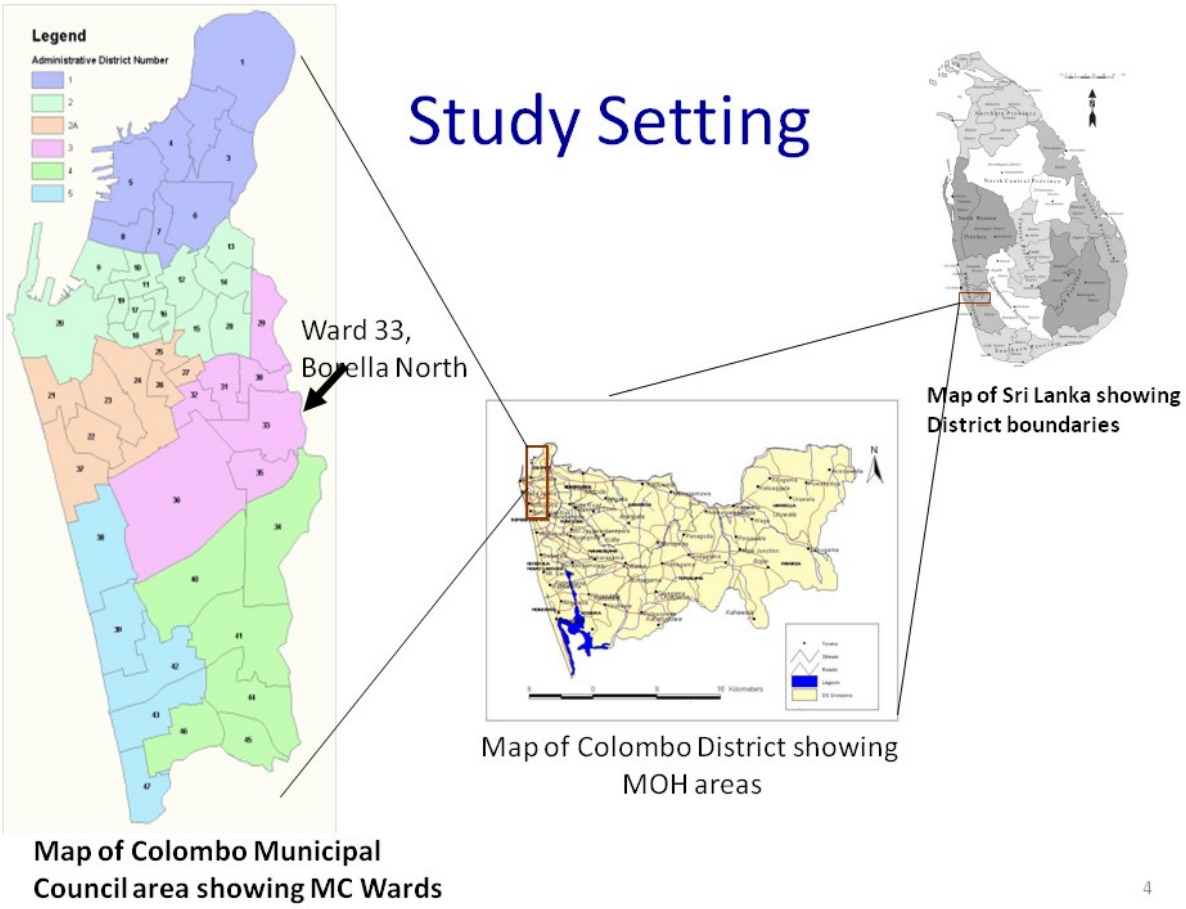


**Figure 2.1. Map and location of study site (Ward 33) in relation to Colombo Municipal Council and Colombo District in Sri Lanka.**

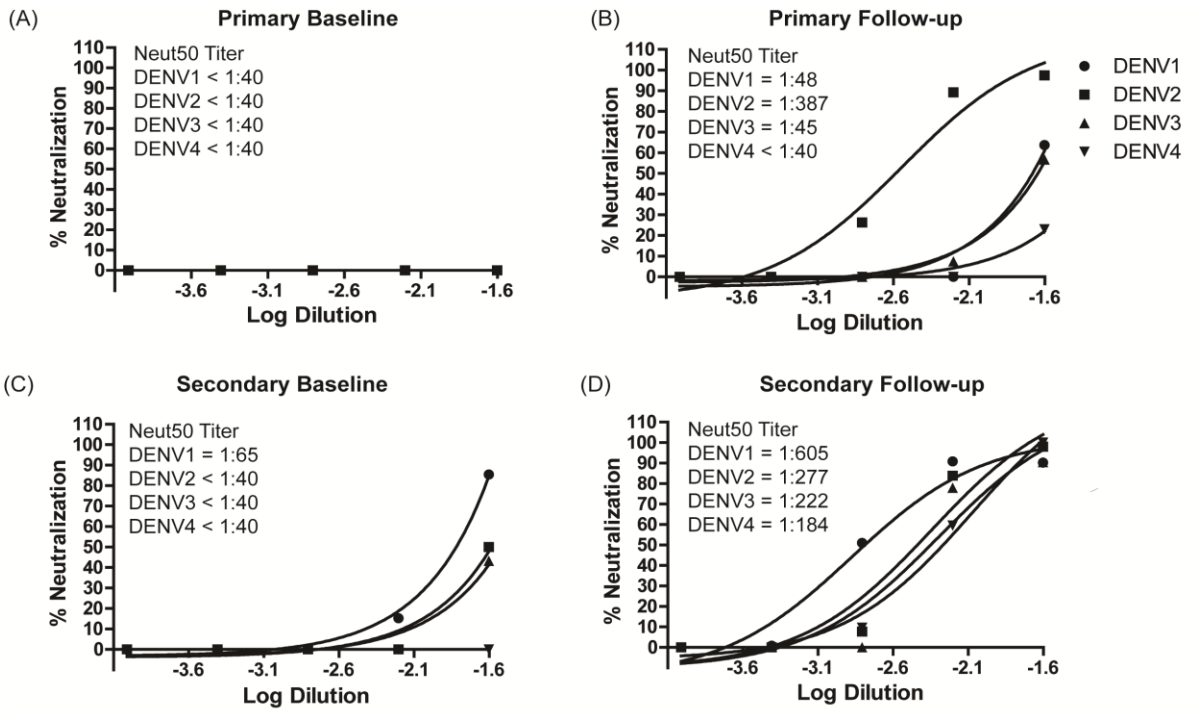


**Figure 2.2. Representative neutralization curves for confirmed inapparent dengue cases.** Graph shows representative cases. Paired baseline (A,C) and follow-up sera (B,D) (when available) from 1° (A-B) and 2° (C-D) dengue cases were subjected to neutralization assay at 1:40 – 1:10240 in 4-fold increments against DENV1-4. Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. These graphs depict temporal sera collected from two representative children (1 primary and 1 secondary).

# CHAPTER THREE

## Temporal Regulation of Antibody Responses Following Dengue Virus Infections in a Sri Lankan Pediatric Cohort [21] [22]

### 3.1 Introduction

Dengue virus (DENV) is a positive-stranded RNA virus that is transmitted to humans via the bite of *Aedes* mosquitos. DENVs exist as four serotypes, DENV1-4, which circulate in tropical and subtropical regions. Currently, over 2/3 of the world's population is at risk of being exposed to DENV [21, 22]. A recent study estimates 390 million DENV infections occur globally each year, rendering dengue the most common mosquito-borne human virus in the world [28].

An individual can be infected and re-infected with DENV. Primary (1°) dengue infection occurs when a naïve individual is exposed to DENV. Repeat dengue infection occurs when a previously-exposed individual is exposed to DENV for the second (secondary), third (tertiary), or fourth (quaternary) time, etc. Natural human DENV infection can result in clinically inapparent or apparent infections. Apparent infections, which account for less than half of total DENV infections, manifest as mild dengue fever (DF), severe dengue hemorrhagic fever (DHF), or potentially fatal dengue shock syndrome (DSS) [28].

---

[21] Portions of this chapter are in press. | **Corbett K.S., Katzelnick L, Tissera H, Amarasinghe A, de Silva A.D., and de Silva A.M.**, *Pre-existing Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort.* **Journal of Infectious Diseases**.

[22] This study was funded by the Pediatric Dengue Vaccine Initiative (Bill and Melinda Gates Foundation Grant #: 23197). Additional funding was provided by UNC Virology Training Grant (NIH Grant #: T32AI007419) and Initiative for Minority Student Development (NIH Grant #: 5R25GM055336).

Viral and host factors have been shown to influence dengue disease outcome [31, 38, 42, 43]. But, the most significant risk factor for severe disease is previous DENV infection [69-73]. Primary DENV infection induces cross-reactive non-neutralizing antibodies that assist in binding and uptake of DENV particles into FcγR-bearing cells upon secondary infection with a heterologous DENV serotype [69, 221]. This phenomenon is coined "antibody-dependent enhancement (ADE)" [221].

Together, a compilation of recent studies suggests that the human DENV antibody response following primary DENV infection is dominantly cross-reactive and weakly neutralizing [81, 99, 222]. Current dogma suggests that following 1° DENV infections neutralizing antibodies are induced in two forms: long-lasting type-specific antibodies and short-lived cross-reactive neutralizing antibodies (reviewed in [115]), but the specifics of temporal regulation of antibody responses following dengue infections remain unclear. To that end, here we utilized sera samples collected during a prospective dengue fever surveillance study in Colombo, Sri Lanka [215, 216] to provide insight on the timing of IgG and neutralizing antibody responses following primary and repeat DENV infections.

## 3.2 Materials and Methods

### Human Subjects Protocol Approval

Ethical approval for this research was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka and the Institutional Research Board (IRB) of the International Vaccine Institute, Seoul, Korea. University of North Carolina IRB determined that this study does not constitute human subject research with respect to UNC investigators' involvement. Only children whose parents or legal guardians provided written informed consent were enrolled in the study.

### Cell Lines and Viruses

U937+DC-SIGN cells were maintained in RPMI media supplemented with 5% fetal bovine serum, 1% L-Glutamine, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and

0.05 mM beta-mercaptoethanol. C6/36-derived WHO reference DENV strains, DENV1 (West Pac 74), DENV2 (S-16803), DENV3 (CH 53598), DENV4 (TVP-376), were used in all infection-based experiments.

**Sample Collection**

Surveillance and sample collection methods were previously detailed [215, 216]. Briefly, between November 2008 and January 2010, in Colombo, Sri Lanka, blood samples were collected from 799 children, ≤ 12 years old, at enrollment (baseline) and 12 months later (follow-up). In addition, children who experienced febrile illness were bled upon fever onset (acute) and ≥ 10 days following fever dissipation (convalescent) [216]. Blood samples were stored as dried blood spots (DBS) on protein saver cards (Whatman, UK & ID Biological Systems, Greenville SC USA) [207, 208] or centrifuged and stored as plasma.

**Elution of Antibodies from DBS**

DBS diluent volume was determined based on standard plasma dilutions in pilot experiments using matched DBS and plasma obtained from our dengue traveler cohort [99]. Antibodies were eluted from DBS by submerging filter paper in diluent appropriate for subsequent assay. DBS/diluent mixtures were incubated at 37°C for 2hrs. Resulting DBS eluates (sera) were used in IgG, IgM, and neutralization assays, as described below.

**Detection of DENV-specific IgG and IgM Antibodies**

Immunoassays for detection of DENV-specific IgG and IgM antibodies were performed as previously described [31, 39]. Sera were used at 1:100 and 1:50 in IgG and IgM ELISAs, respectively. Cutoffs for IgM and IgG positivity were determined based on positive control samples and, where applicable, represented as standard deviations relative to normal human sera (NHS) controls (N = 10, pooled).

**Detection of DENV Neutralizing Antibodies**

Sera were assessed for the presence of neutralizing antibodies against each DENV serotype using a flow cytometry-based neutralization assay with U937 monocytic cells stably

transfected with DC-SIGN (U937+DC-SIGN), as previously described [213, 214]. Sera dilutions used were 1:40 – 1:10240 in 4-fold increments. Samples were run on GUAVA easyCyte HT and analyzed on GUAVASoft software (Millipore, Billerica MA USA). Sigmoidal curves were generated using Prism v4.0 (GraphPad Software, La Jolla CA USA). $Neut_{50}$ titer was calculated as the dilution of a serum that neutralizes 50% of each DENV serotype. The seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution.

**Laboratory Confirmation of DENV Infections**

To identify clinically apparent DENV infections, acute and convalescent samples were collected from children with fever and tested by PCR and paired IgG and IgM serology, as previously described [216]. Refer to chapter two for complete details of how inapparent DENV infections were detected. Briefly, all children were screened for increases in neutralizing antibody levels over the study year by testing paired baseline and 12-month follow up samples (at dilutions of 1:40 and 1:640) by neutralization assay (**Table 2.1**). Cases were then confirmed by neutralization assay with full serum dilution series (**Figure 2.2**). DENV infections occurring in children who did not have a laboratory-confirmed apparent dengue fever during the study year were classified as inapparent DENV infections. Dengue cases were further classified as primary or secondary based on baseline DENV naïve or immune status, respectively. Notably, all DENV cases in this study were confirmed by gold-standard neutralization assay [213, 214].

**Statistical Analyses**

To compare DENV IgG levels or $neut_{50}$ titers between inapparent and apparent groups, we used unpaired student's t-tests. Unpaired student's t-tests were also used to compare $neut_{50}$ titers between IgM- and IgM+ groups. Repeated measure 1-way ANOVA with Tukey's multiple comparison post-test analysis was used to compare DENV IgG levels of paired baseline, acute, convalescent, and follow-up groups. To compare breadth of neutralizing antibody responses between groups, we performed Fisher's exact tests, defining outcome categories as "monotypic" (seroconversion to one serotype) and "heterotypic" (seroconversion to ≥ 2 serotypes). Pearson's

product-moment correlation tests were used to determine the linear relationship between elapsed time and neutralization breadth or magnitude. A 1-way ANOVA, with Tukey's multiple comparison post-test, was used to compare mean # of serotypes neutralized or mean $neut_{50}$ titer at baseline, acute, convalescence, and follow-up for 2° apparent cases. For some assays, sample variability exists due to the limited quantity of sample available for the multiple and repeat analysis of the various assays, not due to loss at follow-up. P-values represent two-tailed analyses, unless otherwise specified. On graphs, each dot shows an individual serum sample measured in duplicate, and, wherever applicable, means are depicted. Generation of graphs and statistical analyses was completed using Prism v4.0.

## 3.3 Results

We used blood samples from a prospective pediatric dengue cohort study in Colombo, Sri Lanka to describe temporal regulation antibody responses in children who acquired primary and secondary dengue infections. The cohort population, study design, surveillance methods, and epidemiological findings are detailed elsewhere [215, 216]. As previously reported, we identified a total of 35 primary and 32 secondary/repeat infections over the 12-month study period (**Table 2.1**) [216]. Forty of the infections (20 primary and 20 secondary/repeat) were classified as inapparent, as they occurred in children who did not have a laboratory-confirmed dengue fever during the 12 month period of active disease surveillance.

### Comparison of antibody responses following primary inapparent and apparent DENV infections

First, we set out to compare acquired DENV-specific antibody responses in children exposed to 1° inapparent and apparent infections. We found no difference in total DENV-binding IgG (**Figure 3.1A**) or the ability of antibodies to neutralize the 4 DENV serotypes (**Figure 3.1B,C**). Both 1° inapparent and apparent DENV infections induced neutralizing antibody responses that were monotypic in some cases and heterotypic in other cases (**Figure 3.1B**). The magnitude of neutralization, as depicted by combined 50% neutralization across all

DENV serotypes, induced by 1° inapparent and apparent DENV infections was also similar (**Figure 3.1C**). Together, these data indicate that children experiencing 1° inapparent and apparent infections develop similar DENV-specific antibody responses; thus regulation of antibody responses following primary infection must be correlated with another factor, such as time or genetics.

**Temporal analysis of antibody responses following primary DENV infections**

Therefore, we went on to characterize temporal regulation of neutralizing antibody responses following primary DENV infections. With apparent infections, for which we knew the date of fever onset, we found a significant negative correlation between time elapsed after infection and neutralization breadth across the 4 serotypes (Pearson's $r = -0.69$, $P < 0.01$); children who experienced recent dengue fever had more broadly neutralizing antibody responses than children who experienced dengue fever early in the study year (**Figure 3.2A**). Although there was a trend towards a negative correlation between time elapsed after 1° apparent infection and neutralization magnitude, it was not statistically significant (**Figure 3.2C**).

Following these observations, for all 1° infections, both inapparent and apparent, we tested follow-up sera for presence of DENV IgM, which are indicative of recent (<6 months) infection [112]. Serotype cross-neutralizing sera were more likely to be IgM-positive than monotypic sera ($P < 0.05$) (**Figure 3.1B**) further confirming the association between recent primary exposure and broad neutralization. Additionally, IgM-positive sera have a significantly higher neutralization magnitude than IgM-negative sera ($P < 0.01$) (**Figure 3.2D**). Together, these data indicate that 1° DENV infection stimulates a DENV-serotype cross neutralizing response that evolves over time into a more monotypic response.

**Temporal analysis of antibody responses following repeat DENV infections**

Seeing temporal changes in antibody responses following primary infections, we set out to measure changes in DENV-specific antibodies as a function of time following repeat DENV infections. We focused on apparent infections for which dates of actual infection were known.

There was a significant increase in DENV IgG at the early convalescent stage (~2-4 weeks after infection), compared to baseline ($p < 0.001$) and acute stages ($p < 0.05$). However, these elevated IgG levels had declined back to pre-infection and acute levels by the end of the study year (follow-up) (**Figure 3.3A**). Neutralization curves of timed sera from apparent re-infections revealed boosting of neutralizing antibody titer against the serotype of previous infection (data not shown). At the convalescent stage, there was a robust increase in neutralizing antibody titer against multiple DENV serotypes, compared to baseline ($P < 0.001$) (**Figure 3.3B,C**). Although the overall magnitude of neutralizing antibody was slightly reduced at follow-up, the response remained broadly cross neutralizing (**Figure 3.3B,C**). Our complete analysis of neutralization breadth in apparent repeat infections revealed a significant and stable increase in neutralization breadth that was maintained through the end of the study (follow-up) compared to pre-infection (baseline) ($p < 0.001$) and acute ($p < 0.05$) levels (**Figure 3.3C**). Thus, for repeat infections, although the overall quantity of DENV-specific IgG antibody increased and then decreased back to pre-infection/acute levels, the magnitude and breath of neutralization was increased and maintained over the duration of the study year.

## 3.4 Discussion

In this study, we used blood samples collected from a Sri Lankan pediatric dengue cohort to investigate temporal regulation of antibody responses in children exposed to inapparent and apparent DENV infections. With respect to primary DENV infections, factors other than pre-existing immunity, such as infecting DENV strain or serotype and/or intrinsic host factors, must influence development of inapparent versus apparent infection [41, 141, 223, 224]. Here, we explored whether inapparent and apparent primary infections stimulated different antibody responses in children and found no differences in DENV-specific IgG or neutralization quality.

Our data also demonstrates that time elapsed following primary DENV infections negatively correlates with breadth of neutralization, suggesting that neutralizing antibody

responses evolve from cross-neutralizing to serotype-specific by 8-12 months after infection. Others have also observed that primary DENV exposure results in cross-neutralizing/protective antibodies that gradually become monotypic as time elapses [106, 222, 225], although mechanisms responsible for the waning of cross-neutralization following primary DENV infections have not been defined. We utilized IgM as a marker of time in those children who got inapparent infections for which we did not know the specific date of infection. In doing so, we observed a strong association between the presence of DENV-specific IgM and cross neutralization, further associating recent infections with broad neutralization. This data also indicates that IgM may, in part, be responsible for cross neutralization. However, IgM cannot be the only explanation because the period of transient cross neutralization extends to 8-12 months, which is well beyond the 3-6 month window after infection when IgM is detected. The magnitude of the neutralization response following primary infections did not wane significantly over time as breadth did, but we saw significantly higher neutralization titers in IgM-positive sera than IgM-negative sera. This alludes to IgM being partially responsible for high neutralization titers following primary infection, similar to a previous study conducted by the Harris group [113], but further studies are needed to understand what role, if any, IgM antibodies have in neutralization and protection.

For repeat apparent infections, we were able to characterize antibody quantity and quality before, during, and after infection. Although we did not observe a significant rise in DENV-specific IgG during the acute stage of infection, by 2-4 weeks after infection most children had elevated levels of IgG compared to pre-infection levels. Surprisingly, the elevation of IgG after repeat infection was not stable; in fact, when follow-up samples were collected several months after re-infection, IgG levels had declined to pre-infection and acute levels. At the acute stage, we observed boosting of neutralizing antibody response to previously-infecting serotype in most repeat infection cases. At 2-4 weeks after a repeat infection, children had high levels of cross-neutralizing antibodies. When follow-up samples were collected several months

later, although the overall magnitude of neutralizing antibody had slightly decreased (though not significantly), the breath of neutralizing antibody remained significantly greater than in the pre-repeat infection sample. We did not follow these children longer than 12 months, but we believe that these cross-neutralizing antibody responses are long-lived, perhaps for a lifetime. Thus, although following a repeat DENV infection, the quantity of DENV-specific IgG returned to pre-infection/acute levels, the quality of the neutralization response was stably altered. Our findings show that antibody responses following primary and repeat infections are temporally regulated in different ways. Understanding how the temporal regulation of these antibody responses correlates with protection from subsequent DENV infection or disease will be important for DENV vaccine development.

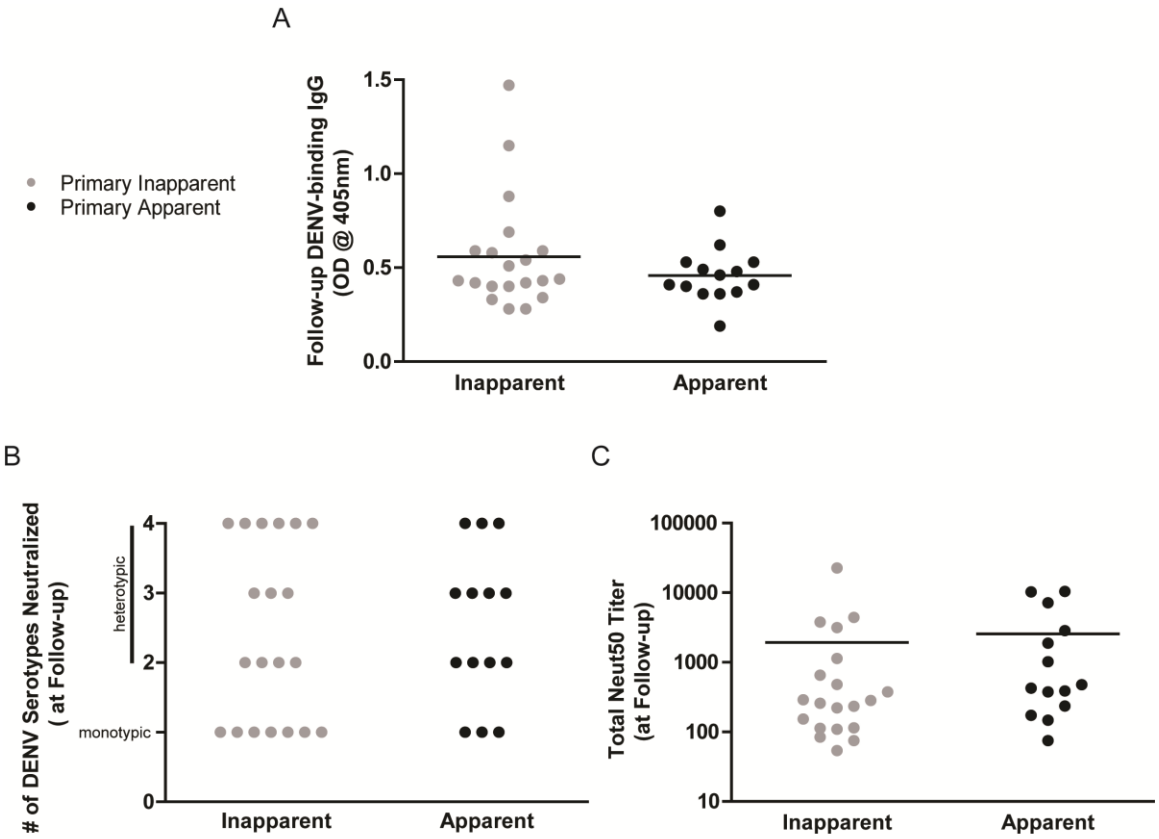


**Figure 3.1. Comparison of antibody responses following primary inapparent and apparent dengue virus infections.** Follow-up sera from children exposed to 1° DENV infections were subjected to (A) IgG ELISA at 1:100 dilution and (B-C) neutralization assays at 1:40 – 1:10240 in 4-fold increments against DENV1-4. (A) Lines represent mean $OD_{405nm}$ values. Means were found to be insignificant by unpaired student's t-test. (B-C) Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. (B) Neutralization/Seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution for each DENV serotype. Groups were compared using Fisher's exact tests, defining outcome categories as "monotypic" (seroconversion to one serotype) and "heterotypic" (seroconversion to ≥ 2 serotypes). (C) Total $neut_{50}$ is a calculation of each serum's combined $neut_{50}$ titer across all serotypes. Lines represent mean total $neut_{50}$ values for each group and were found to be insignificant by unpaired student's t-test. (A-C) Each dot represents mean values of an individual serum tested in duplicate.

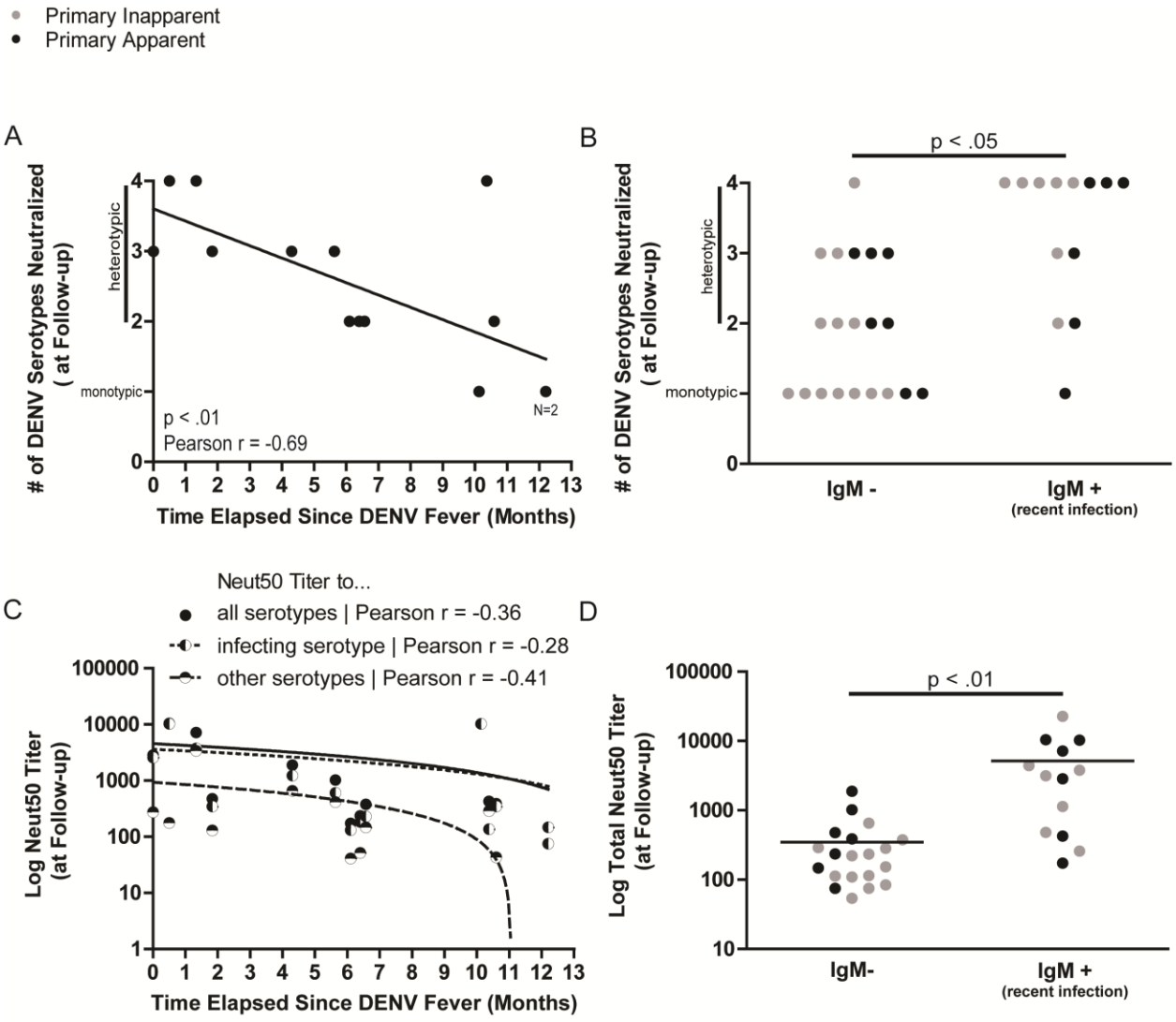


**Figure 3.2. Temporal analysis of neutralizing antibody responses following primary dengue virus infections.** Follow-up sera from children exposed to 1° DENV infections were subjected to neutralization assays at 1:40 – 1:10240 in 4-fold increments against DENV1-4. Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. Neutralization/Seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution for each DENV serotype. $Neut_{50}$ is a calculation of each serum's combined $neut_{50}$ titer across (C,D) all serotypes, (C) the three heterologous serotypes, which were not responsible for infection, or the infecting serotype, as determined by PCR or serotype of highest neutralization response. (B,D) Those sera were also subjected to IgM ELISA at 1:50 dilution; IgM positivity

was determined to be 5 standard deviations over NHS (N=10, pooled). (A,C) For 1° apparent infections, time elapsed since DENV fever was determined as amount of time between date of acute fever onset and date of follow-up sample collection. Line was generated by linear regression analysis. Correlation between (A) neutralization breath or (C) neutralization magnitude and elapsed time was calculated by Pearson's product-moment correlation test. (B) Groups were compared using Fisher's exact tests, defining outcome categories as "monotypic" (seroconversion to one serotype) and "heterotypic" (seroconversion to ≥ 2 serotypes). (D) Lines represent mean total $neut_{50}$ values for each group and were compared using unpaired student's t-test. (A-D) Each dot represents mean values of an individual serum tested in duplicate.

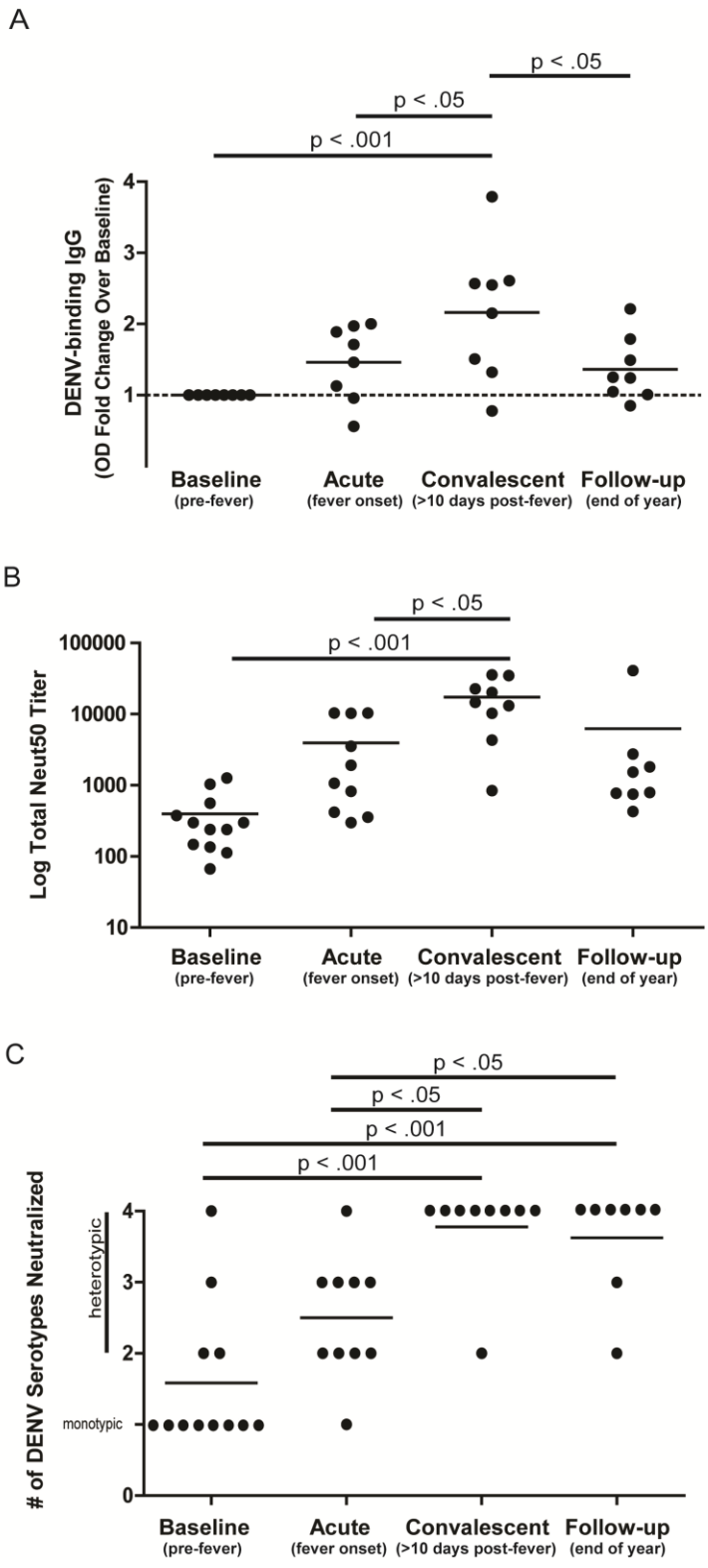


**Figure 3.3. Temporal analysis of antibody responses following repeat dengue virus infections.** Paired baseline, acute, convalescent, and follow-up sera (when available) from

repeat apparent DENV infections were subjected to (A) IgG ELISA at 1:100 dilution, and (B,C) neutralization assay at 1:40 – 1:10240 in 4-fold increments against DENV1-4. (A) DENV IgG OD values were calculated as fold change with respect to baseline OD values. (B-C) Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. (B) Total $Neut_{50}$ is a calculation of each serum's combined $neut_{50}$ titer across all serotypes. (C) The neutralization/seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution for each DENV serotype. (A-C) Each dot represents mean values of an individual serum tested in duplicate. Lines show (A) mean OD values, (B) mean total $neut_{50}$ values, and (C) # of DENV serotypes neutralized at each timepoint; timepoints were compared by 1-way ANOVA, with Tukey's multiple comparison post-test.

# CHAPTER FOUR

## Pre-existing Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort[23] [24]

### 4.1 Introduction

The most significant risk factor for severe disease is previous DENV infection; an individual experiencing secondary (2°) infection with a heterologous DENV serotype faces greater risk of developing severe disease than someone experiencing primary (1°) infection [69-73]. Antibody dependent enhancement (ADE) is the leading explanation for increased severe dengue disease risk following re-infection. ADE theory postulates that 1° DENV infection induces cross-reactive non-neutralizing antibodies that promote entry of DENV particles into FcγR-bearing cells upon 2° infection with a heterologous DENV serotype. This phenomenon is believed to result in increased cellular viral burden and subsequent severe disease [80, 150, 221].

Many studies have been performed to examine the role of antibodies in severe dengue disease [78, 128, 149-152]. A topic that has been less studied is the role of antibodies in clinically inapparent versus apparent DENV infection [38, 113, 116]. The mere fact that 75% of DENV infections occur subclinically in populations where repeat infections are highly likely [28] points to an important role for neutralizing antibodies in protection from repeat apparent infections. In fact, progression to or protection from dengue disease is likely dependent on the

[23]Portions of this chapter are in press. | **Corbett K.S., Katzelnick L, Tissera H, Amarasinghe A, de Silva A.D., and de Silva A.M.**, *Pre-existing Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort.* **Journal of Infectious Diseases**.

[24]This study was funded by the Pediatric Dengue Vaccine Initiative (Bill and Melinda Gates Foundation Grant #: 23197). Additional funding was provided by UNC Virology Training Grant (NIH Grant #: T32AI007419) and Initiative for Minority Student Development (NIH Grant #: 5R25GM055336).

balance between DENV enhancing and neutralizing antibodies [128]. Here we set out to test the hypothesis that antibody responses are linked to the development of inapparent and apparent DENV infections. We did so by utilizing sera collected from naturally-infected children in a prospective dengue fever surveillance cohort study in Colombo, Sri Lanka [216]

## 4.2 Materials and Methods

### Human Subjects Protocol Approval

Ethical approval for this research was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka and the Institutional Research Board (IRB) of the International Vaccine Institute, Seoul, Korea. University of North Carolina IRB determined that this study does not constitute human subject research with respect to UNC investigators' involvement. Only children whose parents or legal guardians provided written informed consent were enrolled in the study.

### Cell Lines and Viruses

U937+DC-SIGN cells were maintained in RPMI media supplemented with 5% fetal bovine serum (FBS), 1% L-Glutamine (L-Gln), 1% Penicillin/Streptomycin (Pen/Strep), 1% non-essential amino acids (NEAA), and 0.05 mM beta-mercaptoethanol. K652 cells were maintained in RPMI media supplemented with 5% FBS, 1% L-Gln, 1% Pen/Strep, and 1% NEAA. C6/36-derived WHO reference DENV strains, DENV1 (West Pac 74), DENV2 (S-16803), DENV3 (CH 53598), DENV4 (TVP-376), were used in all infection-based experiments.

### Sample Collection

Surveillance and sample collection methods were previously detailed [215, 216]. Briefly, between November 2008 and January 2010, in Colombo, Sri Lanka, blood samples were collected from 799 children, ≤ 12 years old, at enrollment (baseline) and 12 months later (follow-up). In addition, children who experienced febrile illness were bled upon fever onset (acute) and ≥ 10 days following fever dissipation (convalescent) [216]. Blood samples were stored as dried

blood spots (DBS) on protein saver cards (Whatman, UK & ID Biological Systems, Greenville SC USA) [207, 208] or centrifuged and stored as plasma.

**Elution of Antibodies from DBS**

DBS diluent volume was determined based on standard plasma dilutions in pilot experiments using matched DBS and plasma obtained from our dengue traveler cohort [99]. Antibodies were eluted from DBS by submerging filter paper in diluent appropriate for subsequent assay. DBS/diluent mixtures were incubated at 37°C for 2hrs. Resulting DBS eluates (sera) were used in IgG, IgM, and neutralization assays, as described below.

**Detection of DENV-specific IgG and IgM Antibodies**

Immunoassays for detection of DENV-specific IgG and IgM antibodies were performed as previously described [31, 39]. Sera were used at 1:100 and 1:50 in IgG and IgM ELISAs, respectively. Cutoffs for IgM and IgG positivity were determined based on positive control samples and, where applicable, represented as standard deviations relative to normal human sera (NHS) controls (N = 10, pooled).

**Detection of DENV Neutralizing Antibodies**

Sera were assessed for the presence of neutralizing antibodies against each DENV serotype using a flow cytometry-based neutralization assay with U937 monocytic cells stably transfected with DC-SIGN (U937+DC-SIGN), as previously described [213, 214]. Sera dilutions used were 1:40 – 1:10240 in 4-fold increments. Samples were run on GUAVA easyCyte HT and analyzed on GUAVASoft software (Millipore, Billerica MA USA). Sigmoidal curves were generated using Prism v4.0 (GraphPad Software, La Jolla CA USA). $Neut_{50}$ titer was calculated as the dilution of a serum that neutralizes 50% of each DENV serotype. The seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution.

**Detection of DENV Enhancing Antibodies**

The presence of DENV enhancing antibodies in sera was determined using a flow cytometry-based enhancement assay with FcγRIIa+ K562 erythroleukemic cells, as previously

described [226]. RPMI media supplemented with 2% FBS, 1% L-Gln, 1% Pen/Strep, and 1% NEAA was used for this assay. 1:40 dilution of sera and each DENV serotype was incubated at 37°C for 1 hour. Virus was added at an MOI of 1, based on titers calculated in Vero81 cells. Cells were added to sera/DENV mixtures and incubated at 37°C for 2 hours. Cells were washed in media twice and incubated at 37°C for 24 hours (from the time of cells being added). As previously described with our flow cytometry based neutralization assay [213, 214], cells were then washed, fixed, permeabilized, and stained with 2H2-Alexa488 to detect DENV-infected cells. 2H2-Alexa488 is a *flavivirus* prM-specific antibody conjugated to Alexa488. Samples were run on GUAVA easyCyte HT and analyzed on GUAVASoft software (Millipore, Billerica MA USA). Percentage of infection was determined. On graphs, % infection is represented at number of standard deviations (SD) above mean % infection of NHS (N=10). Enhancement threshold was set to ≥ 10 SD above mean % infection of NHS (N=10), based on positive controls (N=3) for each DENV serotype.

**Laboratory Confirmation of DENV infections**

To identify clinically apparent DENV infections, acute and convalescent samples were collected from children with fever and tested by PCR and paired IgG and IgM serology, as previously described [216]. Refer to chapter two for complete details of how inapparent DENV infections were detected. Briefly, all children were screened for increases in neutralizing antibody levels over the study year by testing paired baseline and 12-month follow up samples (at dilutions of 1:40 and 1:640) by neutralization assay (**Table 2.1**). Cases were then confirmed by neutralization assay with full serum dilution series (**Figure 2.2**). DENV infections occurring in children who did not have a laboratory-confirmed apparent dengue fever during the study year were classified as inapparent DENV infections. Dengue cases were further classified as primary or secondary based on baseline DENV naïve or immune status, respectively. Notably, all DENV cases in this study were confirmed by gold-standard neutralization assay [213, 214].

**Statistical Analyses**

To compare DENV IgG levels or $neut_{50}$ titers between inapparent and apparent groups, we used unpaired student's t-tests. To compare breadth of neutralizing antibody responses between groups, we performed Fisher's exact tests, defining outcome categories as "monotypic" (seroconversion to one serotype) and "heterotypic" (seroconversion to ≥ 2 serotypes). Enhancement of inapparent and apparent groups were compared for each DENV serotype by Mann Whitney test. Neutralization and enhancement were correlated using Pearson's product moment correlation tests. In the quantitative analysis of neutralization breadth, represented in table 4.1, Wilcoxon rank sum test was used for statistical comparison between groups, as samples were not normally distributed. For some assays, sample variability exists due to the limited quantity of sample available for the multiple and repeat analysis of the various assays, not due to loss at follow-up. P-values represent two-tailed analyses, unless otherwise specified. On graphs, each dot shows an individual serum sample measured in duplicate, and, wherever applicable, means are depicted. Generation of graphs and statistical analyses was completed using Prism v4.0.

## 4.3 Results

We used blood samples from a prospective pediatric dengue cohort study in Colombo, Sri Lanka to compare pre-existing antibody responses between children who developed repeat clinically inapparent and apparent dengue infections. The cohort population, study design, surveillance methods, and epidemiological findings are detailed elsewhere [215, 216]. As previously reported, we identified a total of 35 primary and 32 secondary/repeat infections over the 12-month study period (**Table 2.1**) [216]. Forty of the infections (20 primary and 20 secondary/repeat) were classified as inapparent, as they occurred in children who did not have a laboratory-confirmed dengue fever during the 12 month period of active disease surveillance.

**Comparison of pre-existing neutralizing antibody responses of repeat inapparent and apparent DENV infections**

To compare pre-existing (baseline) antibody responses of children experiencing repeat inapparent and apparent infections, we first looked at total DENV-specific IgG levels and found no difference (**Figure 4.1A**). Then, we compared the magnitude and breadth of pre-existing neutralizing antibody responses. Total 50% neutralization titers, across all DENV serotypes, did not differ (**Figure 4.1B**). However, we found that children who got repeat inapparent DENV infections were more likely to have heterotypic neutralizing responses at baseline compared to children who got repeat apparent infections ($p < 0.05$) (**Figure 4.1C**). To examine whether children who got repeat inapparent infections had experienced very recent past DENV infections, we tested pre-infection sera for the presence of DENV-specific IgM. We did not observe a correlation between the presence of IgM and neutralization breadth or clinical outcome (**Figure 4.1D**). In fact, 17 out of the 20 (85%) baseline sera from children who had inapparent repeat infections were DENV IgM-negative, indicating their prior DENV exposures had not occurred in the past 6 months. These data indicate that neutralization breadth of pre-infection antibodies has significant bearing on clinical outcome of subsequent DENV infection. Furthermore, the difference in neutralization capacity observed comparing repeat inapparent and apparent infections cannot simply be explained by very recent exposure, based on IgM presence.

**Comparison of pre-existing enhancing antibody responses of repeat inapparent and apparent DENV infections**

Showing a difference in pre-existing neutralization capacity between repeat inapparent and apparent DENV infections, we wanted to determine if there was a difference in enhancement ability as well. We saw no difference in the ability of pre-existing antibodies from repeat inapparent and apparent DENV infections to enhance any of the DENV serotypes (**Figure 4.2A**). Because it believed that a balance between enhancing and neutralizing antibodies influences dengue disease outcome, we wanted to compare pre-existing

neutralization to enhancement. Overall, comparing breadth of neutralization to breadth of enhancement, we saw a correlation that was slightly negative but not significant, with a p-value of 0.07 (**Figure 4.2B**). There was a slight opposite relationship between the number of serotypes a child neutralized with the number of serotypes that child enhanced. Suspecting actual levels of neutralization and enhancement may be more telling, we compared pre-existing neutralization magnitude to enhancement magnitude. For each serotype, with the exception of DENV4, there was a negative correlation between neutralization and enhancement magnitudes (DENV1, $p < 0.05$ | DENV2, $p < 0.001$ | DENV3, $p < .001$) (**Figure 4.2C-F**). For DENV1-3, the higher a serum's pre-existing neutralization titer, the weaker that serum's ability to enhance the same serotype. These data indicate that enhancing ability of pre-infection antibodies does not have significant bearing on fever outcome of subsequent DENV infection, in the way that neutralization breadth does.

**Quantitative analysis of differences in neutralizing antibody responses between types**

We conducted a direct analysis of the difference in neutralization of DENV types to delve further into neutralizing antibody responses in the cohort (**Table 4.1**). Breadth of response was measured as the difference between the titer of the dominantly neutralized DENV type, assumed to be of the previous infecting type, and the other three types. Similar to our aforementioned findings, we determined that children experiencing repeat apparent DENV infections had more monotypic baseline neutralizing antibody responses (dominant responses to a single DENV serotype) than those who acquired repeat inapparent infections ($p < 0.05$). Interestingly, a significant difference in post-infection breadth was also observed between inapparent and apparent repeat infections; apparent infections were observed to develop more balanced responses following infection than inapparent infections ($p < 0.05$). Thus, children whose repeat infections were inapparent had relatively conserved patterns of neutralization at baseline and follow-up, while children whose repeat infections were apparent transitioned from mostly type-specific neutralization before infection to broad neutralization after their repeat

DENV exposure. The degree of breadth in this sample was independent of the magnitude of the best neutralized DENV type prior to infection. Further, inapparent and apparent repeat infections had similar maximum titers at baseline and follow-up. Notably, after the study year, both inapparent and apparent repeat infections had higher titers when compared with primary-infected children ($p < 0.05$).

### 4.4 Discussion

Many studies have focused on how antibodies may enhance DENV infections and increase risk of developing DHF [78, 128, 149-152]. While it is well documented that most DENV infections are clinically-inapparent [28], factors responsible for inapparent infections have not been thoroughly investigated. Here, we quantified the magnitude and relative breadth of neutralization before repeat infections. While pre-existing neutralization magnitude did not seem to affect dengue disease outcome, we observed a strong association between breadth of neutralization and manifestation of fever. Children who got repeat inapparent infections had more broadly-neutralizing pre-existing DENV antibodies than children who got apparent re-infections. This is perhaps the most interesting finding of this study. It demonstrates an association between breadth of pre-existing DENV neutralizing antibodies and the likelihood of developing an inapparent versus apparent infection; thus, alluding to an important role of pre-existing neutralizing antibodies in protection from clinical symptom manifestation in repeat dengue infections.

There are different non-exclusive possible explanations for this association. Recent studies based on careful analysis of serial first, second, and third DENV infections in Nicaragua and models based on large dengue data sets from Thailand indicate that the period of cross protection after a primary infection can be up to 2 years after infection [121, 227]. Children in our study who had secondary inapparent infections may have been more recently exposed to their first infection compared to children who had secondary apparent infections. Our data showing IgM absence in baseline samples of nearly all repeat infections (both inapparent and apparent)

demonstrates that these cases were not in children within the first 6 months of their first infection. It remains possible that children who had secondary inapparent infections were still within the 1-2 year window of cross-protection unlike children who had secondary apparent infections who were well beyond this period of cross protection.

Another possibility is that children experiencing repeat apparent infections were exposed to secondary infections whereas most inapparent cases resulted from tertiary or quaternary (post-secondary) exposure. Thus, the broadly neutralizing baseline responses may be due to 2 or more previous DENV infections whereas the children with monotypic responses may have only been exposed to a single serotype. Indeed, a recent study utilizing cohort samples from Iquitos, Peru demonstrated that individuals experiencing post-secondary infections were more likely to develop inapparent infections compared to people exposed to “true” second infections [116]. It is worthwhile to note that while it is possible that children who appeared to have inapparent second infections were actually having post-secondary infections, the degree of neutralization breadth we observed prior to infection in those cases was not significantly different from children with primary infections (**Table 4.1**). It is likely that proximity to previous infection and number of previous infections, together with person-to-person variation, all influence whether someone will experience inapparent or apparent DENV re-infection.

We explored the balance of neutralization and enhancement and found that magnitude of neutralization to DENV1-3 was negatively correlated with magnitude of enhancement responses. However, children who got repeat inapparent and apparent infections had similar levels of pre-existing DENV enhancing antibodies. Together with neutralization data, this data may mean that neutralizing antibodies play a role in protection against DF, but enhancing antibodies do not play a role in inducing DF. This would go along with the field’s idea about ADE; enhancing antibodies are not thought to induce mild DF but severe DHF. Even in the first study that correlated DHF with secondary infection, there was no difference between the occurrences of DF in primary or secondary cases [69]. Our study was not designed to explore

questions about the role of antibodies in DHF, which is typically seen in less than 5% of apparent dengue cases and 1% of dengue cases overall [228]. Even with rising incidence of severe dengue in Sri Lanka [39], our cohort size of 799 children was too small to obtain the number of DHF cases required for such analysis [8].

The role of antibodies in inapparent infections is a neglected topic and increasingly relevant as dengue vaccines enter clinical trials. Recent trial results of a highly-anticipated DENV vaccine candidate signify the importance of understanding the role pre-existing DENV neutralizing antibodies play in protection from subsequent dengue disease and clinical outcome [197]. Our findings point to an important role for heterotypic neutralizing antibodies in protection from dengue disease. Further studies are needed to better define the protective properties of these cross-neutralizing antibodies.

**Table 4.1. Quantitative analysis of breadth of neutralizing antibody responses**[25]

| | | | | Repeat Infections | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Primary Infections | | | Inapparent | | | Apparent | | | P value |
| | Median | *25th* | *75th* | Median | *25th* | *75th* | Median | *25th* | *75th* | |
| Baseline | | | | 3.21 | *2.20* | *4.01* | 5.10 | *3.53* | *5.66* | 0.03[26] |
| Follow-up | 3.91[27] | *2.52* | *4.51* | 3.17 | *1.74* | *4.91* | 1.49 | *0.90* | *2.82* | 0.04[26] |
| | | | | Maximum Titer[28] | | | | | | |
| Baseline | | | | 4.39 | *3.55* | *5.40* | 4.58 | *3.78* | *5.10* | 0.57[26] |
| Follow-up | 4.39 | *3.73* | *6.69* | 6.48 | *5.61* | *7.26* | 5.87 | *5.03* | *6.67* | 0.02[29] |

[25]Breadth of response expressed as $\log_2$(reciprocal $neut_{50}$ titer/10), is a measure of the average difference in titer between the DENV serotype best neutralized by a serum (the maximum titer) and the titers for each of the other three DENV serotypes. A value close to 0 indicates the serum neutralizes all DENV types similarly, while higher values indicate that the serum neutralizes one DENV type significantly better than the other three types.

[26]Wilcoxon rank sum test for difference between secondary inapparent and apparent infections.

[27]Wilcoxon rank sum test for difference in breadth between primary infections and baseline secondary inapparent infection was not significant ($p \cong 0.3$).

[28]Maximum titer for each individual, regardless of the infecting type, is shown.

[29]Kruskal-Wallis test for difference in maximum titer between primary infections, inapparent and apparent repeat infections.

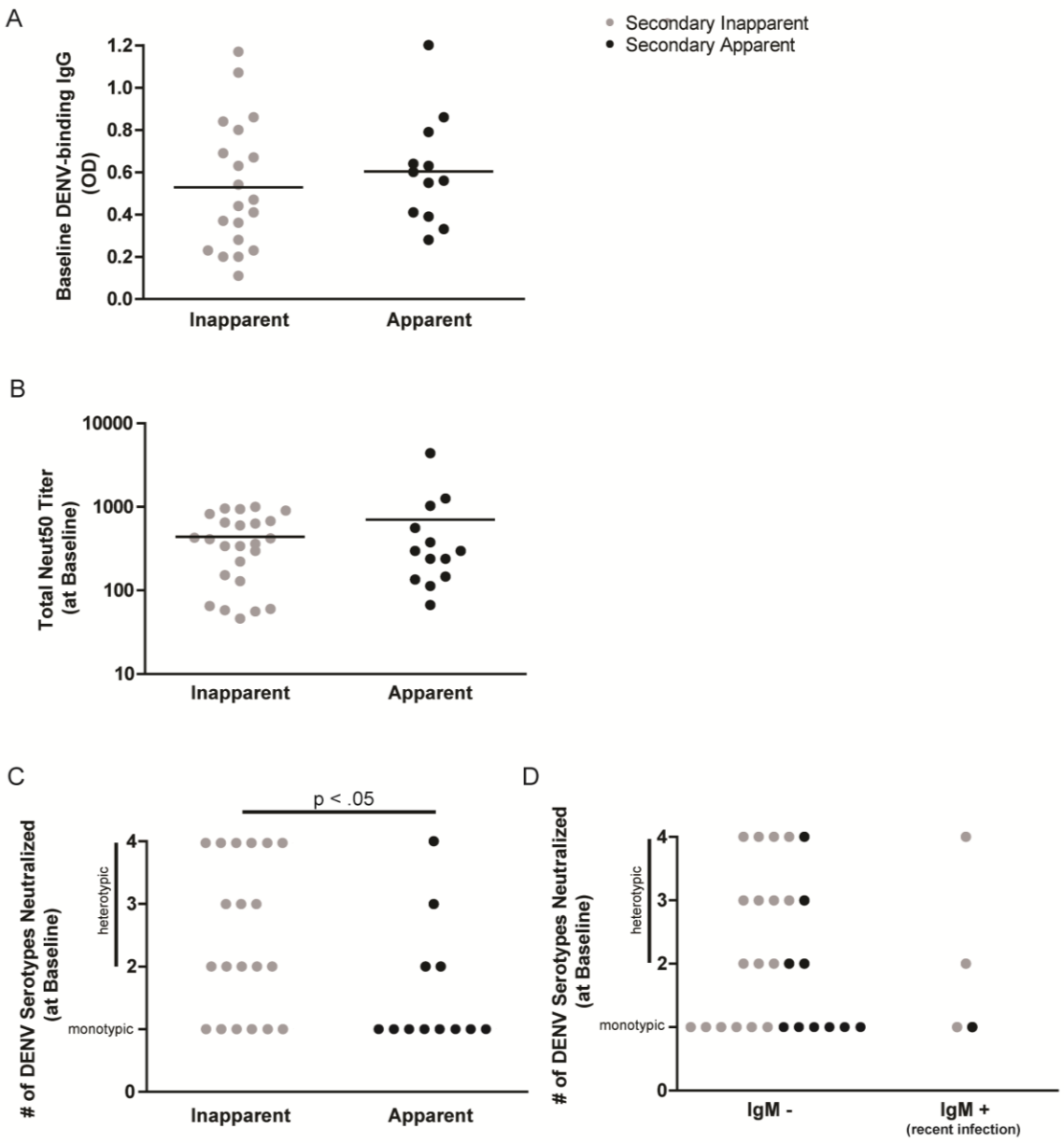


**Figure 4.1. Pre-existing neutralizing antibody responses of repeat inapparent and apparent dengue infections.** Baseline sera (when available) from repeat DENV infections were subjected to (A) IgG ELISA, (B-D) neutralization tests, and (D) IgM capture ELISA. (A) Baseline sera were used at 1:100 dilution. Mean $OD_{405nm}$ values for each group were calculated (represented by lines). Differences in mean OD between inapparent and apparent cases were

found to be insignificant by unpaired student's t-test. (B-D) Baseline sera were subjected to neutralization assays at 1:40 – 1:10240 in 4-fold increments against DENV1-4. Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. (B) Total $neut_{50}$ is a calculation of each serum's combined $neut_{50}$ titer across all serotypes. Lines represent mean total $neut_{50}$ values for each group and were found to be insignificant by unpaired student's t-test. (C-D) Neutralization/Seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution for each DENV serotype. (D) Baseline sera were also subjected to IgM ELISA at 1:50 dilution; IgM positivity was determined to be 5 standard deviations over NHS (N=10, pooled). (C-D) Groups were compared using Fisher's exact tests, defining outcome categories as "monotypic" (seroconversion to one serotype) and "heterotypic" (seroconversion to ≥ 2 serotypes).

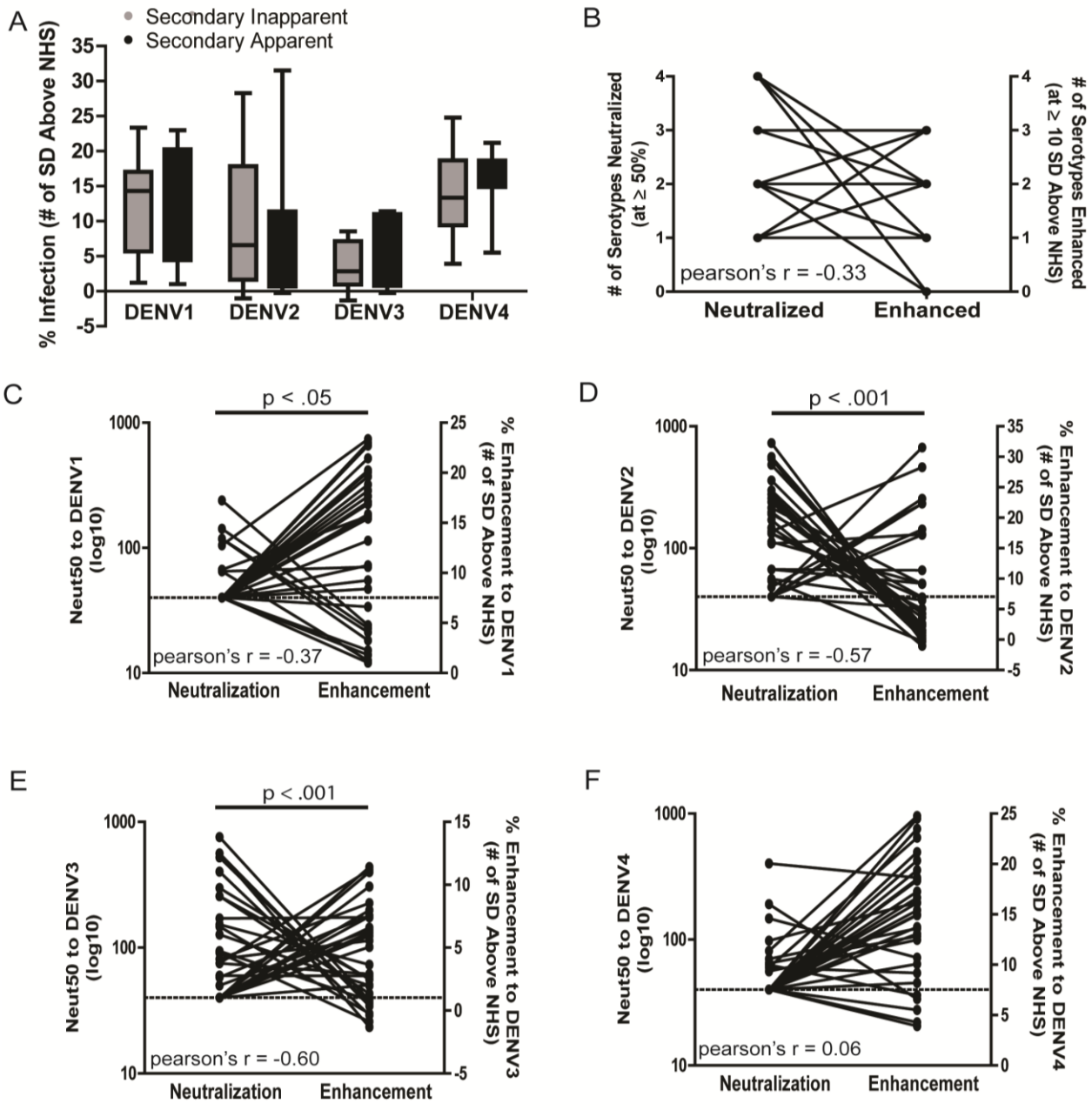


**Figure 4.2. Pre-existing enhancing antibody responses of repeat inapparent and apparent dengue infections.** Baseline sera (when available) from repeat DENV infections were subjected to enhancement and neutralization tests. (A) Baseline sera were used in enhancement assay at 1:40 dilution. Percentage of infection was represented at number of standard deviations (SD) above mean % infection of NHS (N=10). Box and whiskey plots were generated for each group. Inapparent and apparent groups were compared for each DENV

serotype and found to be insignificant by Mann Whitney test. (B) Baseline sera were subjected to neutralization assays at 1:40 – 1:10240 in 4-fold increments against DENV1-4. Sigmoidal curves were generated, and $neut_{50}$ titers were calculated for each serotype. Neutralization/Seroconversion threshold was set to ≥ 50% neutralization at ≥ 1:40 serum dilution for each DENV serotype. Enhancement threshold was set to ≥ 10 SD above mean % infection of NHS (N=10). (C-F) $Neut_{50}$ of each serum against (C) DENV1, (D) DENV2, (E) DENV3, and (4) DENV4 was compared to enhancement ability against the same serotype. (B-F) Neutralization and enhancement were compared by Pearson's product moment correlation test. If no p-value is assigned, statistically analysis revealed an insignificant outcome.

# CHAPTER FIVE

## Conclusions, Discussion, and Future Directions

## 5.1 Overview of Research Purpose

### Research Significance

Dengue virus is estimated to infect about 390 million people per year, representing the greatest epidemiologic and economic burden of all mosquito-borne viruses in the world [24, 28]. Dengue disease manifests with a range of clinical symptoms that WHO traditionally groups into inapparent and apparent infections. Inapparent infections are subclinical asymptomatic infections; apparent infections present as DF, DHF, and DSS [35]. There are implications that both viral and host factors play a role in facilitating severe dengue disease outcome [31, 38, 42, 43]. Antibody dependent enhancement is an immunopathological phenomenon thought to contribute most to dengue disease severity. This theory was formed in the 1980s [69] and has since been intensely studied [74, 78, 79, 88, 90-92, 94, 96, 105, 153, 226]. ADE is essentially a mechanism by which weakly-neutralizing cross-reactive antibodies bind to heterotypic DENV particles upon secondary infection, facilitating the uptake of those DENV particles into FcγR-bearing monotypic cells. Through various mechanisms, viral burden is increased and severe DHF ensues. As with many of the other immune responses to DENV, human antibody responses are considered a double-edged sword of sorts. On one side, there are enhancing responses that increase pathogenesis, and on the other side there are neutralizing responses that induce protection. There is a breadth of research detailing both sides of the antibody double-edged sword, but there is still plenty that we do not understand [74, 77, 78, 88, 90-92,

94, 96, 101-103, 105, 153, 226]. Our lack of understanding about DENV antibody responses was solidified when a prospective DENV vaccine candidate revealed an unpredicted lack of protection following natural challenge [162, 179-182, 184, 186].

### Research Approach

A recent review eloquently pronounces 6 reasons why vaccine researchers could benefit from lessons taught by human cohort studies [138]. To date, prospective human cohorts from around the world have provided us with a glimpse of understanding about antibody responses [64, 70, 118, 121, 131, 138, 140, 142, 143, 148, 152, 154, 155, 160, 168, 182, 197, 229], proving that studying natural DENV infections in human cohorts is a beneficial method of answering some of the field's most daunting questions. Our approach piggybacks on the relevance that human cohorts have to the dengue field. In collaboration with PDVI and investigators from the Ministry of Health in Sri Lanka, our group completed a prospective dengue study of 799 children, ages 0-12 years, who reside in Colombo, Sri Lanka. Using samples collected from the cohort, we addressed the field's three overarching questions about human antibody responses to DENV infection:

(1) What are the differences between antibody responses induced by primary versus secondary infections?

(2) What role do enhancing antibodies play in disease severity?

(3) What role do neutralizing antibodies play in protection from dengue disease?

In this chapter, I will discuss how our findings fit into the context of those three questions, suggest future directions for the Sri Lankan cohort, and close with an overview of the relevance of this work to the field and vaccine development in particular.

## 5.2 Introduction to the Sri Lankan Pediatric Cohort

### Sri Lankan Pediatric Cohort Ideal for Studying Dengue Virus Antibodies

Before delving into the specifics of antibody responses in the Sri Lankan cohort, we first had to characterize DENV cases. Over the course of a year, between 2008 through 2009, 799

children were followed for fever occurrence. Children were bled at enrollment (baseline), and one year later (follow-up). If fever occurred, they were bled at the onset of fever (acute) and ≥ 10 days following fever dissipation (convalescent) [215, 216]. The level of sero-prevalence at baseline in our cohort was 53.1%, meaning half of the children entered the cohort having already been exposed to DENV (**Table 2.3**). For those children who had been exposed to only one DENV serotype, as shown by a monotypic neutralization response at baseline, we determined that all DENV serotypes had previously been introduced to this population (**Table 2.4**). Also, during the study year, children who got repeat infections were exposed to DENV1-4. The high level of initial sero-prevalence to all DENV serotypes and the circulation of all four DENV serotypes during the study year made this population ideal for studying pre-existing antibody responses. There is previous data linking specific serotypes to severe DENV epidemics and correlating sequence of infections to more severe 2° infections [32, 36-40]. A pitfall with our study is that clear conclusions based on serotype specificity of antibody responses could not be made; however, this means that our findings may be generalizable across antibody responses to multiple DENV serotypes.

**Comparison of Inapparent to Apparent Infection Ratios Across Cohorts**

In all, we characterized four classes of DENV infections: (1) 1° inapparent (N=20), (2) 1° apparent (N=15), (3) repeat inapparent (N=20), and (4) repeat apparent (N=12). The inapparent to apparent ratio was 1.48:1 (**Table 2.5**) [216]. There was no difference between the number of primary apparent and repeat apparent cases. Depending on cohort location and severity of the DENV season being monitored, cohorts from around the world have shown inapparent to apparent ratios ranging from 0.9:1 [200] through 13:1 [138, 139] (**Table 1.2**) [139]. Based on what we know about the prevalence of inapparent infections [28], the global ratio would be inferred to be about 3:1. Our ratio represents a few inherent factors of our cohort. Our apparent cases do not include DHF or DSS due to the small number of enrollees. 2008-2009 may have been a year for increased amount of DF cases in Sri Lanka, bringing the ratio closer to 1:1.

Evidence from Sri Lanka [39], and all over the world [230-238], shows that severe epidemics occur in temporal and spatial waves so we can assume that this ratio will change on an epidemic by epidemic basis. Unlike many other cohorts, particularly those recording more apparent cases than inapparent cases, our surveillance methods were not hospital- or school absentee-based. Something is to be said about a possible skewing towards apparent cases if methods include enrolling individuals who present symptoms at a hospital or children who are absent from school due to illness.

**Importance of Using Neutralization Assays to Characterize Dengue Cases**

For those cohorts that have large inapparent to apparent ratios, false characterization of inapparent cases may have occurred due to assay sensitivity and specificity. We are confident that the burden of DENV we detected in our cohort is true because we utilized a gold-standard neutralization assay to detect repeat inapparent cases. Our FACS-based neutralization assay is a high-throughput 96-well plate method that allowed us to screen baseline and follow-up samples from all children for increases in neutralization capacity (**Table 2.1**). Use of such an assay is however tedious; therefore many cohorts also utilize assays to detect secondary inapparent infections [139, 200]. IgG capture ELISA and HAI assay have been shown to detect 1° and 2° DENV infection comparably [239]. We tested the reliability of IgG ELISA to detect repeat inapparent DENV infections in our cohort. Using samples collected one year apart, we found that sensitivity and specificity of detecting repeat infections by IgG were 62.5% and 88.5%, respectively. These statistics were higher for primary infections, due to the positive predictability of IgG ELISA to simply detect seroconversion rather than antibody boosts. Using a virus-like particle neutralization assay [240] with samples from the Nicaraguan dengue cohort[139], researchers show similar sensitivity and specificity for HAI to detect 2° infections [*Harris, Eva. | personal discussion at Dengue Vaccine Consultation Meeting 2013*]. That said, the number of inapparent infections in many cohorts may be overestimated. Some other cohorts present an advantage having collected samples in closer time intervals,

from which basic HAI or IgG assays may be able to detect increases in DENV-specific antibody capacity. But, our data suggest that it is worthwhile to always characterize DENV cases using gold-standard neutralization assays. True estimates of the global burden of DENV, stemming from properly characterized cohorts, will be important when determining vaccine implementation strategies.

**What Understanding True Burden of Dengue Infection in Sri Lanka Means to the Field**

The Asian tropics represent a large portion (67%) of the global DENV infection burden. 37% of that burden is said to come from the Indian subcontinent alone [28]. Sri Lanka, a country in the Indian subcontinent, has a high prevalence of DENV infection as evidenced by our aforementioned sero-prevalence data. Two million DENV infections are said to have occurred in Sri Lanka in 2010, based on surveillance cases reported to the WHO and cartographic estimates of global burden [28]. How accurate is that published estimate of 2 million infections in Sri Lanka per year? How accurate is the estimate of DENV burden for the entire Indian subcontinent? These are questions that only intense surveillance and detection of inapparent cases in a cohort, like the PDVI cohort, can answer. Data on Sri Lankan DF cases shows that even in highest incidence, 15,000 DF cases occurred annually through 2009 [204, 241]. Our characterization of inapparent infections allowed for us to estimate the true burden of DENV infections on the Indian subcontinent, for the first time. It will be interesting to see how our data influences future estimates of the burden of DENV in Sri Lanka.

## 5.3 What are the differences between antibody responses induced by primary versus secondary infections?

**Current Understanding of Temporal Regulation of Dengue Virus Antibody Responses**

Having fully characterized primary and repeat DENV infections in the cohort, we set out to answer the question: What are the differences between antibody responses induced by primary versus secondary infections?. Primary and secondary infections are known to induce different antibody responses (**Figure 1.5**). Primary infections induce IgM responses that last up

to 6 months [112] and IgG responses that are lifelong [120]. We did not analyze IgG responses over time following 1° infections. Recently, when a group looked at IgG avidity over time, they showed IgG avidity waned after primary infections in positive correlation with decreasing neutralization breadth [113]. We found that following repeat infections, IgG responses fluctuated to some extent. From what we currently understand, secondary infections induce IgM responses that are shorter-lived and less robust than primary infections. After a secondary infection, IgG is boosted to a high level that is thought to last indefinitely [113, 114]. Our data shows that the IgG boost that is thought to occur after a secondary infection does in fact occur, particularly through convalescence (**Figure 3.3**). But following the convalescent stage, IgG antibodies return to baseline levels. This means that increases in DENV IgG after repeat infection are is not lifelong, as previously predicted. This may explain why IgG ELISA cannot be used to detect repeat inapparent infections. After a primary infection, IgG levels are increased; this is the level that we see at baseline. After 12 months, even if a dengue infection occurs, the boosted IgG responses have waned back to baseline levels. Therefore looking for significant increases in baseline and follow-up samples do not reveal an repeat inapparent infection.

**Predictions about Waning of Cross-protection**

Following 1° infections, the period of cross-protection is predicted to be anywhere from 3 months, as shown in Sabin's human challenge studies [119], up to 2 years, as shown in naturally-infected people [118, 121]. In our cohort, cross-neutralization is defined as having sero-positivity to ≥ 2 DENV serotypes. Using linear regression analysis, we show neutralization breadth wanes with time elapsed after 1° DENV infection (**Figure 3.2A**). At 10 months post-primary infection, there were still two children who exhibited cross-neutralization, one of which neutralized all serotypes. In the period of 10-12 months, neutralization breadth becomes specific to one DENV serotype. Perhaps, the cross-neutralization capacity of the remaining broad-neutralizers lasts up to 2 years like shown in previous cohorts [118, 121], but we will have to follow the children for longer to determine this to be true.

Interestingly, the period of cross-protection, in our study and others, lasts beyond the known period of IgM presence. That said, other studies have correlated IgM with cross-protection [113]; we did as well (**Figure 3.2B**). IgM positivity even correlated with neutralization titers (**Figure 3.2D**). This has been shown previously, as well [113]. Together, this data would suggest a role for IgM antibodies in early cross-neutralization, but there must be other types of antibodies, besides IgM, responsible for cross-protection beyond the 6 month mark. Following post-secondary infections, lifelong cross-protection is insinuated [116].

What we saw with neutralization responses following repeat infections was markedly different than what we saw with repeat IgG responses. Also, neutralization capacity following re-infections did not wane, as it did following 1° infections, but remained steady through the end of the study year (**Figure 3.3B-C**). This goes along with what we believe about protective antibody responses following secondary infections [116].

**Neutralization Magnitude versus Neutralization Breadth**

Characterizing neutralization breadth is a standard way by which neutralization capacity is determined [116]. But, there is another important aspect of neutralization capacity… magnitude. There are disagreeing data on whether pre-existing neutralization titers correlate with protection from subsequent DENV infection [152, 155] [*Buddhar, Darunee, et al.* | *unpublished data submitted to PLoS Neglected Tropical Diseases*]. Therefore, we felt that it was worthwhile to look at neutralization magnitude in our cohort. Before discussing our findings, it must be noted that there are pitfalls of drawing conclusions from neutralization titers. To date, no specific neutralization titer has been pinpointed as a correlate for protection. Because there are four DENV serotypes, it is quite possible that such a correlate would be different based on serotype, and even genotype. Recent failure of a dengue vaccine to protect against a particular DENV2 genotype draws eminent concern to that point [162, 180]. Also, researchers have their own favorite methods for detecting DENV neutralization [155, 214, 240, 242]. Variability across assays, and even within assays on a day-to-day basis, creates concerns about conclusions

drawn without use of an acceptable number of experimental replicates. All of these things could be reasons why previous data is conflicting.

To satisfy some of those concerns, our FACS-based DENV neutralization assay has been compared to a standard plaque-based neutralization assay [213]. Additionally, in this study, we tested the assay for reproducibility in detecting DENV infections (data not shown), and each confirmed DENV case was tested at least twice to determine $neut_{50}$ titers. We first showed that following 1° infections, while there was a trend towards negative correlation between neutralization breadth and time elapsed, neutralization titers did not decrease significantly over time like neutralization breadth (**Figure 3.2C**). It is worthwhile to note that the most significant of these correlations was that between neutralization titers of serotypes of non-exposure and time elapsed. Essentially, that means that magnitude of cross-neutralizing responses wanes quicker than magnitude of type-specific responses.

In the acute phase following repeat infections, we noticed neutralization magnitude increased to only one serotype, likely the serotype of previous infection. There was an abundance of strong broadly-neutralizing antibodies at the convalescent stage (**Figure 3.3**) (some *data not shown* | *Corbett, Kizzmekia, et. al* | *manuscript in press in Journal of Infectious Diseases*). Another study showed the same type of robustly increasing neutralization through the convalescent stage of infection [113]. At follow-up, we could not determine the relative contribution of neutralization capacity as it related to infection history so we cannot definitively state that the previously infecting serotype is responsible for the highest boosted response. But overall, the temporal trends of neutralization magnitude seen after repeat infections may be associated with original antigenic sin, which has been described for DENV infections [243-247].

## 5.4 What role do enhancing antibodies play in disease severity?

### Using K562 Cells to Determine DENV Enhancement

We then asked: What role do enhancing antibodies play in disease severity? This question is of particular interest with the plethora of data showing DENV enhancing antibodies

can facilitate the uptake of DENV particles into cells to cause an increased viral burden and severe disease [74, 78, 79, 88, 90-92, 94, 96, 105, 153, 226]. We subjected baseline samples from children who acquired repeat DENV infections to a K562 cell-based enhancement assay [226]. This is a pitfall of our study because these cells only contain FcγRIIa, which is considered the stimulatory FcγRII isotype [86, 226]. Without the presence of its inhibitory counterpart, FcγRIIb, FcγRIIa could provide a biologically-irrelevant idea of true DENV enhancement. U937 cells, which contain FcγRIIa and FcγRIIb, were tested but did not induce significant enough enhancement, perhaps due to technical issues. Primary human monocytes, which we also tested (data not shown), are the most relevant cell type to test DENV enhancement, but lack of availability of large batches of these cells called for a more feasible alternative in order to test all of our samples repetitively.

**Dengue Antibody Dependent Enhancement and Mild Disease**

We didn't find a difference in the ability of pre-existing antibodies from repeat inapparent and apparent infections to enhance DENVs (**Figure 4.2**). Without any severe DHF or DSS cases, we cannot draw any strict conclusions about the role of enhancing antibodies on dengue disease severity as a whole. What we can say is that enhancing antibodies did not play a role in inducing DF in our cohort. We were not surprised by this result because enhancing antibodies are not thought to differentiate between inapparent and mild DF [69]. Despite this finding, we were able to negatively correlate enhancement to neutralization; the balance between pathogenic and protective antibody responses which will be discussed later in the chapter.

## 5.5 What role do neutralizing antibodies play in protection from dengue disease?

**Protection from Apparent Dengue Infection Facilitated by Cross-Neutralizing Antibodies**

From a vaccine development perspective, it is of particular importance that we understand the role neutralizing antibodies play in protection from dengue disease. In that aspect, the fact that there were no DHF or DSS cases in our cohort presented an opportunity to examine fine-tuned protection between inapparent and mild DF cases. Starting with simple pre-

existing DENV-binding IgG antibody responses, we did not see a difference between repeat inapparent and apparent cases (**Figure 4.1A**). Our IgG ELISA uses a 1:1:1:1 mixture of DENV1-4 as antigen [31, 39]. There is an abundance of cross-reactive antibodies after DENV infections. Recent studies have shown that most of these cross-reactive antibodies are weakly-neutralizing and even sometimes enhancing [76, 105]. By accessing DENV binding to our antigen mixture, OD readout was just a gauge of DENV cross-reactivity, not a gauge of actual antibody function.

As shown in chapter 3, cross-reactive IgG after 1° infections may exist, but neutralization capacity wanes. That known, we tested baseline sera from children who got repeat infections for DENV-specific neutralization. We didn't find a difference in neutralization magnitude (**Figure 4.1B**). This finding goes along with previous findings that absolute DENV neutralization titers cannot be used a correlate of protection [152, 155]. We did however find that neutralization breadth correlated with clinical outcome of dengue infection. Children who got repeat inapparent infections had more broadly-neutralizing pre-existing DENV antibodies than children who got apparent re-infections (**Figure 4.1C**).

**Quantitative Analysis of Breadth of Neutralizing Antibody Responses**

We quantified neutralization breadth by inserting our neutralization data into a cartography algorithm for determining distance between the best-neutralized DENV serotype and other serotypes; similar methods have been used for influenza studies [248]. To better imagine the concept, think of the 4 DENV serotypes being placed on a map based on their antigenic distances from each other. With the algorithm, each serum tested was placed within that map based on serotype-specificity of neutralization titers. Type-specific sera clustered around one serotype and broadly-neutralizing sera clustered in the middle of multiple serotypes. The placement of groups of sera on said hypothetical map is represented by the numbers shown in **table 4.1.** A value closer to 0 essentially means the sera reside in the middle of the map and neutralize all DENVs similarly.

There are pitfalls to our method though; most notable is that we assumed the DENV serotype that a person neutralized best was the previously infecting serotype. That assumption may not hold true knowing what we know about the differential ability of DENV serotypes to be neutralized in the neutralization assay [214]. For those sera that neutralize more than one serotype, it is difficult to tell which serotype is responsible for the infection. To eliminate much of our concern about this, we compared PCR data from acute infections with neutralization titers at follow-up, following 1° apparent infections; we found our predictions of infecting serotypes based on neutralization titers held true most of the time (data not shown). Another pitfall is the use of a $neut_{50}$ titer of 20 in instances when a serum did not neutralize a serotype. Although the lowest dilution we used in our assays was 1:40, we chose 1:20 based on it being the lowest dilution typically used in neutralization assays [213, 214], but there is no biological basis to justify this cutoff. Use of ≤1:40 dilutions was not feasible due to limited DBS availability. Pitfalls aside, our quantitative analysis further exemplified what we saw when we looked at neutralization breadth: children who got repeat inapparent infections had pre-existing DENV antibodies that were more active against all DENV serotypes than children who got apparent re-infections (**Table 4.1**). With that, we propose that this algorithm be used as a secondary method to determine neutralization breadth in studies of this nature.

**Explanations for Correlation between Breadth of Neutralization and Dengue Disease Outcome**

Our findings demonstrate an association between breadth of pre-existing DENV neutralizing antibodies and the likelihood of developing an inapparent versus apparent infection. For only the second time in the field has a role for pre-existing neutralizing antibodies in protection against apparent dengue infections been shown. In the first study, individuals were extensively followed in Peru; 1°, 2°, and post-secondary infections were characterized [116]. This study showed that breadth of pre-existing neutralization antibodies correlated with protection from post-secondary infections, but not 2° infections. This brings me to a particularly

relevant point when thinking about what our data mean about protection. Because we have yet to decipher the children's infection histories, it is impossible for us to differentiate between 2° and post-secondary infections. We tried to determine prior infection history based on neutralization profiles of baseline samples, but this method did not produce clear results (data not shown). The only indication we have that these infections are "true" secondary infections is that the degree of neutralization breadth observed at baseline for repeat cases was not significantly different from the degree of neutralization breadth at follow-up for 1° cases (**Table 4.1**).

The other non-exclusive possibility for our finding is that individuals who have cross-neutralizing antibodies at baseline got their previous infection recently, within the window of time that cross-neutralization exists. Experimentally, we could not determine if previous infections occurred in the entire 1-2 year window of cross-protection, but we utilized IgM as a marker for determining recent previous infections, within the last 6 months. There was no correlation between neutralization breadth and IgM response in baseline samples from these children (Figure 4.1D). This data suggests that recent infection within the last 6 months cannot be used to explain the association between broad-neutralization and protection. The simplest explanation for this association is that some individuals inherently induce long-lived broadly neutralizing responses after primary infections. Our data showing broad-neutralizing antibody responses up to 10 months after some primary infections would suggest that this as a possibility. All possibilities considered, it is most logical that a combination of time since previous exposure and infection history influence one's ability to neutralize multiple serotypes, consequently influencing protection from apparent dengue disease.

**Balance Between Pathogenic and Protective Antibody Responses**

Progression towards or protection from DENV diseases is likely a matter of the balance between enhancing and neutralizing antibody responses [115]. Comparisons between neutralizing and enhancing antibodies have been made in mouse models and other human

cohorts [137, 249, 250]. We tested the theory of pathogenic versus protective responses using samples from our cohort. For DENV1-3, as a child's neutralization capacity increased, their ability to enhance that serotype decreased (**Figure 4.2C-E**). We did not see this trend with DENV4 (**Figure 4.2F**) which may be due to the fact that the ability of these sera to neutralize DENV4 was typically low. The negative correlation we see with neutralization and enhancement in this cohort justifies the idea that neutralizing and enhancing antibodies are completely different antibodies. An analysis of the correlation between neutralization titer against heterotypic serotypes and enhancing ability would have been nice to further solidify this idea. In just, we found a correlation between neutralization breadth and protective disease outcome, but did not find a correlation with enhancement ability and clinical disease outcome. Therefore, with regards to differentiating between inapparent and mildly apparent dengue cases, pre-existing neutralization capacity is more telling.

## 5.6 Snapshot of Research Findings

**In summary, we drew the following major conclusions from our studies:**

(1) What are the differences between antibody responses induced by primary versus secondary infections?
    a. IgG ELISA was determined to be unreliable for detecting repeat DENV infections utilizing paired samples collected 12 months apart.
    b. Primary inapparent and apparent DENV infections result in similar IgG and neutralization capacity.
    c. After 1° infections, neutralization capacity is negatively correlated with time elapsed since DENV exposure.
    d. Following repeat DENV infections, IgG levels increase during a brief span around febrile illness, but eventually decline to baseline levels. On the other hand, neutralizing antibody capacity increases and remains steady.

(2) What role do enhancing antibodies play in disease severity?

a. There was no correlation between pre-existing enhancement capacity and inapparent or apparent outcome following repeat infection.
b. Pre-existing neutralization capacity and enhancement ability were negatively correlated.

(3) What role do neutralizing antibodies play in protection from dengue disease?

a. Repeat inapparent infections are more likely to occur in children with broadly-neutralizing pre-existing antibody responses than repeat apparent infections, indicating an association between neutralization breath and protection from apparent DENV infection.
b. Breadth of pre-existing neutralizing antibodies is not correlated with IgM level, and thus not indicative of previous DENV exposure within the last 6 months.

Able oFuture Directions

**Dissecting Infection History of Children with Repeat DENV Infections**

Our findings leave a wide-open arena for exploration of specific mechanisms of pre-existing neutralizing antibody responses to DENV infections as they correlate with protection. It will be important to dissect infection history of children with repeat DENV infections to determine whether children exhibiting broadly-neutralizing baseline antibodies have been previously exposed to dengue more than once. This will also allow us to test the hypothesis that serotype specificity of prior DENV exposure influences neutralization/protection or lack thereof (perhaps enhancement) upon repeat exposure. As aforementioned, neutralization responses at baseline do allow us to decisively determine infection history.

Therefore, we propose a couple of options for dissecting infection history using samples from our cohort. One method is to deplete baseline sera with native dengue virions of particular serotypes and then assess neutralization capacity of depleted sera [77]. If a serum contains type-specific neutralizing responses to ≥ 2 serotypes following depletion, that individual has been exposed to DENV multiple times prior to enrollment. The other method is to use a

blockade of binding (BoB) assay. In BoB assays, DENVs are captured on 96-well plates, experimental sera are allowed to bind to DENVs, then a flourochrome-conjugated type-specific mAb is added. If antibodies in a serum block binding by the mAb that means that there are type-specific antibodies in said serum. If a serum contains type-specific responses to ≥ 2 serotypes in the BoB assay, that individual has been exposed to DENV multiple times prior to enrollment.

**Dissecting Epitope-Specificity of Pre-existing Antibodies**

One could take this analysis a step further to dissect epitope-specificity of pre-existing antibodies in children with repeat DENV infections, testing the hypothesis that quantity of pre-infection antibodies that target prM and E fusion loop epitopes (highly-conserved regions) correlate with cross-neutralization [104, 106, 222]. Conversely, we can test the hypothesis that quantity of pre-infection antibodies that target the EDI/II region correlates with type-specificity [76, 77, 102, 103]. A strategy by which to pursue this direction was previously published [106]. Briefly, anti-FL antibodies were determined by capture ELISA using FL-mutated VLPs. The concentration of anti-FL antibodies in each serum was correlated with neutralization capacity. A similar approach can be used for all relative epitopes of DENV. Also we could generate huMAbs from PMBCs collected from these children, as previously described [74]. We could then use neutralizing huMAbs to apply pressure to DENVs in culture to generate escape mutants, which will allow us to map epitope-specificity the huMAbs [76].

**Following Cohort into Year Two of Sample Collection and Possibly into a Vaccine Trial**

Additionally, a second year of samples was collected from 2009 through 2010, which we did not completely analyze for the purposes of this dissertation. Based on our own preliminary analysis and knowledge that a wave of severe DENV1 replaced DENV2/3 in Colombo, Sri Lanka during that year [33], we suspect many interesting findings will come from applying the specific aims from this dissertation to those samples. The most interesting experiments would be a detailed analysis of burden of DENV infection in year 2, from which, we could compare inapparent to apparent infection ratios across two markedly different epidemics and provide the

field with a longer-termed analysis of the burden of DENV infection in Sri Lanka. We could also determine, once infection history is assessed, if there is a particular level of immunity to certain serotypes that render 2° DENV1 infections more or less severe. Cohorts like this provide the ideal setting for vaccine trials because the participants' previous DENV immunity is mapped out in detail. Introducing a dengue vaccine candidate into this cohort could provide the field with an understanding of how pre-existing immunity affects vaccine efficacy.

### 5.8 Closing Statement

This dissertation details the characterization of human antibody responses to DENV infections in a Sri Lankan Pediatric Cohort. We were able to characterize the true burden of DENV infection in the Indian subcontinent, analyze the temporal regulation of antibodies following DENV infections, and, most importantly, correlate DENV neutralization breadth with protection from subsequent apparent DENV infection. This finding implicates neutralization capacity as having an important role in protection against clinical manifestations. Our research is innovative because it is based on existing clinical samples collected from a cohort of children before and after experiencing inapparent and apparent DENV infections. DENV vaccine development is hindered by a gap in understanding of the properties of antibodies that protect or possibly enhance disease. Our results will provide insight into pre-existing antibody responses that correlate with protection. These findings may be applicable to developing and evaluating DENV vaccines in the future.

## REFERENCES


1. **Normile, Dennis, *Surprising New Dengue Virus Throws a Spanner in Disease Control Efforts.* Science, 2013. 342(6157): p. 415.**

2. **Jessie, Kala, Fong, Mun Yik, Devi, Shamala, Lam, Sai Kit, and Wong, K. Thong, *Localization of Dengue Virus in Naturally Infected Human Tissues, by Immunohistochemistry and In Situ Hybridization.* Journal of Infectious Diseases, 2004. 189(8): p. 1411-1418.**

3. **Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M., *Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate.* Nat Med, 1997. 3(8): p. 866-71.**

4. **Martínez-Barragán, José de Jesús and del Angel, Rosa M., *Identification of a Putative Coreceptor on Vero Cells That Participates in Dengue 4 Virus Infection.* Journal of Virology, 2001. 75(17): p. 7818-7827.**

5. **Chen, Yun-Chi, Wang, Sheng-Yuan, and King, Chwan-Chuen, *Bacterial Lipopolysaccharide Inhibits Dengue Virus Infection of Primary Human Monocytes/Macrophages by Blockade of Virus Entry via a CD14-Dependent Mechanism.* Journal of Virology, 1999. 73(4): p. 2650-2657.**

6. **Reyes-del Valle, Jorge, Chávez-Salinas, Salvador, Medina, Fernando, and del Angel, Rosa M., *Heat Shock Protein 90 and Heat Shock Protein 70 Are Components of Dengue Virus Receptor Complex in Human Cells.* Journal of Virology, 2005. 79(8): p. 4557-4567.**

7. **Lozach, Pierre-Yves, Burleigh, Laura, Staropoli, Isabelle, Navarro-Sanchez, Erika, Harriague, Julie, Virelizier, Jean-Louis, Rey, Felix A., Desprès, Philippe, Arenzana-Seisdedos, Fernando, and Amara, Ali, *Dendritic Cell-specific Intercellular Adhesion Molecule 3-grabbing Non-integrin (DC-SIGN)-mediated Enhancement of Dengue Virus Infection Is Independent of DC-SIGN Internalization Signals.* Journal of Biological Chemistry, 2005. 280(25): p. 23698-23708.**

8. **Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J. L., Arenzana-Seisdedos, F., and Despres, P., *Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses.* EMBO Rep, 2003. 4(7): p. 723-8.**

9. **Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Birx, D. L., Steinman, R. M., Schlesinger, S., and Marovich, M. A., *DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells.* J Exp Med, 2003. 197(7): p. 823-9.**

10. **Acosta, Eliana G., Talarico, Laura B., and Damonte, Elsa B., *Cell entry of dengue virus.* Future Virology, 2008. 3(5): p. 471-479.**

11. **van der Schaar, Hilde M., Rust, Michael J., Chen, Chen, van der Ende-Metselaar, Heidi, Wilschut, Jan, Zhuang, Xiaowei, and Smit, Jolanda M., *Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells.* PLoS Pathog, 2008. 4(12): p. e1000244.**

12. **Acosta, Eliana G., Castilla, Viviana, and Damonte, Elsa B., *Alternative infectious entry pathways for dengue virus serotypes into mammalian cells.* Cellular Microbiology, 2009. 11(10): p. 1533-1549.**

13. **Zaitseva, Elena, Yang, Sung-Tae, Melikov, Kamran, Pourmal, Sergei, and Chernomordik, Leonid V., *Dengue Virus Ensures Its Fusion in Late Endosomes Using Compartment-Specific Lipids.* PLoS Pathog, 2010. 6(10): p. e1001131.**

14. **Modis, Y., Ogata, S., Clements, D., and Harrison, S. C., *Structure of the dengue virus envelope protein after membrane fusion.* Nature, 2004. 427(6972): p. 313-9.**

15. **Stiasny, Karin and Heinz, Franz X., *Flavivirus membrane fusion.* Journal of General Virology, 2006. 87(10): p. 2755-2766.**

16. **Clyde, K., Kyle, J. L., and Harris, E., *Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis.* J Virol, 2006. 80(23): p. 11418-31.**

17. **Kuhn, Richard J., Zhang, Wei, Rossmann, Michael G., Pletnev, Sergei V., Corver, Jeroen, Lenches, Edith, Jones, Christopher T., Mukhopadhyay, Suchetana, Chipman, Paul R., Strauss, Ellen G., Baker, Timothy S., and Strauss, James H., *Structure of Dengue Virus.* Cell. 108(5): p. 717-725.**

18. **Yu, I. M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G., and Chen, J., *Structure of the immature dengue virus at low pH primes proteolytic maturation.* Science, 2008. 319(5871): p. 1834-7.**

19. **Rodenhuis-Zybert, Izabela A., van der Schaar, Hilde M., da Silva Voorham, Júlia M., van der Ende-Metselaar, Heidi, Lei, Huan-Yao, Wilschut, Jan, and Smit, Jolanda M., *Immature Dengue Virus: A Veiled Pathogen?* PLoS Pathog, 2010. 6(1): p. e1000718.**

20. **Nelson, S., Jost, C. A., Xu, Q., Ess, J., Martin, J. E., Oliphant, T., Whitehead, S. S., Durbin, A. P., Graham, B. S., Diamond, M. S., and Pierson, T. C., *Maturation of***

***West Nile virus modulates sensitivity to antibody-mediated neutralization.*** **PLoS Pathog, 2008. 4(5): p. e1000060.**

**21. Mackenzie, J. S., Gubler, D. J., and Petersen, L. R., *Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses.* Nat Med, 2004. 10(12 Suppl): p. S98-109.**

**22. Guzman, A. and Isturiz, R. E., *Update on the global spread of dengue.* Int J Antimicrob Agents, 2010. 36 Suppl 1: p. S40-2.**

**23. Jansen, Cassie C. and Beebe, Nigel W., *The dengue vector Aedes aegypti: what comes next.* Microbes and Infection, 2010. 12(4): p. 272-279.**

**24. Gubler, Duane J., *The Economic Burden of Dengue.* The American Journal of Tropical Medicine and Hygiene, 2012. 86(5): p. 743-744.**

**25. Radke, E. G., Gregory, C. J., Kintziger, K. W., Sauber-Schatz, E. K., Hunsperger, E. A., Gallagher, G. R., Barber, J. M., Biggerstaff, B. J., Stanek, D. R., Tomashek, K. M., and Blackmore, C. G., *Dengue outbreak in Key West, Florida, USA, 2009.* Emerg Infect Dis, 2012. 18(1): p. 135-7.**

**26. Bouri, N., Sell, T. K., Franco, C., Adalja, A. A., Henderson, D. A., and Hynes, N. A., *Return of epidemic dengue in the United States: implications for the public health practitioner.* Public Health Rep, 2012. 127(3): p. 259-66.**

**27. Lambrechts, Louis, Scott, Thomas W., and Gubler, Duane J., *Consequences of the Expanding Global Distribution of <italic>Aedes albopictus</italic> for Dengue Virus Transmission.* PLoS Negl Trop Dis, 2010. 4(5): p. e646.**

**28. Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J., and Hay, S. I., *The global distribution and burden of dengue.* Nature, 2013. 496(7446): p. 504-7.**

**29. Vitarana, T., *Viral diseases in Sri Lanka: a national overview*, in *Viral diseases in south east asia and the western pacific*, J.S. Mackenzie, Editor 1982, Academic Press: London. p. 198-204.**

**30. Sirisena, P. D. and Noordeen, F., *Evolution of dengue in Sri Lanka-changes in the virus, vector, and climate.* Int J Infect Dis, 2014. 19: p. 6-12.**

31. Kanakaratne, N., Wahala, W. M., Messer, W. B., Tissera, H. A., Shahani, A., Abeysinghe, N., de-Silva, A. M., and Gunasekera, M., *Severe dengue epidemics in Sri Lanka, 2003-2006.* Emerg Infect Dis, 2009. 15(2): p. 192-9.

32. Messer, W.B., Gubler, D.G., Harris, E., Sivananthan, K., and de Silva, A.M., *Emergence and Global Spread of a Dengue Serotype 3, Subtype III virus.* Emerg Infect Dis, 2003. 9(7): p. 800-809.

33. Tissera, H. A., Ooi, E. E., Gubler, D. J., Tan, Y., Logendra, B., Wahala, W. M., de Silva, A. M., Abeysinghe, M. R., Palihawadana, P., Gunasena, S., Tam, C. C., Amarasinghe, A., Letson, G. W., Margolis, H. S., and De Silva, A. D., *New dengue virus type 1 genotype in Colombo, Sri Lanka.* Emerg Infect Dis, 2011. 17(11): p. 2053-5.

34. *Dengue hemorrhagic fever: diagnosis, treatment, prevention, and control*, 1997, World Health Organization.

35. *Dengue: Guildlines for Diagnosis, Treatment, Prevention, and Control*, 2009, World Health Organization

Special Programme for Research and Training in Tropical Diseases

36. Vaughn, David W., Green, Sharone, Kalayanarooj, Siripen, Innis, Bruce L., Nimmannitya, Suchitra, Suntayakorn, Saroj, Endy, Timothy P., Raengsakulrach, Boonyos, Rothman, Alan L., Ennis, Francis A., and Nisalak, Ananda, *Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity.* Journal of Infectious Diseases, 2000. 181(1): p. 2-9.

37. Fried, J. R., Gibbons, R. V., Kalayanarooj, S., Thomas, S. J., Srikiatkhachorn, A., Yoon, I. K., Jarman, R. G., Green, S., Rothman, A. L., and Cummings, D. A., *Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006.* PLoS Negl Trop Dis, 2010. 4(3): p. e617.

38. OhAinle, Molly, Balmaseda, Angel, Macalalad, Alexander R., Tellez, Yolanda, Zody, Michael C., Saborío, Saira, Nuñez, Andrea, Lennon, Niall J., Birren, Bruce W., Gordon, Aubree, Henn, Matthew R., and Harris, Eva, *Dynamics of Dengue Disease Severity Determined by the Interplay Between Viral Genetics and Serotype-Specific Immunity.* Sci Transl Med, 2011. 3(114): p. 114ra128.

39. Messer, William B, Vitarana, U Tissa, Sivananthan, Kamalanayani, Elvtigala, Jayanthi, Preethimala, L D, Ramesh, R, Withana, Nalini, Gubler, Duane J, and De Silva, Aravinda M, *Epidemiology of dengue in Sri Lanka before and after the*

***emergence of epidemic dengue hemorrhagic fever.*** **Am J Trop Med Hyg, 2002. 66(6): p. 765-73.**

**40. Adams, B., Holmes, E. C., Zhang, C., Mammen, M. P., Nimmannitya, S., Kalayanarooj, S., and Boots, M., *Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok.* Proceedings of the National Academy of Sciences, 2006. 103(38): p. 14234-14239.**

**41. Leitmeyer, Katrin C., Vaughn, David W., Watts, Douglas M., Salas, Rosalba, Villalobos, Iris, Chacon, de, Ramos, Celso, and Rico-Hesse, Rebeca, *Dengue Virus Structural Differences That Correlate with Pathogenesis.* J Virol, 1999. 73(6): p. 4738-4747.**

**42. Tuiskunen, A., Monteil, V., Plumet, S., Boubis, L., Wahlstrom, M., Duong, V., Buchy, P., Lundkvist, A., Tolou, H., and Leparc-Goffart, I., *Phenotypic and genotypic characterization of dengue virus isolates differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome.* Arch Virol, 2011. 156(11): p. 2023-32.**

**43. Mazzon, Michela, Jones, Meleri, Davidson, Andrew, Chain, Benjamin, and Jacobs, Michael, *Dengue Virus NS5 Inhibits Interferon-α Signaling by Blocking Signal Transducer and Activator of Transcription 2 Phosphorylation.* Journal of Infectious Diseases, 2009. 200(8): p. 1261-1270.**

**44. Morrison, J., Aguirre, S., and Fernandez-Sesma, A., *Innate immunity evasion by Dengue virus.* Viruses, 2012. 4(3): p. 397-413.**

**45. Wu, S. J., Grouard-Vogel, G., Sun, W., Mascola, J. R., Brachtel, E., Putvatana, R., Louder, M. K., Filgueira, L., Marovich, M. A., Wong, H. K., Blauvelt, A., Murphy, G. S., Robb, M. L., Innes, B. L., Birx, D. L., Hayes, C. G., and Frankel, S. S., *Human skin Langerhans cells are targets of dengue virus infection.* Nat Med, 2000. 6(7): p. 816-20.**

**46. Libraty, Daniel H., Pichyangkul, Sathit, Ajariyakhajorn, Chuanpis, Endy, Timothy P., and Ennis, Francis A., *Human Dendritic Cells Are Activated by Dengue Virus Infection: Enhancement by Gamma Interferon and Implications for Disease Pathogenesis.* Journal of Virology, 2001. 75(8): p. 3501-3508.**

**47. Warke, Rajas V., Becerra, Aniuska, Zawadzka, Agatha, Schmidt, Diane J., Martin, Katherine J., Giaya, Kris, Dinsmore, Jonathan H., Woda, Marcia, Hendricks, Gregory, Levine, Tracy, Rothman, Alan L., and Bosch, Irene, *Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells.* Journal of General Virology, 2008. 89(7): p. 1605-1615.**

48. **Fink, J., Gu, F., Ling, L., Tolfvenstam, T., Olfat, F., Chin, K. C., Aw, P., George, J., Kuznetsov, V. A., Schreiber, M., Vasudevan, S. G., and Hibberd, M. L., *Host gene expression profiling of dengue virus infection in cell lines and patients.* PLoS Negl Trop Dis, 2007. 1(2): p. e86.**

49. **Moreno-Altamirano, M. M., Romano, M., Legorreta-Herrera, M., Sanchez-Garcia, F. J., and Colston, M. J., *Gene expression in human macrophages infected with dengue virus serotype-2.* Scand J Immunol, 2004. 60(6): p. 631-8.**

50. **Warke, R. V., Xhaja, K., Martin, K. J., Fournier, M. F., Shaw, S. K., Brizuela, N., de Bosch, N., Lapointe, D., Ennis, F. A., Rothman, A. L., and Bosch, I., *Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells.* J Virol, 2003. 77(21): p. 11822-32.**

51. **Sariol, Carlos A., Muñoz-Jordán, Jorge L., Abel, Kristina, Rosado, Lymarie C., Pantoja, Petraleigh, Giavedoni, Luis, Rodriguez, Idia Vanessa, White, Laura J., Martínez, Melween, Arana, Teresa, and Kraiselburd, Edmundo N., *Transcriptional Activation of Interferon-Stimulated Genes but Not of Cytokine Genes after Primary Infection of Rhesus Macaques with Dengue Virus Type 1.* Clinical and Vaccine Immunology, 2007. 14(6): p. 756-766.**

52. **Navarro-Sanchez, E., Despres, P., and Cedillo-Barron, L., *Innate immune responses to dengue virus.* Arch Med Res, 2005. 36(5): p. 425-35.**

53. **Green, Angela M., Beatty, P. Robert, Hadjilaou, Alexandros, and Harris, Eva, *Innate Immunity to Dengue Virus Infection and Subversion of Antiviral Responses.* Journal of Molecular Biology, 2014. 426(6): p. 1148-1160.**

54. **Kurane, I., Innis, B. L., Nimmannitya, S., Nisalak, A., Rothman, A. L., Livingston, P. G., Janus, J., and Ennis, F. A., *Human immune responses to dengue viruses.* Southeast Asian J Trop Med Public Health, 1990. 21(4): p. 658-62.**

55. **Rizvi, N., Chaturvedi, U. C., and Mathur, A., *Obligatory role of macrophages in dengue virus antigen presentation to B lymphocytes.* Immunology, 1989. 67(1): p. 38-43.**

56. **Rizvi, N., Chaturvedi, U. C., and Mathur, A., *Inhibition of the presentation of dengue virus antigen by macrophages to B cells by serine-protease inhibitors.* Int J Exp Pathol, 1991. 72(1): p. 23-9.**

57. **Chaturvedi, U. C., Nagar, R., and Shrivastava, R., *Macrophage and dengue virus: friend or foe?* Indian J Med Res, 2006. 124(1): p. 23-40.**

**58.** **Kurane, I., Innis, B. L., Nisalak, A., Hoke, C., Nimmannitya, S., Meager, A., and Ennis, F. A., *Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production.* J Clin Invest, 1989. 83(2): p. 506-13.**

**59.** **Loke, Hsin, Bethell, Delia B., Phuong, C. X. T., Dung, Minh, Schneider, Joerg, White, Nicholas J., Day, Nicholas P., Farrar, Jeremy, and Hill, Adrian V. S., *Strong HLA Class I–Restricted T Cell Responses in Dengue Hemorrhagic Fever: A Double-Edged Sword?* Journal of Infectious Diseases, 2001. 184(11): p. 1369-1373.**

**60.** **Simmons, C. P., Dong, T., Chau, N. V., Dung, N. T., Chau, T. N., Thao le, T. T., Hien, T. T., Rowland-Jones, S., and Farrar, J., *Early T-cell responses to dengue virus epitopes in Vietnamese adults with secondary dengue virus infections.* J Virol, 2005. 79(9): p. 5665-75.**

**61.** **Luhn, K., Simmons, C. P., Moran, E., Dung, N. T., Chau, T. N., Quyen, N. T., Thao le, T. T., Van Ngoc, T., Dung, N. M., Wills, B., Farrar, J., McMichael, A. J., Dong, T., and Rowland-Jones, S., *Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection.* J Exp Med, 2007. 204(5): p. 979-85.**

**62.** **Pang, Tikki, Cardosa, Mary Jane, and Guzman, Maria G., *Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF//DSS).* Immunol Cell Biol, 2006. 85(1): p. 43-45.**

**63.** **Rathakrishnan, Anusyah, Wang, Seok Mui, Hu, Yongli, Khan, Asif M., Ponnampalavanar, Sasheela, Lum, Lucy Chai See, Manikam, Rishya, and Sekaran, Shamala Devi, *Cytokine Expression Profile of Dengue Patients at Different Phases of Illness.* PLoS One, 2012. 7(12): p. e52215.**

**64.** **Mongkolsapaya, Juthathip, Duangchinda, Thaneeya, Dejnirattisai, Wanwisa, Vasanawathana, Sirijit, Avirutnan, Panisadee, Jairungsri, Aroonroong, Khemnu, Nuanpan, Tangthawornchaikul, Nattaya, Chotiyarnwong, Pojchong, Sae-Jang, Kanokwan, Koch, Michael, Jones, Yvonne, McMichael, Andrew, Xu, Xiaoning, Malasit, Prida, and Screaton, Gavin, *T Cell Responses in Dengue Hemorrhagic Fever: Are Cross-Reactive T Cells Suboptimal?* The Journal of Immunology, 2006. 176(6): p. 3821-3829.**

**65.** **Yamanaka, Atsushi, Hendrianto, Eryk, Mulyatno, Kris C., Susilowati, Helen, Ginting, Amor P., Sary, Dian D., Soegijanto, Soegeng, and Konishi, Eiji, *Correlation between Complement Component Levels and Disease Severity in Dengue Patients in Indonesia.* Japanese Journal of Infectious Diseases, 2013. 66(5): p. 366-374.**

**66.** **Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., Jairungsri, A., Kanlaya, R., Tangthawornchaikul, N.,**

**Puttikhunt, C., Pattanakitsakul, S. N., Yenchitsomanus, P. T., Mongkolsapaya, J., Kasinrerk, W., Sittisombut, N., Husmann, M., Blettner, M., Vasanawathana, S., Bhakdi, S., and Malasit, P., *Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement.* J Infect Dis, 2006. 193(8): p. 1078-88.**

**67. Nascimento, Eduardo J. M., Silva, Ana M., Cordeiro, Marli T., Brito, Carlos A., Gil, Laura H. V. G., Braga-Neto, Ulisses, and Marques, Ernesto T. A., *Alternative Complement Pathway Deregulation Is Correlated with Dengue Severity.* PLoS One, 2009. 4(8): p. e6782.**

**68. Cardosa, M. J., Porterfield, J. S., and Gordon, S., *Complement receptor mediates enhanced flavivirus replication in macrophages.* J Exp Med, 1983. 158(1): p. 258-63.**

**69. Burke, D. S., Nisalak, A., Johnson, D. E., and Scott, R. M., *A prospective study of dengue infections in Bangkok.* Am J Trop Med Hyg, 1988. 38(1): p. 172-80.**

**70. Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., Phanthumachinda, B., and Halstead, S. B., *Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak.* Am J Epidemiol, 1984. 120(5): p. 653-69.**

**71. Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S., and Morier, L., *Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study.* Am J Trop Med Hyg, 1990. 42(2): p. 179-84.**

**72. Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazques, S., Delgado, I., and Halstead, S. B., *Epidemiologic studies on Dengue in Santiago de Cuba, 1997.* Am J Epidemiol, 2000. 152(9): p. 793-9; discussion 804.**

**73. Guzman, Maria G., Alvarez, Angel, Vazquez, Susana, Alvarez, Mayling, Rosario, Delfina, Pelaez, Otto, Cruz, Guillermo, Rodriguez, Rosmari, Pavon, Alequis, Gonzalez, Annia, Morier, Luis, Ruiz, Dydie, Kouri, Gustavo, and Halstead, Scott B., *Epidemiological studies on dengue virus type 3 in Playa municipality, Havana, Cuba, 2001–2002.* IJID, 2012. 16(3): p. e198-e203.**

**74. Beltramello, M., Williams, K. L., Simmons, C. P., Macagno, A., Simonelli, L., Quyen, N. T., Sukupolvi-Petty, S., Navarro-Sanchez, E., Young, P. R., de Silva, A. M., Rey, F. A., Varani, L., Whitehead, S. S., Diamond, M. S., Harris, E., Lanzavecchia, A., and Sallusto, F., *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. 271-83.**

**75.** **Schieffelin, J. S., Costin, J. M., Nicholson, C. O., Orgeron, N. M., Fontaine, K. A., Isern, S., Michael, S. F., and Robinson, J. E., *Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient.* Virol J, 2010. 7: p. 28.**

**76.** **de Alwis, Ruklanthi, Beltramello, Martina, Messer, William B., Sukupolvi-Petty, Soila, Wahala, Wahala M. P. B., Kraus, Annette, Olivarez, Nicholas P., Pham, Quang, Brian, James, Tsai, Wen-Yang, Wang, Wei-Kung, Halstead, Scott, Kliks, Srisakul, Diamond, Michael S., Baric, Ralph, Lanzavecchia, Antonio, Sallusto, Federica, and de Silva, Aravinda M., *In-Depth Analysis of the Antibody Response of Individuals Exposed to Primary Dengue Virus Infection.* PLoS Negl Trop Dis, 2011. 5(6): p. e1188.**

**77.** **de Alwis, Ruklanthi, Smith, Scott A., Olivarez, Nicholas P., Messer, William B., Huynh, Jeremy P., Wahala, Wahala M. P. B., White, Laura J., Diamond, Michael S., Baric, Ralph S., Crowe, James E., and de Silva, Aravinda M., *Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions.* Proceedings of the National Academy of Sciences, 2012. 109(19): p. 7439-7444.**

**78.** **Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L., and Burke, D. S., *Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever.* Am J Trop Med Hyg, 1989. 40(4): p. 444-51.**

**79.** **Wahala, W. M. and Silva, A. M., *The human antibody response to dengue virus infection.* Viruses, 2011. 3(12): p. 2374-95.**

**80.** **Flipse, Jacky, Wilschut, Jan, and Smit, Jolanda M., *Molecular Mechanisms Involved in Antibody-Dependent Enhancement of Dengue Virus Infection in Humans.* Traffic, 2013. 14(1): p. 25-35.**

**81.** **Dejnirattisai, W., Jumnainsong, A., Onsirisakul, N., Fitton, P., Vasanawathana, S., Limpitikul, W., Puttikhunt, C., Edwards, C., Duangchinda, T., Supasa, S., Chawansuntati, K., Malasit, P., Mongkolsapaya, J., and Screaton, G., *Cross-reacting antibodies enhance dengue virus infection in humans.* Science, 2010. 328(5979): p. 745-8.**

**82.** **Falconar, A. K., *Identification of an epitope on the dengue virus membrane (M) protein defined by cross-protective monoclonal antibodies: design of an improved epitope sequence based on common determinants present in both envelope (E and M) proteins.* Arch Virol, 1999. 144(12): p. 2313-30.**

**83.** **Kuno, G., Vorndam, A. V., Gubler, D. J., and Gomez, I., *Study of anti-dengue NS1 antibody by western blot.* J Med Virol, 1990. 32(2): p. 102-8.**

**84.** **Shu, P. Y., Chen, L. K., Chang, S. F., Yueh, Y. Y., Chow, L., Chien, L. J., Chin, C., Lin, T. H., and Huang, J. H., *Dengue NS1-specific antibody responses: isotype distribution and serotyping in patients with Dengue fever and Dengue hemorrhagic fever.* J Med Virol, 2000. 62(2): p. 224-32.**

**85.** **Littaua, R, Kurane, I, and Ennis, F A, *Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection.* The Journal of Immunology, 1990. 144(8): p. 3183-6.**

**86.** **Boonnak, K., Slike, B. M., Donofrio, G. C., and Marovich, M. A., *Human FcgammaRII cytoplasmic domains differentially influence antibody-mediated dengue virus infection.* J Immunol, 2013. 190(11): p. 5659-65.**

**87.** **Chan, K. R., Zhang, S. L., Tan, H. C., Chan, Y. K., Chow, A., Lim, A. P., Vasudevan, S. G., Hanson, B. J., and Ooi, E. E., *Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection.* Proc Natl Acad Sci U S A, 2011. 108(30): p. 12479-84.**

**88.** **Goncalvez, A. P., Engle, R. E., St Claire, M., Purcell, R. H., and Lai, C. J., *Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention.* Proc Natl Acad Sci U S A, 2007. 104(22): p. 9422-7.**

**89.** **Boonnak, Kobporn, Dambach, Kaitlyn M., Donofrio, Gina C., Tassaneetrithep, Boonrat, and Marovich, Mary A., *Cell Type Specificity and Host Genetic Polymorphisms Influence Antibody-Dependent Enhancement of Dengue Virus Infection.* Journal of Virology, 2011. 85(4): p. 1671-1683.**

**90.** **Yamanaka, Atsushi, Kosugi, Saori, and Konishi, Eiji, *Infection-Enhancing and -Neutralizing Activities of Mouse Monoclonal Antibodies against Dengue Type 2 and 4 Viruses Are Controlled by Complement Levels.* Journal of Virology, 2008. 82(2): p. 927-937.**

**91.** **Moi, Meng Ling, Lim, Chang-Kweng, Chua, Kaw Bing, Takasaki, Tomohiko, and Kurane, Ichiro, *Dengue Virus Infection-Enhancing Activity in Serum Samples with Neutralizing Activity as Determined by Using FcγR-Expressing Cells.* PLoS Negl Trop Dis, 2012. 6(2): p. e1536.**

**92.** **Chareonsirisuthigul, Takol, Kalayanarooj, Siripen, and Ubol, Sukathida, *Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells.* Journal of General Virology, 2007. 88(2): p. 365-375.**

**93.** **Espada-Murao, L. A. and Morita, K., *Dengue and soluble mediators of the innate immune system.* Trop Med Health, 2011. 39(4 Suppl): p. 53-62.**

**94.** **Modhiran, N., Kalayanarooj, S., and Ubol, S., *Subversion of innate defenses by the interplay between DENV and pre-existing enhancing antibodies: TLRs signaling collapse.* PLoS Negl Trop Dis, 2010. 4(12): p. e924.**

**95.** **Kou, Z., Lim, J. Y., Beltramello, M., Quinn, M., Chen, H., Liu, S., Martinez-Sobrido, L., Diamond, M. S., Schlesinger, J. J., de Silva, A., Sallusto, F., and Jin, X., *Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes.* Virology, 2011. 410(1): p. 240-7.**

**96.** **Ubol, S., Phuklia, W., Kalayanarooj, S., and Modhiran, N., *Mechanisms of immune evasion induced by a complex of dengue virus and preexisting enhancing antibodies.* J Infect Dis, 2010. 201(6): p. 923-35.**

**97.** **Rolph, M. S., Zaid, A., Rulli, N. E., and Mahalingam, S., *Downregulation of interferon-beta in antibody-dependent enhancement of dengue viral infections of human macrophages is dependent on interleukin-6.* J Infect Dis, 2011. 204(3): p. 489-91.**

**98.** **Crill, Wayne D. and Roehrig, John T., *Monoclonal Antibodies That Bind to Domain III of Dengue Virus E Glycoprotein Are the Most Efficient Blockers of Virus Adsorption to Vero Cells.* Journal of Virology, 2001. 75(16): p. 7769-7773.**

**99.** **Wahala, W. M. P. B., Kraus, Annette A., Haymore, Laura B., Accavitti-Loper, Mary Ann, and de Silva, Aravinda M., *Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody.* Virology, 2009. 392(1): p. 103-113.**

**100.** **VanBlargan, Laura A., Mukherjee, Swati, Dowd, Kimberly A., Durbin, Anna P., Whitehead, Stephen S., and Pierson, Theodore C., *The Type-Specific Neutralizing Antibody Response Elicited by a Dengue Vaccine Candidate Is Focused on Two Amino Acids of the Envelope Protein.* PLoS Pathog, 2013. 9(12): p. e1003761.**

**101.** **Smith, S. A., de Alwis, R., Kose, N., Durbin, A. P., Whitehead, S. S., de Silva, A. M., and Crowe, J. E., Jr., *Human monoclonal antibodies derived from memory B cells following live attenuated dengue virus vaccination or natural infection exhibit similar characteristics.* J Infect Dis, 2013. 207(12): p. 1898-908.**

**102.** **Fibriansah, G., Tan, J. L., Smith, S. A., de Alwis, A. R., Ng, T. S., Kostyuchenko, V. A., Ibarra, K. D., Wang, J., Harris, E., de Silva, A., Crowe, J. E., Jr., and Lok, S. M., *A***

***potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface.*** **EMBO Mol Med, 2014. 6(3): p. 358-71.**

**103. Messer, W. B., de Alwis, R., Yount, B. L., Royal, S. R., Huynh, J. P., Smith, S. A., Crowe, J. E., Jr., Doranz, B. J., Kahle, K. M., Pfaff, J. M., White, L. J., Sariol, C. A., de Silva, A. M., and Baric, R. S., *Dengue virus envelope protein domain I/II hinge determines long-lived serotype-specific dengue immunity.* Proc Natl Acad Sci U S A, 2014. 111(5): p. 1939-44.**

**104. Costin, J. M., Zaitseva, E., Kahle, K. M., Nicholson, C. O., Rowe, D. K., Graham, A. S., Bazzone, L. E., Hogancamp, G., Figueroa Sierra, M., Fong, R. H., Yang, S. T., Lin, L., Robinson, J. E., Doranz, B. J., Chernomordik, L. V., Michael, S. F., Schieffelin, J. S., and Isern, S., *Mechanistic study of broadly neutralizing human monoclonal antibodies against dengue virus that target the fusion loop.* J Virol, 2013. 87(1): p. 52-66.**

**105. Smith, Scott A., de Alwis, A. Ruklanthi, Kose, Nurgun, Harris, Eva, Ibarra, Kristie D., Kahle, Kristen M., Pfaff, Jennifer M., Xiang, Xiaoxiao, Doranz, Benjamin J., de Silva, Aravinda M., Austin, S. Kyle, Sukupolvi-Petty, Soila, Diamond, Michael S., and Crowe, James E., *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).**

**106. Lai, Chih-Yun, Williams, Katherine L., Wu, Yi-Chieh, Knight, Sarah, Balmaseda, Angel, Harris, Eva, and Wang, Wei-Kung, *Analysis of Cross-Reactive Antibodies Recognizing the Fusion Loop of Envelope Protein and Correlation with Neutralizing Antibody Titers in Nicaraguan Dengue Cases.* PLoS Negl Trop Dis, 2013. 7(9): p. e2451.**

**107. Vaughn, David W., Green, Sharone, Kalayanarooj, Siripen, Innis, Bruce L., Nimmannitya, Suchitra, Suntayakorn, Saroj, Rothman, Alan L., Ennis, Francis A., and Nisalak, Ananda, *Dengue in the Early Febrile Phase: Viremia and Antibody Responses.* Journal of Infectious Diseases, 1997. 176(2): p. 322-330.**

**108. Kowitdamrong, E., Thammaborvorn, R., Semboonlor, L., Mungmee, V., and Bhattarakosol, P., *Detection of dengue HI and IgM antibody: is it diagnostically useful? when and how?* J Med Assoc Thai, 2001. 84 Suppl 1: p. S148-54.**

**109. Bessoff, K., Delorey, M., Sun, W., and Hunsperger, E., *Comparison of two commercially available dengue virus (DENV) NS1 capture enzyme-linked immunosorbent assays using a single clinical sample for diagnosis of acute DENV infection.* Clin Vaccine Immunol, 2008. 15(10): p. 1513-8.**

**110. McBride, W. J., *Evaluation of dengue NS1 test kits for the diagnosis of dengue fever.* Diagn Microbiol Infect Dis, 2009. 64(1): p. 31-6.**

**111. Chua, K. B., Mustafa, B., Abdul Wahab, A. H., Chem, Y. K., Khairul, A. H., Kumarasamy, V., Mariam, M., Nurhasmimi, H., and Abdul Rasid, K., *A comparative evaluation of dengue diagnostic tests based on single-acute serum samples for laboratory confirmation of acute dengue.* Malays J Pathol, 2011. 33(1): p. 13-20.**

**112. Prince, Harry E. and Matud, Jose L., *Estimation of Dengue Virus IgM Persistence Using Regression Analysis.* Clinical and Vaccine Immunology, 2011. 18(12): p. 2183-2185.**

**113. Puschnik, Andreas, Lau, Louis, Cromwell, Elizabeth A., Balmaseda, Angel, Zompi, Simona, and Harris, Eva, *Correlation between Dengue-Specific Neutralizing Antibodies and Serum Avidity in Primary and Secondary Dengue Virus 3 Natural Infections in Humans.* PLoS Negl Trop Dis, 2013. 7(6): p. e2274.**

**114. Vazquez, S., Lozano, C., Perez, A. B., Castellanos, Y., Ruiz, D., Calzada, N., and Guzman, M. G., *Dengue specific immunoglobulins M, A, and E in primary and secondary dengue 4 infected Salvadorian children.* J Med Virol, 2014. 86(9): p. 1576-83.**

**115. Rothman, A. L., *Dengue: defining protective versus pathologic immunity.* J Clin Invest, 2004. 113(7): p. 946-51.**

**116. Olkowski, S., Forshey, B. M., Morrison, A. C., Rocha, C., Vilcarromero, S., Halsey, E. S., Kochel, T. J., Scott, T. W., and Stoddard, S. T., *Reduced risk of disease during postsecondary dengue virus infections.* J Infect Dis, 2013. 208(6): p. 1026-33.**

**117. Wikramaratna, Paul S., Simmons, Cameron P., Gupta, Sunetra, and Recker, Mario, *The Effects of Tertiary and Quaternary Infections on the Epidemiology of Dengue.* PLoS One, 2010. 5(8): p. e12347.**

**118. Anderson, Kathryn B., Gibbons, Robert V., Cummings, Derek A.T., Nisalak, Ananda, Green, Sharone, Libraty, Daniel H., Jarman, Richard G., Srikiatkhachorn, Anon, Mammen, Mammen P., Darunee, Buddhari, Yoon, In-Kyu, and Endy, Timothy P., *A Shorter Time Interval Between First and Second Dengue Infections Is Associated With Protection From Clinical Illness in a School-based Cohort in Thailand.* Journal of Infectious Diseases, 2014. 209(3): p. 360-368.**

**119. Sabin, Albert B., *Research on Dengue during World War II.* The American Journal of Tropical Medicine and Hygiene, 1952. 1(1): p. 30-50.**

120. Imrie, A., Meeks, J., Gurary, A., Sukhbaatar, M., Truong, T. T., Cropp, C. B., and Effler, P., *Antibody to dengue 1 detected more than 60 years after infection.* Viral Immunol, 2007. 20(4): p. 672-5.

121. Montoya, Magelda, Gresh, Lionel, Mercado, Juan Carlos, Williams, Katherine L., Vargas, Maria José, Gutierrez, Gamaliel, Kuan, Guillermina, Gordon, Aubree, Balmaseda, Angel, and Harris, Eva, *Symptomatic Versus Inapparent Outcome in Repeat Dengue Virus Infections Is Influenced by the Time Interval between Infections and Study Year.* PLoS Negl Trop Dis, 2013. 7(8): p. e2357.

122. Zompi, S. and Harris, E., *Animal models of dengue virus infection.* Viruses, 2012. 4(1): p. 62-82.

123. Shresta, S., Kyle, J. L., Robert Beatty, P., and Harris, E., *Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice.* Virology, 2004. 319(2): p. 262-73.

124. Raut, C. G., Deolankar, R. P., Kolhapure, R. M., and Goverdhan, M. K., *Susceptibility of laboratory-bred rodents to the experimental infection with dengue virus type 2.* Acta Virol, 1996. 40(3): p. 143-6.

125. Chen, S., Yu, M., Jiang, T., Deng, Y., Qin, C., and Qin, E., *Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein.* DNA Cell Biol, 2007. 26(6): p. 361-7.

126. Mota, Javier and Rico-Hesse, Rebeca, *Dengue Virus Tropism in Humanized Mice Recapitulates Human Dengue Fever.* PLoS One, 2011. 6(6): p. e20762.

127. Mota, Javier and Rico-Hesse, Rebeca, *Humanized Mice Show Clinical Signs of Dengue Fever according to Infecting Virus Genotype.* Journal of Virology, 2009. 83(17): p. 8638-8645.

128. Balsitis, Scott J., Williams, Katherine L., Lachica, Ruben, Flores, Diana, Kyle, Jennifer L., Mehlhop, Erin, Johnson, Syd, Diamond, Michael S., Beatty, P. Robert, and Harris, Eva, *Lethal Antibody Enhancement of Dengue Disease in Mice Is Prevented by Fc Modification.* PLoS Pathog, 2010. 6(2): p. e1000790.

129. Zellweger, Raphaël M., Prestwood, Tyler R., and Shresta, Sujan, *Enhanced Infection of Liver Sinusoidal Endothelial Cells in a Mouse Model of Antibody-Induced Severe Dengue Disease.* Cell Host Microbe, 2010. 7(2): p. 128-139.

**130. Marchette, N. J. and Halstead, S. B., *Immunopathogenesis of dengue infection in the rhesus monkey.* Transplant Proc, 1974. 6(2): p. 197-201.**

**131. Ambuel, Y., Young, G., Brewoo, J. N., Paykel, J., Weisgrau, K. L., Rakasz, E. G., Haller, A. A., Royals, M., Huang, C. Y., Capuano, S., Stinchcomb, D. T., Partidos, C. D., and Osorio, J. E., *A rapid immunization strategy with a live-attenuated tetravalent dengue vaccine elicits protective neutralizing antibody responses in non-human primates.* Front Immunol, 2014. 5: p. 263.**

**132. Coller, B. A., Clements, D. E., Bett, A. J., Sagar, S. L., and Ter Meulen, J. H., *The development of recombinant subunit envelope-based vaccines to protect against dengue virus induced disease.* Vaccine, 2011. 29(42): p. 7267-75.**

**133. Danko, Janine R., Beckett, Charmagne G., and Porter, Kevin R., *Development of dengue DNA vaccines.* Vaccine, 2011. 29(42): p. 7261-7266.**

**134. Koraka, P., Benton, S., van Amerongen, G., Stittelaar, K. J., and Osterhaus, A. D., *Efficacy of a live attenuated tetravalent candidate dengue vaccine in naive and previously infected cynomolgus macaques.* Vaccine, 2007. 25(29): p. 5409-16.**

**135. Osorio, J. E., Brewoo, J. N., Silengo, S. J., Arguello, J., Moldovan, I. R., Tary-Lehmann, M., Powell, T. D., Livengood, J. A., Kinney, R. M., Huang, C. Y., and Stinchcomb, D. T., *Efficacy of a tetravalent chimeric dengue vaccine (DENVax) in Cynomolgus macaques.* Am J Trop Med Hyg, 2011. 84(6): p. 978-87.**

**136. Wright, P. F., Ankrah, S., Henderson, S. E., Durbin, A. P., Speicher, J., Whitehead, S. S., Murphy, B. R., and Pletnev, A. G., *Evaluation of the Langat/dengue 4 chimeric virus as a live attenuated tick-borne encephalitis vaccine for safety and immunogenicity in healthy adult volunteers.* Vaccine, 2008. 26(7): p. 882-90.**

**137. Williams, K. L., Zompi, S., Beatty, P. R., and Harris, E., *A mouse model for studying dengue virus pathogenesis and immune response.* Ann N Y Acad Sci, 2009. 1171 Suppl 1: p. E12-23.**

**138. Endy, Timothy P, *Human Immune Responses to Dengue Virus Infection: lessons learned from prospective cohort studies.* Front Immunol, 2014. 5.**

**139. Kuan, Guillermina, Gordon, Aubree, Avilés, William, Ortega, Oscar, Hammond, Samantha N., Elizondo, Douglas, Nuñez, Andrea, Coloma, Josefina, Balmaseda, Angel, and Harris, Eva, *The Nicaraguan Pediatric Dengue Cohort Study: Study Design, Methods, Use of Information Technology, and Extension to Other Infectious Diseases.* Am J Epidemiol, 2009. 170(1): p. 120-129.**

**140. Sirivichayakul, C., Limkittikul, K., Chanthavanich, P., Jiwariyavej, V., Chokejindachai, W., Pengsaa, K., Suvannadabba, S., Dulyachai, W., Letson, G. W., and Sabchareon, A., *Dengue infection in children in Ratchaburi, Thailand: a cohort study. II. Clinical manifestations.* PLoS Negl Trop Dis, 2012. 6(2): p. e1520.**

**141. Endy, Timothy P., Anderson, Kathryn B., Nisalak, Ananda, Yoon, In-Kyu, Green, Sharone, Rothman, Alan L., Thomas, Stephen J., Jarman, Richard G., Libraty, Daniel H., and Gibbons, Robert V., *Determinants of Inapparent and Symptomatic Dengue Infection in a Prospective Study of Primary School Children in Kamphaeng Phet, Thailand.* PLoS Negl Trop Dis, 2011. 5(3): p. e975.**

**142. Dinh The, Trung, Le Thi Thu, Thao, Nguyen Minh, Dung, Tran Van, Ngoc, Tran Tinh, Hien, Nguyen Van Vinh, Chau, Wolbers, Marcel, Dong Thi Hoai, Tam, Farrar, Jeremy, Simmons, Cameron, and Wills, Bridget, *Clinical Features of Dengue in a Large Vietnamese Cohort: Intrinsically Lower Platelet Counts and Greater Risk for Bleeding in Adults than Children.* PLoS Negl Trop Dis, 2012. 6(6): p. e1679.**

**143. Yacoub, S., Griffiths, A., Chau, T. T., Simmons, C. P., Wills, B., Hien, T. T., Henein, M., and Farrar, J., *Cardiac function in Vietnamese patients with different dengue severity grades.* Crit Care Med, 2012. 40(2): p. 477-83.**

**144. Alexander, Neal, Balmaseda, Angel, Coelho, Ivo C. B., Dimaano, Efren, Hien, Tran T., Hung, Nguyen T., Jänisch, Thomas, Kroeger, Axel, Lum, Lucy C. S., Martinez, Eric, Siqueira, Joao B., Thuy, Tran T., Villalobos, Iris, Villegas, Elci, Wills, Bridget, and on behalf of the European Union, World Health Organization supported Denco Study Group, *Multicentre prospective study on dengue classification in four South-east Asian and three Latin American countries***

***Etude prospective multicentrique sur la classification de la dengue dans quatre pays d'Asie du sud-est et trois d'Amérique latine.* Tropical Medicine & International Health, 2011. 16(8): p. 936-948.**

**145. Cavalcanti, L. P., Mota, L. A., Lustosa, G. P., Fortes, M. C., Mota, D. A., Lima, A. A., Coelho, I. C., and Mourao, M. P., *Evaluation of the WHO classification of dengue disease severity during an epidemic in 2011 in the state of Ceara, Brazil.* Mem Inst Oswaldo Cruz, 2014. 109(1): p. 93-8.**

**146. Chaterji, S., Allen, J. C., Jr., Chow, A., Leo, Y. S., and Ooi, E. E., *Evaluation of the NS1 rapid test and the WHO dengue classification schemes for use as bedside diagnosis of acute dengue fever in adults.* Am J Trop Med Hyg, 2011. 84(2): p. 224-8.**

**147. Jayaratne, S. D., Atukorale, V., Gomes, L., Chang, T., Wijesinghe, T., Fernando, S., Ogg, G. S., and Malavige, G. N., *Evaluation of the WHO revised criteria for***

***classification of clinical disease severity in acute adult dengue infection.*** **BMC Res Notes, 2012. 5: p. 645.**

**148. van de Weg, C. A., van Gorp, E. C., Supriatna, M., Soemantri, A., Osterhaus, A. D., and Martina, B. E., *Evaluation of the 2009 WHO dengue case classification in an Indonesian pediatric cohort.* Am J Trop Med Hyg, 2012. 86(1): p. 166-70.**

**149. Teerasut, C., Lei, H. Y., Natthakorn, K., Jittmittraphap, A., Thammapalo, S., Witayathawornwong, P., Lin, Y. T., Jarman, R. G., Sawanpanyalert, P., Jampangern, W., and Limkittikul, K., *The serotype-independent but concentration-dependent enhancing antibodies among Thai dengue patients.* Southeast Asian J Trop Med Public Health, 2012. 43(3): p. 624-33.**

**150. Guzman, MariaG, Alvarez, Mayling, and Halstead, ScottB, *Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection.* Arch Virol, 2013. 158(7): p. 1445-1459.**

**151. Halstead, Scott B., Porterfield, James S., and O'Rourke, Edward J., *Enhancement of Dengue Virus Infection in Monocytes by Flavivirus Antisera.* Am J Trop Med Hyg, 1980. 29(4): p. 638-642.**

**152. Endy, Timothy P., Nisalak, Ananda, Chunsuttitwat, Supamit, Vaughn, David W., Green, Sharone, Ennis, Francis A., Rothman, Alan L., and Libraty, Daniel H., *Relationship of Preexisting Dengue Virus (DV) Neutralizing Antibody Levels to Viremia and Severity of Disease in a Prospective Cohort Study of DV Infection in Thailand.* J Infect Dis, 2004. 189(6): p. 990-1000.**

**153. Chau, T. N., Quyen, N. T., Thuy, T. T., Tuan, N. M., Hoang, D. M., Dung, N. T., Lien le, B., Quy, N. T., Hieu, N. T., Hieu, L. T., Hien, T. T., Hung, N. T., Farrar, J., and Simmons, C. P., *Dengue in Vietnamese infants--results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity.* J Infect Dis, 2008. 198(4): p. 516-24.**

**154. Chau, T. N., Hieu, N. T., Anders, K. L., Wolbers, M., Lien le, B., Hieu, L. T., Hien, T. T., Hung, N. T., Farrar, J., Whitehead, S., and Simmons, C. P., *Dengue virus infections and maternal antibody decay in a prospective birth cohort study of Vietnamese infants.* J Infect Dis, 2009. 200(12): p. 1893-900.**

**155. Sirivichayakul, C., Sabchareon, A., Limkittikul, K., and Yoksan, S., *Plaque reduction neutralization antibody test does not accurately predict protection against dengue infection in Ratchaburi cohort, Thailand.* Virol J, 2014. 11: p. 48.**

156. **Babu, J. Pradeep, Pattnaik, Priyabrata, Gupta, Nimesh, Shrivastava, Ambuj, Khan, Mohsin, and Rao, P. V. Lakshmana, *Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice.* Vaccine, 2008. 26(36): p. 4655-4663.**

157. **Khalil, Syed Muaz, Tonkin, Daniel R., Mattocks, Melissa D., Snead, Andrew T., Johnston, Robert E., and White, Laura J., *A tetravalent alphavirus-vector based dengue vaccine provides effective immunity in an early life mouse model.* Vaccine, 2014. 32(32): p. 4068-4074.**

158. **Vaughn, David W., Hoke Jr, Charles H., Yoksan, Sutee, LaChance, Rebecca, Innis, Bruce L., Rice, Robert M., and Bhamarapravati, Natth, *Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in ten American volunteers.* Vaccine, 1996. 14(4): p. 329-336.**

159. **Thomas, Stephen J., Eckels, Kenneth H., Carletti, Isabelle, De La Barrera, Rafael, Dessy, Francis, Fernandez, Stefan, Putnak, Robert, Toussaint, Jean-Francois, Sun, Wellington, Bauer, Kristen, Gibbons, Robert V., and Innis, Bruce L., *A Phase II, Randomized, Safety and Immunogenicity Study of a Re-Derived, Live-Attenuated Dengue Virus Vaccine in Healthy Adults.* The American Journal of Tropical Medicine and Hygiene, 2013. 88(1): p. 73-88.**

160. **Osorio, Jorge E., Velez, Ivan D., Thomson, Cynthia, Lopez, Liliana, Jimenez, Alejandra, Haller, Aurelia A., Silengo, Shawn, Scott, Jaclyn, Boroughs, Karen L., Stovall, Janae L., Luy, Betty E., Arguello, John, Beatty, Mark E., Santangelo, Joseph, Gordon, Gilad S., Huang, Claire Y. H., and Stinchcomb, Dan T., *Safety and immunogenicity of a recombinant live attenuated tetravalent dengue vaccine (DENVax) in flavivirus-naive healthy adults in Colombia: a randomised, placebo-controlled, phase 1 study.* The Lancet Infectious Diseases, 2014. 14(9): p. 830-838.**

161. **Briggs, C. M., Smith, K. M., Piper, A., Huitt, E., Spears, C. J., Quiles, M., Ribeiro, M., Thomas, M. E., Brown, D. T., and Hernandez, R., *Live Attenuated Tetravalent Dengue Virus Host Range Vaccine Is Immunogenic in African Green Monkeys following a Single Vaccination.* J Virol, 2014. 88(12): p. 6729-42.**

162. **Sabchareon, A., Wallace, D., Sirivichayakul, C., Limkittikul, K., Chanthavanich, P., Suvannadabba, S., Jiwariyavej, V., Dulyachai, W., Pengsaa, K., Wartel, T. A., Moureau, A., Saville, M., Bouckenooghe, A., Viviani, S., Tornieporth, N. G., and Lang, J., *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. 1559-67.**

163. **Watanaveeradej, V., Simasathien, S., Nisalak, A., Endy, T. P., Jarman, R. G., Innis, B. L., Thomas, S. J., Gibbons, R. V., Hengprasert, S., Samakoses, R., Kerdpanich,**

**A., Vaughn, D. W., Putnak, J. R., Eckels, K. H., Barrera Rde, L., and Mammen, M. P., Jr., *Safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in flavivirus-naive infants.* Am J Trop Med Hyg, 2011. 85(2): p. 341-51.**

**164. Capeding, R. Z., Luna, I. A., Bomasang, E., Lupisan, S., Lang, J., Forrat, R., Wartel, A., and Crevat, D., *Live-attenuated, tetravalent dengue vaccine in children, adolescents and adults in a dengue endemic country: randomized controlled phase I trial in the Philippines.* Vaccine, 2011. 29(22): p. 3863-72.**

**165. Poo, J., Galan, F., Forrat, R., Zambrano, B., Lang, J., and Dayan, G. H., *Live-attenuated Tetravalent Dengue Vaccine in Dengue-naive Children, Adolescents, and Adults in Mexico City: Randomized Controlled Phase 1 Trial of Safety and Immunogenicity.* Pediatr Infect Dis J, 2010.**

**166. Sun, W., Cunningham, D., Wasserman, S. S., Perry, J., Putnak, J. R., Eckels, K. H., Vaughn, D. W., Thomas, S. J., Kanesa-Thasan, N., Innis, B. L., and Edelman, R., *Phase 2 clinical trial of three formulations of tetravalent live-attenuated dengue vaccine in flavivirus-naive adults.* Hum Vaccin, 2009. 5(1): p. 33-40.**

**167. Simasathien, S., Thomas, S. J., Watanaveeradej, V., Nisalak, A., Barberousse, C., Innis, B. L., Sun, W., Putnak, J. R., Eckels, K. H., Hutagalung, Y., Gibbons, R. V., Zhang, C., De La Barrera, R., Jarman, R. G., Chawachalasai, W., and Mammen, M. P., Jr., *Safety and immunogenicity of a tetravalent live-attenuated dengue vaccine in flavivirus naive children.* Am J Trop Med Hyg, 2008. 78(3): p. 426-33.**

**168. Chanthavanich, P., Luxemburger, C., Sirivichayakul, C., Lapphra, K., Pengsaa, K., Yoksan, S., Sabchareon, A., and Lang, J., *Short report: immune response and occurrence of dengue infection in thai children three to eight years after vaccination with live attenuated tetravalent dengue vaccine.* Am J Trop Med Hyg, 2006. 75(1): p. 26-8.**

**169. Kitchener, S., Nissen, M., Nasveld, P., Forrat, R., Yoksan, S., Lang, J., and Saluzzo, J. F., *Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults.* Vaccine, 2006. 24(9): p. 1238-41.**

**170. Blaney, J. E., Jr., Matro, J. M., Murphy, B. R., and Whitehead, S. S., *Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys.* J Virol, 2005. 79(9): p. 5516-28.**

**171. Guy, B., Chanthavanich, P., Gimenez, S., Sirivichayakul, C., Sabchareon, A., Begue, S., Yoksan, S., Luxemburger, C., and Lang, J., *Evaluation by flow cytometry of antibody-dependent enhancement (ADE) of dengue infection by sera***

***from Thai children immunized with a live-attenuated tetravalent dengue vaccine.*** **Vaccine, 2004. 22(27-28): p. 3563-74.**

**172. Innis, B. L. and Eckels, K. H., *Progress in development of a live-attenuated, tetravalent dengue virus vaccine by the United States Army Medical Research and Materiel Command.* Am J Trop Med Hyg, 2003. 69(6 Suppl): p. 1-4.**

**173. Edelman, R., Wasserman, S. S., Bodison, S. A., Putnak, R. J., Eckels, K. H., Tang, D., Kanesa-Thasan, N., Vaughn, D. W., Innis, B. L., and Sun, W., *Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine.* Am J Trop Med Hyg, 2003. 69(6 Suppl): p. 48-60.**

**174. Sun, W., Edelman, R., Kanesa-Thasan, N., Eckels, K. H., Putnak, J. R., King, A. D., Houng, H. S., Tang, D., Scherer, J. M., Hoke, C. H., Jr., and Innis, B. L., *Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates.* Am J Trop Med Hyg, 2003. 69(6 Suppl): p. 24-31.**

**175. Rothman, A. L., Kanesa-thasan, N., West, K., Janus, J., Saluzzo, J. F., and Ennis, F. A., *Induction of T lymphocyte responses to dengue virus by a candidate tetravalent live attenuated dengue virus vaccine.* Vaccine, 2001. 19(32): p. 4694-9.**

**176. Bhamarapravati, N. and Sutee, Y., *Live attenuated tetravalent dengue vaccine.* Vaccine, 2000. 18 Suppl 2: p. 44-7.**

**177. Jirakanjanakit, N., Khin, M. M., Yoksan, S., and Bhamarapravati, N., *The use of Toxorhynchites splendens for identification and quantitation of serotypes contained in the tetravalent live attenuated dengue vaccine.* Vaccine, 1999. 17(6): p. 597-601.**

**178. Sun, Wellington, Eckels, Kenneth H., Putnak, J. Robert, Lyons, Arthur G., Thomas, Stephen J., Vaughn, David W., Gibbons, Robert V., Fernandez, Stefan, Gunther, Vicky J., Mammen, Mammen P., Statler, John D., and Innis, Bruce L., *Experimental Dengue Virus Challenge of Human Subjects Previously Vaccinated With Live Attenuated Tetravalent Dengue Vaccines.* Journal of Infectious Diseases, 2013. 207(5): p. 700-708.**

**179. Guy, B., Barrere, B., Malinowski, C., Saville, M., Teyssou, R., and Lang, J., *From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine.* Vaccine, 2011. 29(42): p. 7229-41.**

**180. da Costa, Vivaldo G., Marques-Silva, Ariany C., Floriano, Vitor G., and Moreli, Marcos L., *Safety, immunogenicity and efficacy of a recombinant tetravalent***

***dengue vaccine: A meta-analysis of randomized trials.*** **Vaccine, 2014. 32(39): p. 4885-4892.**

**181. Capeding, M. R., Tran, N. H., Hadinegoro, S. R., Ismail, H. I., Chotpitayasunondh, T., Chua, M. N., Luong, C. Q., Rusmil, K., Wirawan, D. N., Nallusamy, R., Pitisuttithum, P., Thisyakorn, U., Yoon, I. K., van der Vliet, D., Langevin, E., Laot, T., Hutagalung, Y., Frago, C., Boaz, M., Wartel, T. A., Tornieporth, N. G., Saville, M., and Bouckenooghe, A., *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.**

**182. Dayan, G. H., Garbes, P., Noriega, F., Izoton de Sadovsky, A. D., Rodrigues, P. M., Giuberti, C., and Dietze, R., *Immunogenicity and safety of a recombinant tetravalent dengue vaccine in children and adolescents ages 9-16 years in Brazil.* Am J Trop Med Hyg, 2013. 89(6): p. 1058-65.**

**183. Hss, A. S., Koh, M. T., Tan, K. K., Chan, L. G., Zhou, L., Bouckenooghe, A., Crevat, D., and Hutagalung, Y., *Safety and immunogenicity of a tetravalent dengue vaccine in healthy children aged 2-11 years in Malaysia: a randomized, placebo-controlled, Phase III study.* Vaccine, 2013. 31(49): p. 5814-21.**

**184. Villar, L. A., Rivera-Medina, D. M., Arredondo-Garcia, J. L., Boaz, M., Starr-Spires, L., Thakur, M., Zambrano, B., Miranda, M. C., Rivas, E., and Dayan, G. H., *Safety and immunogenicity of a recombinant tetravalent dengue vaccine in 9-16 year olds: a randomized, controlled, phase II trial in Latin America.* Pediatr Infect Dis J, 2013. 32(10): p. 1102-9.**

**185. Dayan, G. H., Thakur, M., Boaz, M., and Johnson, C., *Safety and immunogenicity of three tetravalent dengue vaccine formulations in healthy adults in the USA.* Vaccine, 2013. 31(44): p. 5047-54.**

**186. Leo, Y. S., Wilder-Smith, A., Archuleta, S., Shek, L. P., Chong, C. Y., Leong, H. N., Low, C. Y., Oh, M. L., Bouckenooghe, A., Wartel, T. A., and Crevat, D., *Immunogenicity and safety of recombinant tetravalent dengue vaccine (CYD-TDV) in individuals aged 2-45 y: Phase II randomized controlled trial in Singapore.* Hum Vaccin Immunother, 2012. 8(9): p. 1259-71.**

**187. Lanata, C. F., Andrade, T., Gil, A. I., Terrones, C., Valladolid, O., Zambrano, B., Saville, M., and Crevat, D., *Immunogenicity and safety of tetravalent dengue vaccine in 2-11 year-olds previously vaccinated against yellow fever: randomized, controlled, phase II study in Piura, Peru.* Vaccine, 2012. 30(41): p. 5935-41.**

**188. Pierson, Ted. *Laboratory of Viral Diseases*. 2012 November 2, 2012 [cited 2014 September 2, 2014]; Available from:**

**http://www.niaid.nih.gov/labsandresources/labs/aboutlabs/lvd/viralpathogenesissection/Pages/default.aspx.**

**189. Rothman, Alan L., *Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms.* Nat Rev Immunol, 2011. 11(8): p. 532-543.**

**190. Mairiang, D., Zhang, H., Sodja, A., Murali, T., Suriyaphol, P., Malasit, P., Limjindaporn, T., and Finley, R. L., Jr., *Identification of new protein interactions between dengue fever virus and its hosts, human and mosquito.* PLoS One, 2013. 8(1): p. e53535.**

**191. Whitehead, S. S., Blaney, J. E., Durbin, A. P., and Murphy, B. R., *Prospects for a dengue virus vaccine.* Nat Rev Microbiol, 2007. 5(7): p. 518-28.**

**192. Peeling, R. W., Artsob, H., Pelegrino, J. L., Buchy, P., Cardosa, M. J., Devi, S., Enria, D. A., Farrar, J., Gubler, D. J., Guzman, M. G., Halstead, S. B., Hunsperger, E., Kliks, S., Margolis, H. S., Nathanson, C. M., Nguyen, V. C., Rizzo, N., Vazquez, S., and Yoksan, S., *Evaluation of diagnostic tests: dengue.* Nat Rev Microbiol, 2010. 8(12 Suppl): p. S30-8.**

**193. Halstead, S. B., *Dengue.* Lancet, 2007. 370(9599): p. 1644-52.**

**194. Endy, T. P., Chunsuttiwat, S., Nisalak, A., Libraty, D. H., Green, S., Rothman, A. L., Vaughn, D. W., and Ennis, F. A., *Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand.* Am J Epidemiol, 2002. 156(1): p. 40-51.**

**195. Vong, S., Goyet, S., Ly, S., Ngan, C., Huy, R., Duong, V., Wichmann, O., Letson, G. W., Margolis, H. S., and Buchy, P., *Under-recognition and reporting of dengue in Cambodia: a capture-recapture analysis of the National Dengue Surveillance System.* Epidemiol Infect, 2012. 140(3): p. 491-9.**

**196. Yoon, I. K., Rothman, A. L., Tannitisupawong, D., Srikiatkhachorn, A., Jarman, R. G., Aldstadt, J., Nisalak, A., Mammen, M. P., Jr., Thammapalo, S., Green, S., Libraty, D. H., Gibbons, R. V., Getis, A., Endy, T., Jones, J. W., Koenraadt, C. J., Morrison, A. C., Fansiri, T., Pimgate, C., and Scott, T. W., *Underrecognized mildly symptomatic viremic dengue virus infections in rural Thai schools and villages.* J Infect Dis, 2012. 206(3): p. 389-98.**

**197. Sabchareon, A., Sirivichayakul, C., Limkittikul, K., Chanthavanich, P., Suvannadabba, S., Jiwariyavej, V., Dulyachai, W., Pengsaa, K., Margolis, H. S., and Letson, G. W., *Dengue infection in children in Ratchaburi, Thailand: a cohort***

***study. I. Epidemiology of symptomatic acute dengue infection in children, 2006-2009.*** **PLoS Negl Trop Dis, 2012. 6(7): p. e1732.**

**198. Tien, N. T., Luxemburger, C., Toan, N. T., Pollissard-Gadroy, L., Huong, V. T., Van Be, P., Rang, N. N., Wartel, T. A., and Lang, J., *A prospective cohort study of dengue infection in schoolchildren in Long Xuyen, Viet Nam.* Trans R Soc Trop Med Hyg, 2010. 104(9): p. 592-600.**

**199. Balmaseda, A., Standish, K., Mercado, J. C., Matute, J. C., Tellez, Y., Saborio, S., Hammond, S. N., Nunez, A., Aviles, W., Henn, M. R., Holmes, E. C., Gordon, A., Coloma, J., Kuan, G., and Harris, E., *Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua.* J Infect Dis, 2010. 201(1): p. 5-14.**

**200. Morrison, A. C., Minnick, S. L., Rocha, C., Forshey, B. M., Stoddard, S. T., Getis, A., Focks, D. A., Russell, K. L., Olson, J. G., Blair, P. J., Watts, D. M., Sihuincha, M., Scott, T. W., and Kochel, T. J., *Epidemiology of dengue virus in Iquitos, Peru 1999 to 2005: interepidemic and epidemic patterns of transmission.* PLoS Negl Trop Dis, 2010. 4(5): p. e670.**

**201. Ramos, M. M., Arguello, D. F., Luxemburger, C., Quinones, L., Munoz, J. L., Beatty, M., Lang, J., and Tomashek, K. M., *Epidemiological and clinical observations on patients with dengue in Puerto Rico: results from the first year of enhanced surveillance--June 2005-May 2006.* Am J Trop Med Hyg, 2008. 79(1): p. 123-7.**

**202. Tam, C. C., Tissera, H., de Silva, A. M., De Silva, A. D., Margolis, H. S., and Amarasinge, A., *Estimates of dengue force of infection in children in Colombo, Sri Lanka.* PLoS Negl Trop Dis, 2013. 7(6): p. e2259.**

**203. Chakravarti, A., Arora, R., and Luxemburger, C., *Fifty years of dengue in India.* Trans R Soc Trop Med Hyg, 2012. 106(5): p. 273-82.**

**204. Raheel, U., Faheem, M., Riaz, M. N., Kanwal, N., Javed, F., Zaidi, Nu, and Qadri, I., *Dengue fever in the Indian Subcontinent: an overview.* J Infect Dev Ctries, 2011. 5(4): p. 239-47.**

**205. Anonymous., *Population and Housing Data City of Colombo (Colombo Municipal Council), Sri Lanka Census of Population and Housing 2001.* , D.o.C.a. Statistics., Editor 2001: Colombo, Sri Lanka.**

**206. Tissera, Hasitha A., Amarasinghe, Ananda, Silva, Aravinda M. de, Tam, Clarence C., DeSilva, Aruna Dharshan, Letson, G. William, and Margolis, Harold S.,**

***Surveillance of dengue in a community cohort in Metropolitan Colombo, Sri Lanka: Methods and study population.*** **WHO Bulletin, 2013. 91.**

**207. Snijdewind, I. J., van Kampen, J. J., Fraaij, P. L., van der Ende, M. E., Osterhaus, A. D., and Gruters, R. A., *Current and future applications of dried blood spots in viral disease management.* Antiviral Res, 2012. 93(3): p. 309-21.**

**208. Balmaseda, A., Saborio, S., Tellez, Y., Mercado, J. C., Perez, L., Hammond, S. N., Rocha, C., Kuan, G., and Harris, E., *Evaluation of immunological markers in serum, filter-paper blood spots, and saliva for dengue diagnosis and epidemiological studies.* J Clin Virol, 2008. 43(3): p. 287-91.**

**209. Wahala, W. M., Kraus, A. A., Haymore, L. B., Accavitti-Loper, M. A., and de Silva, A. M., *Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody.* Virology, 2009. 392(1): p. 103-13.**

**210. Anders, K. L., Nguyet, N. M., Quyen, N. T., Ngoc, T. V., Tram, T. V., Gan, T. T., Tung, N. T., Dung, N. T., Chau, N. V., Wills, B., and Simmons, C. P., *An evaluation of dried blood spots and oral swabs as alternative specimens for the diagnosis of dengue and screening for past dengue virus exposure.* Am J Trop Med Hyg, 2012. 87(1): p. 165-70.**

**211. Kuno, G., Gomez, I., and Gubler, D. J., *Detecting artificial anti-dengue IgM immune complexes using an enzyme-linked immunosorbent assay.* Am J Trop Med Hyg, 1987. 36(1): p. 153-9.**

**212. Chungue, E., Marche, G., Plichart, R., Boutin, J. P., and Roux, J., *Comparison of immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA) and haemagglutination inhibition (HI) test for the detection of dengue antibodies. Prevalence of dengue IgG-ELISA antibodies in Tahiti.* Trans R Soc Trop Med Hyg, 1989. 83(5): p. 708-11.**

**213. Kraus, A. A., Messer, W., Haymore, L. B., and de Silva, A. M., *Comparison of plaque- and flow cytometry-based methods for measuring dengue virus neutralization.* J Clin Microbiol, 2007. 45(11): p. 3777-80.**

**214. de Alwis, Ruklanthi and de Silva, AravindaM, *Measuring Antibody Neutralization of Dengue Virus (DENV) Using a Flow Cytometry-Based Technique*, in *Dengue*, R. Padmanabhan and S.G. Vasudevan, Editors. 2014, Springer New York. p. 27-39.**

**215. Tissera, H., Amarasinge, A., de Silva, Aravinda M, Tam, C. C., de Silva, A. D., Letson, G. W., and Margolis, H. S., *Surveillance of dengue in a community cohort***

*in Metropolitan Colombo, Sri Lanka: part I methods and study population.* **WHO Dengue Bulletin, 2013. 37: p. 141-154.**

**216. Tissera, Hasitha, Amarasinghe, Ananda, De Silva, Aruna Dharshan, Kariyawasam, Pradeep, Corbett, Kizzmekia S., Katzelnick, Leah, Tam, Clarence, Letson, G. William, Margolis, Harold S., and de Silva, Aravinda M., *Burden of Dengue Infection and Disease in a Pediatric Cohort in Urban Sri Lanka.* Am J Trop Med Hyg, 2014. 91(1): p. 132-7.**

**217. Beatty, M. E., Stone, A., Fitzsimons, D. W., Hanna, J. N., Lam, S. K., Vong, S., Guzman, M. G., Mendez-Galvan, J. F., Halstead, S. B., Letson, G. W., Kuritsky, J., Mahoney, R., and Margolis, H. S., *Best practices in dengue surveillance: a report from the Asia-Pacific and Americas Dengue Prevention Boards.* PLoS Negl Trop Dis, 2010. 4(11): p. e890.**

**218. Wichmann, O., Yoon, I. K., Vong, S., Limkittikul, K., Gibbons, R. V., Mammen, M. P., Ly, S., Buchy, P., Sirivichayakul, C., Buathong, R., Huy, R., Letson, G. W., and Sabchareon, A., *Dengue in Thailand and Cambodia: an assessment of the degree of underrecognized disease burden based on reported cases.* PLoS Negl Trop Dis, 2011. 5(3): p. e996.**

**219. Tran, Hau P., Adams, Jon, Jeffery, Jason A.L., Nguyen, Yen T., Vu, Nam S., Kutcher, Simon C., Kay, Brian H., and Ryan, Peter A., *Householder perspectives and preferences on water storage and use, with reference to dengue, in the Mekong Delta, southern Vietnam.* Int Health, 2010. 2(2): p. 136-142.**

**220. Thai, Khoa T. D., Binh, Tran Quang, Giao, Phan Trong, Phuong, Hoang Lan, Hung, Le Quoc, Nam, Nguyen Van, Nga, Tran Thanh, Groen, Jan, Nagelkerke, Nico, and de Vries, Peter J., *Seroprevalence of dengue antibodies, annual incidence and risk factors among children in southern Vietnam.* Tropical Medicine & International Health, 2005. 10(4): p. 379-386.**

**221. Halstead, S. B., *Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion.* Yale J Biol Med, 1970. 42(5): p. 350-62.**

**222. Lai, Chih-Yun, Tsai, Wen-Yang, Lin, Su-Ru, Kao, Chuan-Liang, Hu, Hsien-Ping, King, Chwan-Chuen, Wu, Han-Chung, Chang, Gwong-Jen, and Wang, Wei-Kung, *Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II.* J Virol, 2008. 82(13): p. 6631-6643.**

**223. Popper, Stephen J., Gordon, Aubree, Liu, Minghsun, Balmaseda, Angel, Harris, Eva, and Relman, David A., *Temporal Dynamics of the Transcriptional Response***

*to Dengue Virus Infection in Nicaraguan Children.* **PLoS Negl Trop Dis, 2012. 6(12): p. e1966.**

**224. Cobra, Claudine, Rigau-Pérez, José G., Kuno, Goro, and Vomdam, Vance, *Symptoms of Dengue Fever in Relation to Host Immunologic Response and Virus Serotype, Puerto Rico, 1990–1991.* Am J Epidemiol, 1995. 142(11): p. 1204-1211.**

**225. Hu, D., Li, J., Wang, D., Di, B., Qiu, L., Wang, Y., Ding, X., and Che, X., *[Analysis of serum neutralizing antibody response in patients with primary dengue virus type 1 infection].* Nan Fang Yi Ke Da Xue Xue Bao, 2012. 32(12): p. 1773-6, 1791.**

**226. Konishi, Eiji, Tabuchi, Yuko, and Yamanaka, Atsushi, *A simple assay system for infection-enhancing and -neutralizing antibodies to dengue type 2 virus using layers of semi-adherent K562 cells.* Journal of Virological Methods, 2010. 163(2): p. 360-367.**

**227. Reich, Nicholas G., Shrestha, Sourya, King, Aaron A., Rohani, Pejman, Lessler, Justin, Kalayanarooj, Siripen, Yoon, In-Kyu, Gibbons, Robert V., Burke, Donald S., and Cummings, Derek A. T., *Interactions between serotypes of dengue highlight epidemiological impact of cross-immunity.* J R Soc Interface, 2013. 10(86).**

**228. Gubler, D. J., *Dengue/dengue haemorrhagic fever: history and current status.* Novartis Found Symp, 2006. 277: p. 3-16; discussion 16-22, 71-3, 251-3.**

**229. Durbin, A. P., Whitehead, S. S., McArthur, J., Perreault, J. R., Blaney, J. E., Jr., Thumar, B., Murphy, B. R., and Karron, R. A., *rDEN4delta30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, and highly infectious in healthy adult volunteers.* J Infect Dis, 2005. 191(5): p. 710-8.**

**230. Restrepo, A. C., Baker, P., and Clements, A. C., *National spatial and temporal patterns of notified dengue cases, Colombia 2007-2010.* Trop Med Int Health, 2014. 19(7): p. 863-71.**

**231. Naish, S., Dale, P., Mackenzie, J. S., McBride, J., Mengersen, K., and Tong, S., *Spatial and temporal patterns of locally-acquired dengue transmission in northern Queensland, Australia, 1993-2012.* PLoS One, 2014. 9(4): p. e92524.**

**232. Wang, C., Yang, W., Fan, J., Wang, F., Jiang, B., and Liu, Q., *Spatial and Temporal Patterns of Dengue in Guangdong Province of China.* Asia Pac J Public Health, 2013.**

**233. Seidahmed, O. M., Hassan, S. A., Soghaier, M. A., Siam, H. A., Ahmed, F. T., Elkarsany, M. M., and Sulaiman, S. M., *Spatial and temporal patterns of dengue transmission along a Red Sea coastline: a longitudinal entomological and serological survey in Port Sudan city.* PLoS Negl Trop Dis, 2012. 6(9): p. e1821.**

**234. Wen, T. H., Lin, N. H., Chao, D. Y., Hwang, K. P., Kan, C. C., Lin, K. C., Wu, J. T., Huang, S. Y., Fan, I. C., and King, C. C., *Spatial-temporal patterns of dengue in areas at risk of dengue hemorrhagic fever in Kaohsiung, Taiwan, 2002.* Int J Infect Dis, 2010. 14(4): p. e334-43.**

**235. Mammen, M. P., Pimgate, C., Koenraadt, C. J., Rothman, A. L., Aldstadt, J., Nisalak, A., Jarman, R. G., Jones, J. W., Srikiatkhachorn, A., Ypil-Butac, C. A., Getis, A., Thammapalo, S., Morrison, A. C., Libraty, D. H., Green, S., and Scott, T. W., *Spatial and temporal clustering of dengue virus transmission in Thai villages.* PLoS Med, 2008. 5(11): p. e205.**

**236. Chowell, G., Torre, C. A., Munayco-Escate, C., Suarez-Ognio, L., Lopez-Cruz, R., Hyman, J. M., and Castillo-Chavez, C., *Spatial and temporal dynamics of dengue fever in Peru: 1994-2006.* Epidemiol Infect, 2008. 136(12): p. 1667-77.**

**237. Tran, A., Deparis, X., Dussart, P., Morvan, J., Rabarison, P., Remy, F., Polidori, L., and Gardon, J., *Dengue spatial and temporal patterns, French Guiana, 2001.* Emerg Infect Dis, 2004. 10(4): p. 615-21.**

**238. Endy, T. P., Nisalak, A., Chunsuttiwat, S., Libraty, D. H., Green, S., Rothman, A. L., Vaughn, D. W., and Ennis, F. A., *Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand.* Am J Epidemiol, 2002. 156(1): p. 52-9.**

**239. Sang, Chew Theng, Cuzzubbo, Andrea J., and Devine, Peter L., *Evaluation of a Commercial Capture Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin M and G Antibodies Produced during Dengue Infection.* Clinical and Diagnostic Laboratory Immunology, 1998. 5(1): p. 7-10.**

**240. Mattia, Kimberly, Puffer, Bridget A., Williams, Katherine L., Gonzalez, Ritela, Murray, Meredith, Sluzas, Emily, Pagano, Dan, Ajith, Sandya, Bower, Megan, Berdougo, Eli, Harris, Eva, and Doranz, Benjamin J., *Dengue Reporter Virus Particles for Measuring Neutralizing Antibodies against Each of the Four Dengue Serotypes.* PLoS One, 2011. 6(11): p. e27252.**

**241. Kularatnea, S.A.M., Seneviratneb, S.L., Malavigec, G.N., Fernandoc, S., Velathanthiric, V.G.N.S., Ranatungad, P.K., Wijewickramae, E.S., Gurugamaf, P.N., Karunatilakad, D.H., Aaskovg, J.G., and Jayaratnee, S.D., *Synopsis of Findings from Recent Studies on Dengue in Sri Lanka.* WHO Dengue Bulletin, 2006. 30: p. 7.**

**242.** **Thomas, S. J., Nisalak, A., Anderson, K. B., Libraty, D. H., Kalayanarooj, S., Vaughn, D. W., Putnak, R., Gibbons, R. V., Jarman, R., and Endy, T. P., *Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions impact performance.* Am J Trop Med Hyg, 2009. 81(5): p. 825-33.**

**243.** **Zompi, S. and Harris, E., *Original antigenic sin in dengue revisited.* Proc Natl Acad Sci U S A, 2013. 110(22): p. 8761-2.**

**244.** **Midgley, C. M., Bajwa-Joseph, M., Vasanawathana, S., Limpitikul, W., Wills, B., Flanagan, A., Waiyaiya, E., Tran, H. B., Cowper, A. E., Chotiyarnwong, P., Grimes, J. M., Yoksan, S., Malasit, P., Simmons, C. P., Mongkolsapaya, J., and Screaton, G. R., *An in-depth analysis of original antigenic sin in dengue virus infection.* J Virol, 2011. 85(1): p. 410-21.**

**245.** **Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., Sawasdivorn, S., Duangchinda, T., Dong, T., Rowland-Jones, S., Yenchitsomanus, P. T., McMichael, A., Malasit, P., and Screaton, G., *Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever.* Nat Med, 2003. 9(7): p. 921-7.**

**246.** **Kuno, G., Gubler, D. J., and Oliver, A., *Use of 'original antigenic sin' theory to determine the serotypes of previous dengue infections.* Trans R Soc Trop Med Hyg, 1993. 87(1): p. 103-5.**

**247.** **Halstead, S. B., Rojanasuphot, S., and Sangkawibha, N., *Original antigenic sin in dengue.* Am J Trop Med Hyg, 1983. 32(1): p. 154-6.**

**248.** **Fonville, J. M., Burke, D. F., Lewis, N. S., Katzelnick, L. C., and Russell, C. A., *Quantifying the fitness advantage of polymerase substitutions in Influenza A/H7N9 viruses during adaptation to humans.* PLoS One, 2013. 8(9): p. e76047.**

**249.** **Yamanaka, A., Tabuchi, Y., Mulyatno, K. C., Susilowati, H., Hendrianto, E., Soegijanto, S., and Konishi, E., *Dengue virus infection-enhancing and neutralizing antibody balance in children of the Philippines and Indonesia.* Microbes Infect, 2012. 14(13): p. 1152-9.**

**250.** **Konishi, E. and Miyagawa, Y., *Balance of infection-enhancing and neutralizing antibodies induced by a dengue tetravalent DNA vaccine in a mouse model.* Microbes Infect, 2011. 13(12-13): p. 1091-8.**