Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Kathryn B. Anderson Date

Sero-epidemiology of dengue viruses: The influence of preexisting immunity on disease severity and implications for vaccine development

By

Kathryn B. Anderson, MSPH
Doctor of Philosophy

Epidemiology

Ruth L. Berkelman, MD
Advisor

Derek AT Cummings, PhD MSc
Committee Member

Timothy P. Endy, MD MPH
Committee Member

W. Dana Flanders, MD DSc
Committee Member

John McGowan, MD
Committee Member

Alan L. Rothman, MD
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

Sero-epidemiology of dengue viruses: The influence of preexisting immunity on disease severity and implications for vaccine development

By

Kathryn B. Anderson
MSPH

Advisor: Ruth L Berkelman, MD

An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Epidemiology, 2011

Abstract

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are important causes of morbidity and mortality globally. The four dengue virus serotypes (DENV-1 - DENV-4) co-circulate and cause annual epidemics in endemic regions such as Southeast Asia. Cross-reactive DENV antibodies have been linked to DHF in multiple epidemiological and immunological studies. However, cross-reactive antibodies have also been associated with protection from illness. The complex factors involved in whether a non-primary infection with DENV will be subclinical, DF, or develop into DHF are poorly understood. This remains a central issue in DENV immunology, and one that looms large as DENV vaccines approach licensure and implementation. These studies sought to address important knowledge gaps regarding DENV immunity in three complementary ways. The first study assessed how immunity to Japanese encephalitis virus (JEV), a related flavivirus, may influence the clinical severity of DENV infections, using data from a prospective, school-based cohort for asymptomatic and symptomatic DENV infections in Thailand that was conducted from 1998-2002. The study found a positive association between preexisting JEV antibodies and symptomatic (versus asymptomatic) DENV infection. The second study considered whether time between DENV infections was a significant predictor of DENV severity, using time as a proxy for the decay of cross-reactive antibodies. The same prospective cohort data were used as in study one, in addition to a second phase conducted from 2004-2007. There was evidence of a temporal trend in disease risk with time between first and second infections, with asymptomatic infections occurring at shorter intervals and DHF at longer intervals. The final study evaluated the nature of serotype interactions in multivalent DENV vaccines, using data from a factorial design clinical trial that evaluated all combinations of high and low dose serotype strains in tetravalent formulations. Analyses considered how adjustments in the dose of a serotype affected seroconversion to that and other serotypes. Both immunological interference and facilitation were observed to occur between DENV serotypes with respect to seroconversion as well as the occurrence of adverse events. Together, these three studies provide novel insights into the complex nature of DENV immunity in the setting of multiple serotype exposures.

Sero-epidemiology of dengue viruses: The influence of preexisting immunity on disease severity and implications for vaccine development

By

Kathryn B. Anderson
MSPH

Advisor: Ruth L Berkelman, MD

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Epidemiology, 2011

Dedication

This dissertation is dedicated to my parents, John and Jeri Anderson, for their tireless support throughout this process. I am particularly grateful to my father for sharing his experience and insights from many years of service in academia; I couldn't have finished this dissertation without your guidance. I'm incredibly fortunate to call you Mom and Dad.

Acknowledgements

This dissertation would not have been possible without the support of my committee, my colleagues and friends in the US Army, funding support from the CDC, and my family and friends.

I would like to offer my deepest gratitude to my advisor and chair Ruth Berkelman, for taking on the challenges of a project that was based outside of Emory University and a student that was often in the field. Your support, expertise, patience, and caring helped me through this process. I am incredibly grateful for your generosity and look forward to continuing our relationship, as colleagues and friends, in the years to come.

I would like to thank John McGowan for his mentorship and support throughout my ten (!) years at Emory, and most especially during my PhD. You have pushed me to excel and I am a better scientist for your efforts. Thank you for generously sharing your time and expertise these years. I would like to thank Dana Flanders for sharing his analytical expertise and for his kind support throughout this process. Derek Cummings provided invaluable perspectives on dengue epidemiology and modeling opportunities and I am grateful for his participation on the committee.

My heartfelt thanks to Tim Endy, Robert Gibbons, Alan Rothman, and others that have facilitated opportunities to work with the Armed Forces Institute of Medical Sciences and the Walter Reed Army Institute of Research. I consider myself incredibly fortunate to be a

member of the 'team' and look forward to more opportunities to work together in the years ahead.

This work would not have been possible without the financial support of the Centers for Disease Control (CDC) Grants for Public Health Research Dissertation (R36), which facilitated data collection in the field and provided for travel costs.

Finally, my thanks to my parents, siblings, and friends for their support throughout this process. This dissertation was a significant challenge but I knew you were there for me through the ups and downs.

TABLE OF CONTENTS

Abstract……

Acknowledgements.....

Dedication .....

List of Figures .....

List of Tables .....

List of Abbreviations.....

I. INTRODUCTION.....1

II. BACKGROUND

A. The Virus.....8

B. Transmission

(i) The Vectors 10

(ii) Transmission Cycles 13

C. Epidemiology

(i) History.....16

(ii) Present Distribution and Burden of Disease 18

(iii) Epidemic Characteristics 19

(iv) Prevention and Control 20

D. Clinical Aspects of DENV Infection 21

E. Mechanisms of DHF

(i) DHF and Secondary Infection.....23

(ii) Antibody-Dependent Enhancement 24

(iii) Original Antigenic Sin 26

(iv) Viral Factors 26

(v) Host Factors 27

F. The Antibody Response to Infection 27

(i) Dose-dependence of Cross-protection and Enhancement 29

(ii) Temporal Shifts in Cross-protection and Risk of Enhancement 30

(iii) Proposed Model of Trends in the Risk of Illness with Secondary DENV Infection 31

G. DENV Serological Assays – Methods, Capabilities, and Limitations 33

(i) Plaque Reduction Neutralization Titers (PRNTs) 34

(ii) Hemagglutination Inhibition (HI) 36

(iii) Enzyme-linked Immunosorbent Assays (ELISAs) 36

H. The Future – Dengue vaccines 36

I. References 38

III. STUDY ONE: PREEXISTING IMMUNITY TO JAPANESE ENCEPHALITIS VIRUS AND THE INCREASED OCCURRENCE OF SYMPTOMATIC DENGUE INFECTION

A. Abstract 47

B. Introduction 48

C. Methods

(i) Study Population 50

(ii) Study Methods 50

(iii) Characterization of Symptomatic Illness 50

(iv) Characterization of Asymptomatic Infections 51

(v) Characterization of DENV and JEV Antibody Status 52

(vi) Inferring the Infecting Serotype 53

(vii) Statistical Analyses 53

(viii) Human Subjects Research Approval 54

D. Results

(i) Descriptive Statistics 55

(ii) Factors Associated with Symptomatic Illness 55

(iii) Factors Associated with JEV Positivity 56

(iv) Associations of JEV NAb Positivity with DENV Illness 56

(v) Duration of Illness 58

(vi) Titer of JEV NAbs 58

(vii) Role of Infecting DENV Serotype 58

(viii) Multivariate Model of JEV NAb Positivity and Symptomatic DENV Infection 59

(viii) Serological Cross-reactivity Between JEV and DENV 59

(ix) Relative Risk of Symptomatic Infection with JEV Antibodies 59

E. Discussion 61

F. Acknowledgements 66

G. References 67

H. Tables 71

I. Figures 77

J. Supplemental Tables 81

IV. STUDY TWO: A SHORTER TIME INTERVAL BETWEEN FIRST AND SECOND DENGUE INFECTIONS IS ASSOCIATED WITH PROTECTION FROM CLINICAL ILLNESS IN A PROSPECTIVE SCHOOL-BASED COHORT IN THAILAND

A. Abstract 85

B. Introduction 87

C. Methods

(i) Study Population 91

(ii) Acute Illness Specimens and Active Fever Surveillance 91

(iii) Routine Blood Specimens and Detection of Seroconversions 92

(iv) Characterization of First-Detected and Second-Detected Infections 93

(v) Defining Baseline DENV Immunity and Other Immunological Parameters 94

(vi) Immunological Parameters 95

(vii) Inferring the Infecting Serotype 95

(viii) Statistical Analyses 96

(ix) Human Subjects Research Approval 97

D. Results 98

(i) Time from First to Second-Detected Infection and Clinical

Severity 98

(ii) Predictors of the Time to Infection and the Severity of the Second-Detected Infection 99

(iii) Immunological Correlates of Infection Severity.....100

(iv) Modeling the Probability of Asymptomatic Infection over Time.....100

E. Discussion.....102

G. References 107

H. Tables 110

I. Figures 113

J. Appendices 120

V. STUDY THREE: IMMUNOLOGICAL INTERFERENCE AND FACILITATION BETWEEN DENGUE SEROTYPES IN TETRAVALENT FORMULATIONS

A. Abstract 130

B. Introduction 132

C. Methods

(i) Data Sources: Monovalent and Tetravalent Clinical Trials 135

(ii) Quantification of Immunogenicity – Serological Methods.....136

(iii) Quantification of Reactogenicity 136

(iv) Statistical Analyses 137

(v) Human Subjects Clearance.....138

D. Results

(i) Descriptive Results....139
(ii) Comparison of Monovalent and Tetravalent Formulations....139
(iii) Bivariate Analysis: Effects of Dose on Homologous and Heterologous Serotypes in Tetravalent Formulations....140
(iv) Multivariate Analysis: Effects of Dose on Homologous and Heterologous Serotypes in Tetravalent Formulations....141
(v) Effects of Dose on Multitypic Immunogenicity....141
(vi) Effects of Dose on Vaccine Reactogenicity....141
E. Discussion....143
F. Acknowledgements....147
G. References....148
H. Tables....151
I. Figures....155

VI. CONCLUSIONS....163
A. Dissertation Studies
a. Study One....165
b. Study Two....167
c. Study Three....170
B. Limitations....172
C. Strengths....175
D. Public Health Contributions
a. Interactions between Heterologous Flaviviruses....177

b. Evidence of Cross-Protection and a Temporal Aspect to Disease Risk.....180

c. Identifying Interactions in Multivalent Vaccines 183

E. References.....186

VII. REFERENCES.....190

List of Tables

STUDY ONE

Table 1. Cohort characteristics associated with symptomatic DENV illness……..……..….79

Table 2. Factors associated with JEV seropositivity prior to DENV infection…..……..….81

Table 3. Crude associations of DENV illness severity and JEV antibody status……..….83

Table 4. Duration of DENV illness (days) by JEV NAb status……84

Supplemental Table 1. Association between JEV NAb status and asymptomatic (Asx) DENV infection, stratified on reported receipt of the JEV vaccine…….....90

Supplemental Table 2. Relative risk of symptomatic DENV infection with JEV hemagglutination inhibition (HI) antibodies* time of enrollment into the cohort study…...91

STUDY TWO

Appendix 1. Time-course of HII titers, from 1st infection to 2nd infection (6 examples from individuals)……...…….131

Appendix 2. Time to infection – restricting to HI-negatives……..135

Appendix 4. Multivariate logistic model output……138

STUDY THREE

Table 1. Dengue vaccine formulations…….…….164

Table 2. Multivariate logistic regression models for seroconversion to DENV-1 – DENV-4 (four individual models were constructed). Seroconversion is defined as a PRNT50 titer of >=10 after one dose of a tetravalent formulation…….....166

Table 3. Logistic regression model comparing seroconversion to three or more serotypes to seroconversion to zero-two dengue serotypes following a single dose of tetravalent vaccine……..167

Table 4. Multivariate linear regression model of reactogenicity index following a single dose of tetravalent vaccine…….……..168

List of Figures

BACKGROUND

Figure 1. Flavivirus Classification……..……..…11

Figure 2. World distribution of dengue viruses and their mosquito vector, *Aedes aegypti*..
From: (CDC)……...……...……...14

Figure 3. Distribution of *Aedes albopictus* in the United States by county, 2000.
From: (CDC)…..……..……...…..…….…….14

Figure 4. Figure 4. Transmission cycles of flaviviruses that cause disease in humans. From:
(S. C. Weaver & Barrett, 2004)……..…….…….17

Figure 5. Increasing co-circulation of DENV serotypes, from 1970 (top) to 2004 (bottom)
From: (Mackenzie, Gubler, & Petersen, 2004)……...…….……20

Figure 6. The 'iceberg' of DENV infection severity……..…….…….24

Figure 7. Example: generation of specific and cross-reactive antibodies following DENV
infection……..……31

Figure 8. The waning of maternal antibodies and shifts in the risk of DHF in infants.
From: (Halstead, et al., 2002)…….…….....32

Figure 9. Temporal trends in the titers of specific and cross-reactive antibodies generated in
response to a primary DENV infection (in this example, DENV-1) over time…….……..…35

STUDY ONE

Figures 1a-1c. The proportion of dengue (DENV) infections developing symptomatic illness (1a), hospitalized illness (1b), and dengue hemorrhagic fever (1c)……..…….85-6

Figure 2. Distribution of JEV neutralizing antibody titers among those with detectable JEV antibodies, by DENV illness severity…….87

Figures 3a-3c. Proportions of infections developing symptomatic illness (3a) and hospitalized illness (3b and 3c) by infecting DENV serotype……88

STUDY TWO

Figure 1. Flowchart of the total starting population for this study (children that experienced at least one DENV infection), detailing the numbers remaining at risk for a second infection each year, the numbers of cases, and their severities……124

Figure 2. Total numbers of infections, and the severity of infections, detected each year of the cohort studies……....126

Figure 3. Probability of asymptomatic infection by year since the first-detected infection in the cohort studies, by whether a child had detectable HI antibodies at enrollment (HI-positive) or was negative by HI at enrollment (HI-negative…….128

Figure 4. Odds ratios (ORs) for the risk of symptomatic infection in HI-negatives given that a child was still at risk for a second infection during an interval…….....129

Figure 5. Odds ratios (ORs) for experiencing a symptomatic versus an asymptomatic second infection, given that a second infection occurred…….130

Appendix 3. Associations of immune parameters with clinical severity of 2nd-detected DENV infections, stratified by whether a child was hemagglutination inhibition (HI) antibody positive or HI-negative for DENV serotypes on enrollment……136

STUDY THREE

Figure 1. Mean reactogenicity index (lower right y-axis) and total # of serotypes with seroconversion at day 28 (upper left y-axis), sorted in descending order of immunogenicity…….169

Figure 2. Proportion of individuals seroconverting to DENV-1 – DENV-4 (Figure 2a) and the geometric mean titer (GMT) of the PRNT50 for DENV-1 – DENV-4 (among individuals that seroconverted to that serotype) (Figure 2b), by whether they received each serotype as a high-dose monovalent vaccine or as a high-dose component of a tetravalent vaccine……...172

Figure 3. Proportion of individuals, across all tetravalent formulations, seroconverting to DENV-1 – DENV-4 (x-axis) depending upon whether DENV-1 (Figure 3a), DENV-2 (Figure 3b), DENV-3 (Figure 3c), or DENV-4 (Figure 3d) were high or low dose in the formulation……175

List of Abbreviations

Ab	Antibody
ADE	Antibody-dependent enhancement
ANOVA	Analysis of variance analysis
Asx	Asymptomatic
C	Capsid (protein)
CDC	Centers for Disease Control (and Prevention)
CI	Confidence interval
DALYs	Disability adjusted life-years
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DI-DIII	Domains I-III (of E protein)
DSS	Dengue shock syndrome
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Program for Immunization
FRhL	Fetal rhesus lung
GMT	Geometric mean titer
HI	Hemagglutination inhibition
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G

IgM	Immunoglobulin M
JEV	Japanese encephalitis virus
JEVAX	Commercial name for an inactivated, mouse-brain derived JEV vaccine
KPS	Kamphaeng Phet Study
MMR	Measles-mumps-rubella
MVEV	Murray Valley encephalitis virus
N	Number of individuals
NAb	Neutralizing antibody
Neg	Negative
NIAID	National Institute for Allergy and Infectious Disease
NS	Non-structural (protein)
OPV	Oral polio vaccine
OR	Odds ratio
PCR	Polymerase chain reaction
PDK	Primary dog kidney
PFU	Plaque-forming unit
Pos	Positive
PrM	Membrane (protein)
PRNT	Plaque reduction neutralization titer
PRNT50	The concentration of plasma that resulted in a 50% reduction in plaque formation
RI	Reactogenicity index
RR	Risk ratio

RT-PCR	Reverse-transcriptase polymerase chain reaction
SD	Standard deviation
SLEV	St. Louis encephalitis virus
Sx	Symptomatic
U.S	United States
WHO	World Health Organization
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
YFV	Yellow fever virus

CHAPTER 1 – INTRODUCTION

Dengue is the most important mosquito-borne virus causing human disease in tropical and subtropical regions of the world and both the geographic range and the magnitude and severity of epidemics are increasing each year ("NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report.,"). 2.5 billion people are estimated to live in dengue endemic areas and are at risk for severe disease, with an estimated 50 million cases of DF occurring each year and several hundred thousand cases of DHF ("Strategic direction for dengue research: Disease burden and epidemiological trends.," Feb 2002.). The burden of disease is greatest in SE Asia, where DHF and the severe complication dengue shock syndrome (DSS) are a significant cause of childhood mortality, resulting in a loss of 465 disability adjusted life-years (DALYs) per million population on average and 954 DALYs/million/year during peak epidemic years, which is of the same order of magnitude as the impact of meningitis, hepatitis B, and tropical diseases (which include schistosomiasis, trypanosomiasis, and leishmaniasis) (Anderson, et al., 2007). As no effective treatment exists for dengue infection, prevention efforts necessarily focus on mosquito control and the development of tetravalent dengue vaccines (DeRoeck, Deen, & Clemens, 2003).

There are four dengue virus serotypes (DENV-1 – DENV-4), which are responsible for annual epidemics in endemic regions such as Southeast Asia. The co-circulation of DENV serotypes is associated with the emergence of the severe disease dengue hemorrhagic fever (DHF), which is hypothesized to result when preexisting, cross-reactive antibodies from a previous DENV exposure facilitate subsequent infection with a different DENV serotype, resulting in an increased viral load, increased immune activation, and ultimately plasma leakage and shock (Halstead & Simasathien, 1970). However, a cross-protective role of

preexisting DENV immunity has also been demonstrated (Sabin, 1952) and the complex immunological factors involved in whether an infection with DENV will be subclinical, mild, or develop into DHF are poorly understood. The ability to discriminate between 'pathologic' immune profiles, those predisposing to illness with subsequent infection, and 'protective' immune profiles, those reducing risk of illness with subsequent infection, remains a central issue in DENV immunology, and one that looms large as DENV vaccines approach licensure and implementation.

The following three studies seek to address important knowledge gaps in the relationships between preexisting immunity and the clinical outcome of DENV infection (antibody – virus associations) and between tetravalent vaccination and the serological responses to vaccination (virus – antibody associations). The hypotheses were: 1) The preexistence of Japanese encephalitis virus (JEV) neutralizing antibodies is protective against illness with subsequent DENV infection, 2) Time between DENV infections is associated with the risk of DENV illness, with exposures that occur within a shorter interval of time following an infection being more likely to result in asymptomatic infection, 3) Evidence of immunological interference and facilitation can be detected between DENV serotypes in tetravalent vaccine formulations.

Studies one and two used data from longitudinal, school-based cohort studies for DENV infection that took place in Kamphaeng Phet, Thailand, from 1998-2002 and from 2004-2007 (T. P. Endy, Chunsuttiwat, et al., 2002; Mammen, et al., 2008). These cohort studies were unique in that the fever surveillance system was designed to capture illnesses across a range of clinical severities, from mild to hospitalized, and asymptomatic infections were

detected as seroconversions in the routine specimens that were collected from all study participants. These routine sera further allowed the unique characterization of a child's immune state prior to experiencing an infection. Study three used data from a factorial design clinical trial of 16 tetravalent DENV formulations, which evaluated all combinations of high and low dose for each serotype (Edelman, et al., 2003). Monovalent clinical trial data for each serotype were also available for comparison with tetravalent formulations (Kanesa-Thasan, et al., 2003).

Paper 1: Preexisting immunity to Japanese encephalitis virus and the increased occurrence of symptomatic dengue infection

DENV and Japanese Encephalitis virus (JEV) co-circulate in Southeast Asia, where they are both important causes of human morbidity and mortality (T. P. Endy & Nisalak, 2002). They have significant cross-reactivity in serological assays, but the possible clinical implications of this remain poorly understood. Some previous studies have suggested at least a transient protective effect of JEV immunization against DHF (Hoke, et al., 1988). The association between pre-existing JEV neutralizing antibodies (NAbs) and the clinical severity of subsequent DENV infection was evaluated for a cohort of Thai children who were followed for the occurrence of both symptomatic and asymptomatic infections from 1998-2002. Covariates considered included age, epidemic year, baseline DENV antibody status, and infecting DENV serotype. An improved understanding of possible interactions between JEV and DENV is important as large-scale vaccine trials will soon commence in areas where JEV co-circulates with DENV or where JEV vaccination is common.

Paper 2: Temporal trends in the probability of asymptomatic infection and DHF with secondary DENV infection in a prospective school-based cohort study in Thailand

Despite the association between preexisting, cross-reactive DENV antibodies and the occurrence of DHF, the majority of secondary infections are in fact asymptomatic or dengue fever (DF). Other studies have presented evidence of a possible protective effect of cross-protective immunity (T. P. Endy, et al., 2004; Sabin, 1952). Thus, the relationship between preexisting, cross-reactive DENV antibodies and the clinical severity of secondary infections remains unclear, though some studies have suggested a possible titer-dependent and time-dependent role of cross-protective dengue DENV antibodies (T. P. Endy, et al., 2004; M. G. Guzman, et al., 2000; Halstead, et al., 2002; Sabin, 1952). This study used data from two prospective cohort studies for asymptomatic and symptomatic DENV infections in Thai children, from 1998-2002 (T. P. Endy, Chunsuttiwat, et al., 2002) and 2004-2007 (Mammen, et al., 2008). This study sought to evaluate whether time between first and second dengue infections in the cohort studies was a significant predictor of the clinical severity of infection, as a possible proxy for a decline in cross-reactive antibodies over time. Hemagglutination inhibition (HI) antibody titers were used to characterize a child's DENV antibody profiles prior to the first infection, immediately following the first infection, and prior to the second infection. This study was the first to investigate temporal shifts in disease risk using longitudinal human infection data since Sabin's early reports of a 'window of protection' following DENV infection (Sabin, 1952).

Paper 3: Immunological interference and facilitation in the neutralizing antibody response to tetravalent DENV vaccination

There is no specific antiviral treatment for DENV infection and modern vector control programs have been largely unsuccessful in containing DENV transmission (Gubler, 1997). The development of safe and effective DENV vaccines is presently thought to be the best option for long-term reductions in DENV infections and disease (DeRoeck, et al., 2003). The co-circulation of DENV serotypes in many DENV-endemic locales and the association between preexisting DENV immunity and DHF has warranted the development of tetravalent vaccines. However, the generation of strong, balanced responses with multivalent virus vaccines has historically proven to be a significant challenge (Berger & Just, 1988 Oct; Patriarca, Wright, & John, 1991); DENV vaccines are no exception. To identify possible interactions between DENV serotypes in tetravalent formulations, this study analyzed data from a factorial design clinical trial that evaluated all combinations of high or low doses of each DENV serotype (16 formulations, n=64). Interactions between DENV serotypes were identified by evaluating how changes in the dose of each serotype in tetravalent formulations affected the antibody response to all four serotypes as well as the reactogenicity of each formulation. Such information may lead to an improved understanding of how DENV serotypes may interfere or facilitate seroconversion in tetravalent vaccine formulations. This in turn may inform the development of safe and immunogenic vaccines.

REFERENCES

Anderson, K. B., Chunsuttiwat, S., Nisalak, A., Mammen, M. P., Libraty, D. H., Rothman, A. L., et al. (2007). Burden of symptomatic dengue infection in children at primary school in Thailand: a prospective study. *Lancet, 369*(9571), 1452-1459.

Berger, R., & Just, M. (1988 Oct). Interference between strains in live virus vaccines. II: Combined vaccination with varicella and measles-mumps-rubella vaccine. *J Biol Stand, 16*(4), 275-279.

DeRoeck, D., Deen, J., & Clemens, J. D. (2003). Policymakers' views on dengue fever/dengue haemorrhagic fever and the need for dengue vaccines in four southeast Asian countries. *Vaccine, 22*(1), 121-129.

Edelman, R., Wasserman, S. S., Bodison, S. A., Putnak, R. J., Eckels, K. H., Tang, D., et al. (2003). Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. *Am J Trop Med Hyg, 69*(6 Suppl), 48-60.

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

Endy, T. P., & Nisalak, A. (2002). Japanese encephalitis virus: ecology and epidemiology. *Curr Top Microbiol Immunol, 267*, 11-48.

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever: Its history and resurgence as a global public health problem. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 1-22). New York: CAB International.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazquez, S., et al. (2000). Epidemiologic studies on dengue in Santiago de Cuba, 1997. *Am J Epidemiol, 152*(9), 793-799.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., & Simasathien, P. (1970). Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. *Yale J Biol Med, 42*, 276-292.

Hoke, C. H., Jr., Nisalak, A., Sangawhipa, N., Jatanasen, S., Laorakpongse, T., Innis, B. L., et al. (1988). Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med, 319*, 608-614.

Kanesa-Thasan, N., Edelman, R., Tacket, C. O., Wasserman, S. S., Vaughn, D. W., Coster, T. S., et al. (2003). Phase 1 studies of Walter Reed Army Institute of Research candidate attenuated dengue vaccines: selection of safe and immunogenic monovalent vaccines. *Am J Trop Med Hyg, 69*(6 Suppl), 17-23.

Mammen, M. P., Pimgate, C., Koenraadt, C. J., Rothman, A. L., Aldstadt, J., Nisalak, A., et al. (2008). Spatial and temporal clustering of dengue virus transmission in Thai villages. *PLoS Med, 5*(11), e205.

NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report. *U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Allergy and Infectious Disease.*

Patriarca, P., Wright, P., & John, T. (1991). Factors affecting immunogenicity of oral poliovirus vaccine in developing countries: review. *Rev Infect Dis, 13*(926-939).

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Strategic direction for dengue research: Disease burden and epidemiological trends. (Feb 2002.). *World Health Organization.* .

CHAPTER 2 - BACKGROUND

I. The virus.

The dengue viruses are single-strand, enveloped, positive-sense RNA viruses in the virus family *Flaviviridae*. Thirty-four of 74 identified flaviviruses are transmitted by mosquitoes and 40 of 74 have been shown to infect humans (Burke & Monath, 2001). The most significant arboviral causes of human morbidity and mortality across the globe are members of this family, namely dengue viruses (DENV), tick-borne encephalitis virus, yellow fever virus (YFV), and Japanese encephalitis virus (JEV). There are four dengue serotypes (DENV-1, -2, -3, and -4), which together comprise the dengue antigenic family of flaviviruses. All four serotypes have been linked to human disease. On the basis of the amino acids within the envelope (E) protein, DENV-1 and DENV-3 share the greatest sequence homology (i.e., are the most closely related, with 77% homology), then DENV-2 (69%), then DENV-3 (62%) (3). Notably, the homology of different virus isolates within a single serotype have been shown to be highly variable, with up to 10% differences in the amino acid composition of the E protein (Lewis, et al., 1993). Extending beyond the DENV antigenic complex, the DENVs are most closely related to JEV, sharing 46-53% of their amino acids (Blok, et al., 1992). Other arboviruses contained within the JEV antigenic complex and closely related to DENVs include West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV) (Figure 1) (Kuno, Chang, Tsuchiya, Karabatsos, & Cropp, 1998).

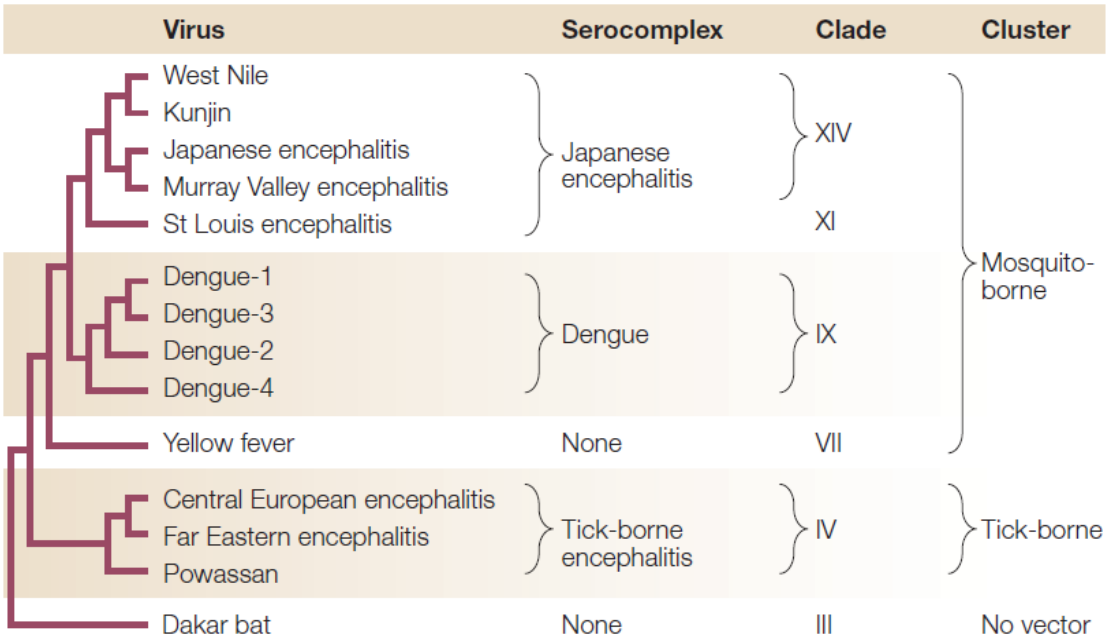


Figure 1 | **Flavivirus classification.** The relationships between selected flaviviruses are shown in the dendrogram on the left. Evolutionary distance is not represented in this figure. The serological (serocomplex) and phylogenetical (clade and cluster) classifications of these flaviviruses are shown on the right. Reproduced with permission from REF. 2 © (2002) Lippincott Williams and Wilkins.

* From (Mukhopadhyay, Kuhn, & Rossmann, 2005)

The DENV genome is approximately 11 kilobases in length, encoding three structural proteins (E, C, PrM) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). The E protein is the primary target for neutralizing antibodies. Characterization of different antigenic domains within the E protein (DI, DII, and DIII) has identified important differences in the serotype specificities and protective capability of neutralizing antibodies elicited against each of the domains. Antibodies against DIII are the most serotype-specific and most protective against infection (Wahala, et al., 2010); antibodies against DIII also tend to represent a small fraction of the total response to infection. In contrast, antibodies against DI and DII neutralize more poorly, exhibit broader serotype cross-reactivity, and tend to dominate the response to infection (Oliphant, et al., 2006). The

non-structural protein NS1 is also thought to play an important role in virulence. NS1 is a glycoprotein that is both secreted from cells and presented on the surface of cells during DENV infection (Avirutnan, et al., 2006). NS1 is thought to be important in stimulating the immune system to recognize and clear infected cells but soluble NS1 is also, in ways that remain poorly understand, thought to contribute to the vascular leakage observed in severe DENV infections (Avirutnan, et al., 2006).

DENVs exhibit significant genetic diversity and each serotype is further divided into three to five genotypes. The virulence of different genotypes has been shown to vary; for example, the introduction of the Asian genotype of DENV-2 into Cuba was suggested to be associated with epidemics of higher virulence than epidemics of the American genotype (Cologna & Rico-Hesse, 2003; Rico-Hesse, 1990). The severity of infection with different DENV strains may vary as a complex result of epidemiological factors (e.g., the sequence and timing in which different serotypes caused epidemics in a region), host factors (preexisting immunity), vector factors (increased or decreased selection driving evolution), or genetic drift with nucleotide changes associated with changes in virulence (e.g., within E-protein domains) (S.C. Weaver & Vasilakis, 2009).

II. Transmission.

The Vectors

Dengue is a mosquito-borne virus. This link, interestingly, was identified by experimental inoculation of human volunteers in the 1920s, years prior to the isolation of DENV in the 1940s. In these experiments, mosquitoes were fed upon individuals showing clinical signs of dengue fever and then allowed to feed on healthy volunteers at various time intervals after

the initial (infective) blood meal. These studies provided early evidence of the association between *Aedes aegypti* and dengue transmission and provided estimation of the intrinsic and extrinsic incubation periods (Siler, Hall, & Hitchens, 1926).

Since that time, several mosquito species in the subgenus *Stegomyia* have been shown to be capable of transmitting DENV (Rodhain & Rosen, 1997). The primary vector is *Aedes aegypti*, which is widely distributed throughout tropical and subtropical regions of the world (Figure 2). Notably, *Aedes aegypti* mosquitoes are also present in southern and south-eastern regions of the United States, providing a potential niche for the reintroduction of DENV (Figure 3). *Aedes aegypti* are primarily daytime feeders, and they are most commonly found domestically or peridomestically. Their close association with human habitations is in part due to their preferred breeding habitats: these mosquitoes lay their eggs in standing water, and many human-made habitats allow these insects to thrive (water storage jugs within the home, old tires, flower pots, etc.). *Aedes aegypti* are particularly efficient vectors for DENV transmission because they feed almost exclusively on humans and they tend to feed on multiple individuals during a single gonotropic cycle (Yasuno & Tonn, 1970).

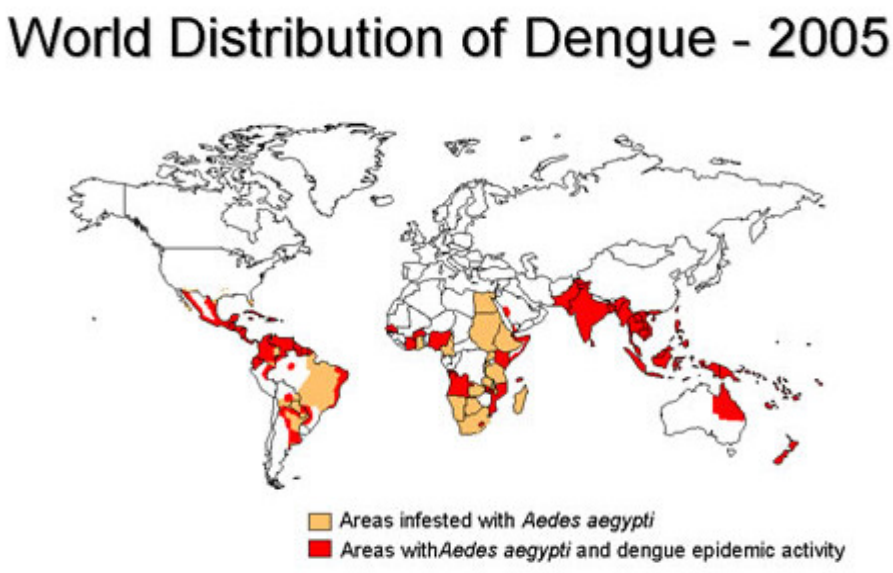


Figure 2. World distribution of dengue viruses and their mosquito vector, *Aedes aegypti*.

* From: (CDC) [Link: http://img.medscape.com/pi/emed/ckb/pediatrics_general/960757-963213-3287.jpg; Accessed October 24, 2010 09:00]

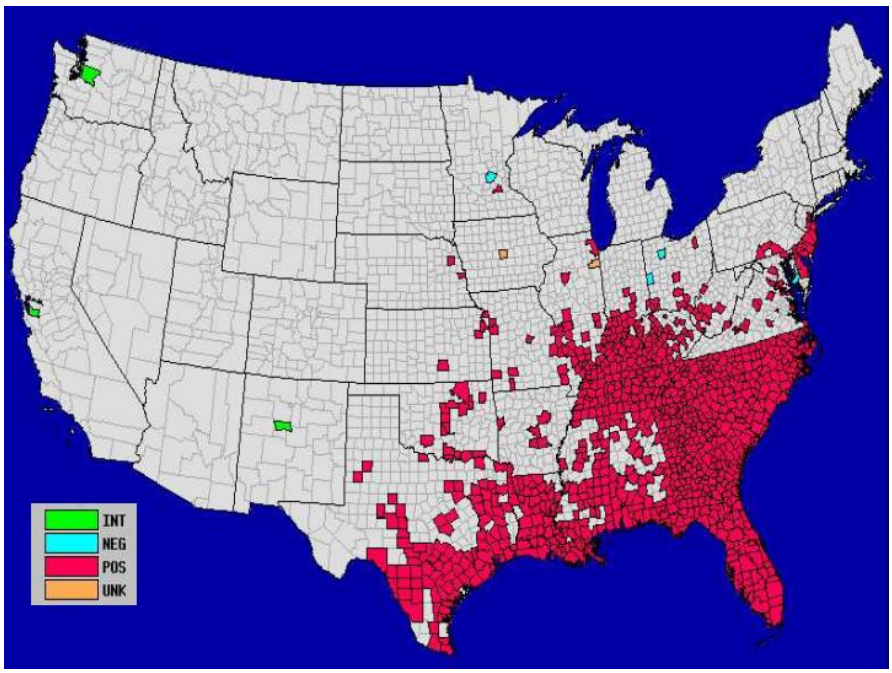


Figure 3. Distribution of *Aedes albopictus* in the United States by county, 2000.

* From: (CDC) [Link: http://www.cdc.gov/ncidod/dvbid/arbor/images/us-map-ae-albo-2000.jpg; Accessed October 24, 2010 09:00]

There is concern regarding the transmission potential of a more temperate vector, *Aedes albopictus*, which has been rapidly expanding its range in the Americas, Africa, the Middle East, and Europe. *Aedes albopictus* was first identified in the United States in 1985, likely transported in truck tires shipped from Asia (Hawley, Reiter, Copeland, Pumpuni, & Craig, 1987). Since that time, it has established itself as an endemic mosquito species throughout vast areas of the Americas, including the southeastern United States (Figure 3). The mosquito has long been identified as a relatively efficient vector of dengue and Chikungunya viruses in Asia but the increase in dengue transmission that was predicted to occur in the United States because of *Aedes albopictus* has not yet been realized, for reasons that remain unclear (Gratz, 2004).

Transmission cycles.

The primary transmission cycle of DENV is thought to be quite simple, cycling from humans to mosquitoes to humans (Figure 4). This is in contrast to Japanese encephalitis virus, in which humans are dead-end hosts in a primarily zoonotic cycle involving horses, pigs, water fowl, and mosquitoes, and in contrast to yellow fever virus, in which urban cycles (involving humans and mosquitoes) are stoked by the introduction of viruses from sylvatic cycles (involving primates and mosquitoes and occasionally humans).

The extrinsic incubation period for DENV (time elapsed from infectious blood meal until the mosquito is itself infectious to humans) is estimated to be eight to twelve days. A bite

from an infected female mosquito can then transmit the virus to humans. The intrinsic incubation period (time elapsed from when an individual receives an infectious bite until he/she develops symptoms) averages four to seven days. Notably, viremia typically develops before the onset of symptoms. Chronic DENV infection in humans does not occur and the virus is cleared from the bloodstream after an average of four to five days of viremia. These estimates of the intrinsic and extrinsic incubation periods and the duration of viremia are derived from two experimental infection studies that took place in 1924-5 and 1929-30 (Nishiura & Halstead, 2007; Siler, et al., 1926; Simmons, St John, & Reynolds, 1931). Trans-ovarial transmission (i.e., infection of the egg by a pregnant, infected mosquito) has been documented to occur but appears to be rare (Watts, Harrison, Pantuwatana, Klein, & Burke, 1985).

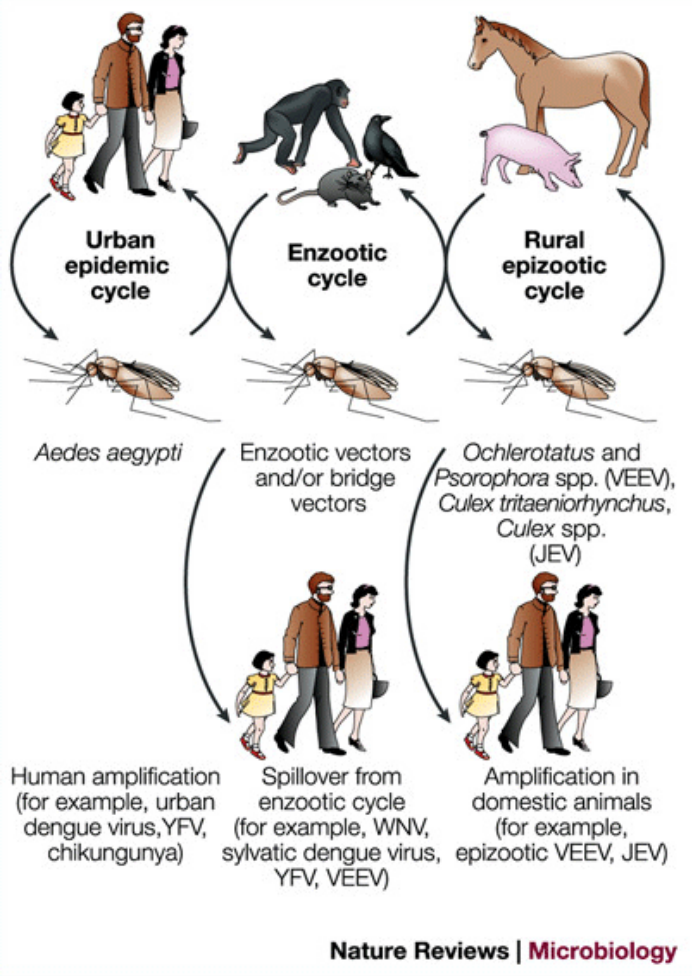


Figure 4. Transmission cycles of flaviviruses that cause disease in humans.

* From: (S. C. Weaver & Barrett, 2004)

Sylvatic cycles of dengue transmission occur but have historically received little attention. Recently, it was shown that many species of neotropical marsupials, bats, and rodents were capable of becoming infected with DENV and that, when trapped near sites of active DENV epidemics in human populations, the isolated viruses from the animals were very similar to the viruses isolated from human cases (de Thoisy, et al., 2009). A sylvatic DENV-2 virus was recently isolated from a man with DHF in Malaysia, suggesting that these viruses have pathogenic potential in humans (Cardosa, et al., 2009). The importance of these

sylvatic cycles in contributing significantly to human DENV infections and disease remains to be seen.

III. Epidemiology.

History.

Reports of epidemics of dengue-like illnesses can be traced back several centuries (Gubler, 1997) Large epidemics in disparate parts of the globe began to emerge in the 1700s, which has led some to speculate that the slave trade facilitated the global dispersal of *Aedes aegypti* and the introduction of DENV into the New World (Smith, 1956). A large epidemic took place in Philadelphia in 1780, which contributed to some of the earliest detailed clinical descriptions of dengue fever (DF) and dengue hemorrhagic fever (DHF) (Rush, 1789). The first isolates of DENV were made in 1943, during which time many soldiers stationed in the Pacific and Asia were becoming infected (Kimura & Hotta, 1944).

DENV is presently considered an important emerging pathogen due to dramatic increases in incidence of infection in the past 100 years and the emergence of DHF as a serious cause of global morbidity and mortality (Gubler & Clark, 1995). This emergence can be linked to several developments over the past century. *Aedes aegypti* is an urban vector, and urbanization and population growth have fostered increased DENV transmission. Increased international travel, particularly air travel, has facilitated the rapid spread of DENV serotypes to new regions. In Latin America, the emergence of DENV can more aptly be labeled a resurgence. Efforts to control the vector *Aedes aegypti* had strong support in the 1940s and 1950s throughout Latin America and these campaigns were largely successful; enthusiasm

waned in the 1970s and a steady re-establishment of *Aedes aegypti* mosquitoes, and DENV serotypes, can be seen each year (P. Russell, 1978).

World War II heralded a large shift in the epidemiology of DENV. This war was associated with the massive mobilization of individuals to distant regions. *Aedes aegypti* and DENV viruses were introduced to new populations and some areas already endemic for DENV became endemic for multiple DENV serotypes (i.e., became 'hyperendemic') (Gubler, 2002b). This shift from a single DENV serotype in circulation to hyperendemicity is important for two reasons. First, infection with a given serotype provides lifelong protection against that serotype but not against infection with any of the other serotypes (Sabin, 1952). Instead of a single DENV infection, an individual living in a hyperendemic region can experience two or more infections over the course of their lifetime. The increased circulation of DENV serotypes in a region is therefore linked with an increased incidence of infection. Second, the co-circulation of DENV serotypes is associated with the increased occurrence of the severe outcome dengue hemorrhagic fever (DHF) (Halstead, 1997). Today, many nations in Southeast Asia have all four dengue viruses in circulation and this region exhibits both the highest rates of DENV infection and the highest rates of DHF. Latin America has lagged Southeast Asia in the co-circulation of DENV serotypes but each year there are reports of novel introductions into the Americas (Figure 5).

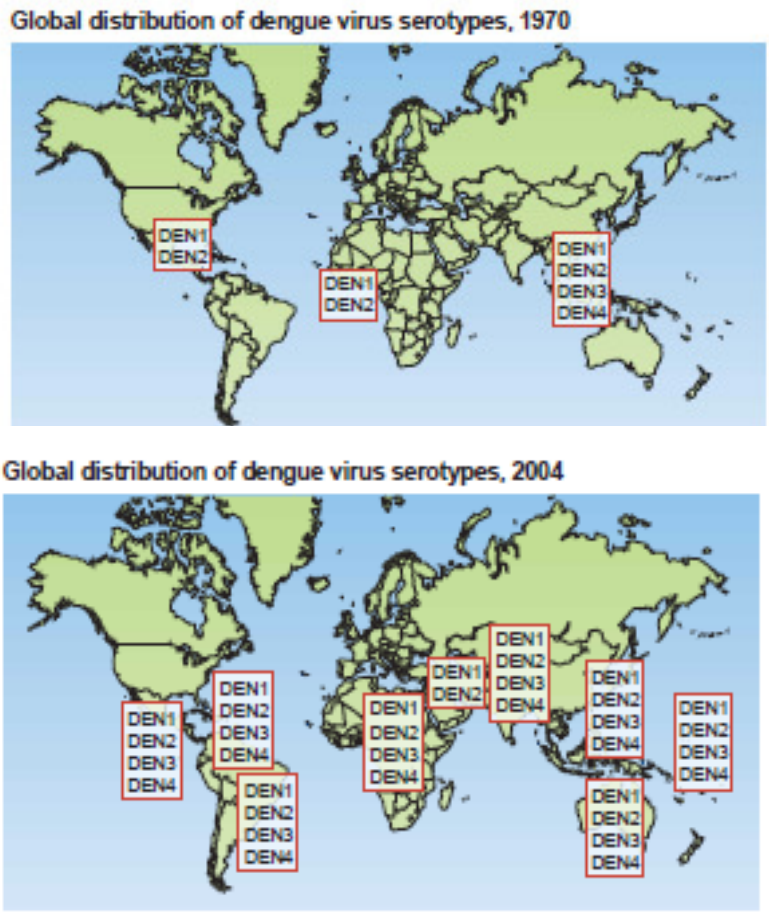


Figure 5. Increasing co-circulation of DENV serotypes, from 1970 (top) to 2004 (bottom).

* From: (Mackenzie, Gubler, & Petersen, 2004)

Present distribution and burden of disease

Dengue is the most important mosquito-borne virus causing human disease in tropical and subtropical regions of the world (U.S. Department of Health and Human Services). More than 100 countries in Asia, Africa, the Americas, the Pacific, and the Caribbean have been reported as DENV-endemic (Division of Vector-Borne Infectious Diseases, 1997). DENV infections in the United States have historically been associated with immigrants or international travelers; despite the endemicity of *Aedes aegypti* in large regions of the U.S, sustained local transmission was not maintained ("From the Centers for Disease Control and

Prevention. Dengue fever at the US-Mexico border, 1995-1996," 1996). Investigations of DENV transmission in two proximal communities on either side of the US/Mexico border suggested that indicators of infrastructure and higher socioeconomic status such as the availability of street drainage, screened windows, and air-conditioning were associated with decreased transmission (Ramos, et al., 2008). Recently, local transmission of DENV-1 was confirmed in Key West, Florida; whether the virus will become endemic in this region remains to be seen ("Locally Acquired Dengue --- Key West, Florida, 2009--2010," 2010).

The World Health Organization estimates that 2.5 billion people live in dengue endemic areas and are at risk for severe disease, with an estimated 50 million cases of DF occurring each year and several hundred thousand cases of DHF ("Strategic direction for dengue research: Disease burden and epidemiological trends.," Feb 2002.). The burden of disease is greatest in SE Asia, where DHF and the severe complication dengue shock syndrome (DSS) are a significant cause of childhood morbidity and mortality, resulting in a loss of 465 disability adjusted life-years (DALYs) per million population on average and 954 DALYs/million/year during peak epidemic years, which is of the same order of magnitude as the impact of meningitis, hepatitis B, and tropical diseases (which include schistosomiasis, trypanosomiasis, and leishmaniasis) (Anderson, et al., 2007).

Epidemic characteristics

In DENV-endemic locales, annual transmission of DENVs typically occurs within the period of maximal mosquito densities. In countries such as Thailand and Vietnam, annual epidemics occur predictably during the rainy season (approximately June – October). However, these annual epidemics vary quite unpredictably with respect to severity, incidence,

and serotype composition. A study of hospitalized DENV illnesses conducted at Queen Sirikit National Institute of Child Health in Bangkok from 1973 to 1999 revealed cyclical fluctuations in DENV incidence and the transient dominance of different serotypes for multi-year intervals (Nisalak, et al., 2003). A prospective, school-based cohort study of symptomatic and asymptomatic DENV infection also described peaks and valleys in incidence and severity (T. P. Endy, Nisalak, et al., 2002).

These fluctuations are likely due to a complex milieu of factors. Some DENV serotypes are more likely to cause severe disease than others; for example, secondary infection with DENV-2 results in a higher rate of DHF than does DENV-4 (Fried, et al., 2010). Shifts in serotype-specific, population-level immunity may drive shifts in serotype dominance. Similarly, as cross-reactive antibodies have been associated with both attenuation of infection as well as enhancement of infection, population-level shifts in cross-reactive immunity may also influence the severity of epidemics (T. Endy, et al., 2010). There is some evidence that the sequence of infection with different serotypes may be associated with the severity of secondary infection; for example, serological studies of a severe outbreak of DENV-3 in Cuba in 2001-2002 suggested that the majority of DHF cases had evidence of prior exposure to DENV-1 and none had evidence of prior exposure to DENV-2 (M. G. Guzman, et al., 2002). Data on the importance of the sequence of infection remain limited because identifying the previous infecting virus is unreliable using currently available serological methods. Finally, climatic factors such as temperature and rainfall can affect mosquito densities, which in turn may influence transmission rates (Nagao, et al., 2003).

Prevention and control

There is no specific antiviral treatment for dengue and no vaccine available at present. Supportive care has been remarkably effective in decreasing case fatality rates in regions with ample resources and experienced medical teams. Fatality rates remain high in many parts of the developed world however, particularly during high incidence periods when available hospital resources can be readily overwhelmed. Control measures consequently rely upon mosquito control, most commonly the spraying of insecticides and efforts to reduce or eliminate larval habitats. Neither of these methods has been wholly effective, particularly in the long-term (Shope, 1997). Personal protective measures are relatively effective, though perhaps are not practical for long-term exposure (Gambel, et al., 1999). There has been some utilization of larval predators, such as fish or predacious copepods; these methods are limited by factors such as availability and sustainability (dos Santos & de Andrade, 1997). Significant research has investigated the potential for transgenic mosquitoes that are either resistant to dengue infection or unable to transmit the virus, but this technology remains at early stages of development and has questionable sustainability in the field (Irvin, Hoddle, O'Brochta, Carey, & Atkinson, 2004). The greatest hope for long-term control of dengue, at present, lies in the development of tetravalent dengue vaccines (DeRoeck, et al., 2003). Several are in development, and are discussed below.

I V. Clinical aspects of DENV infection.

The frequency of clinical outcomes with dengue viruses follows the "iceberg" analogy of infectious diseases (Figure 6). The majority of DENV infections, 60-70% in one study, are asymptomatic (T. P. Endy, Chunsuttiwat, et al., 2002). The proportion of infections that are

asymptomatic versus symptomatic appears to vary spatially and temporally (T. P. Endy, Nisalak, et al., 2002).

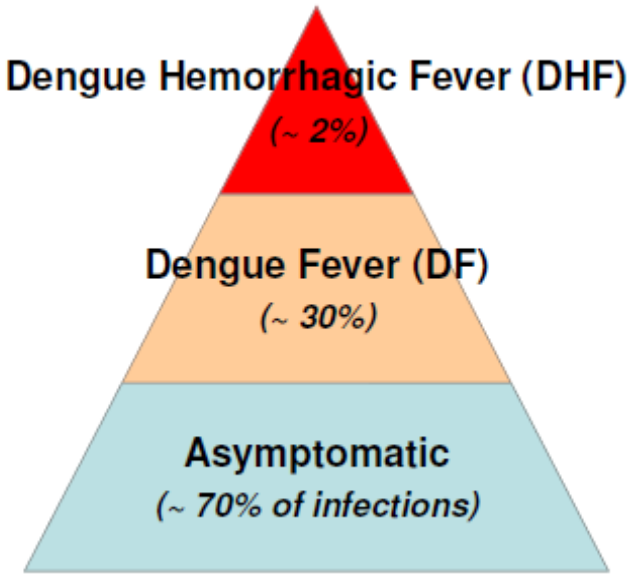


Figure 6. The 'iceberg' of DENV infection severity.

The majority of symptomatic infections manifest as dengue fever (DF), a debilitating but largely non-fatal disease. The case definition for DF proposed by the World Health Organization requires an acute febrile illness of 2-7 days duration plus two or more of the following: headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, or leucopenia ("Case definitions. Dengue fever," 2000).

More rarely and more critically, an infection will progress to dengue hemorrhagic fever (DHF) (approximately 5% of clinical illnesses), a severe clinical syndrome characterized by the rapid onset of plasma leakage and hemorrhage (Nimmannitya, 1997). The present WHO case definition for DHF involves the following criteria: a high continuous fever for 2 to 7 days, hemorrhagic diasthesis, hepatomegaly, thrombocytopenia (platelets ≤100,000 mm^3)

with hemoconcentration (a 20% increase in hematocrit), and/or shock (WHO, 1986). DHF is further classified according to severity: Grades I and II involve early signs of plasma leakage in the absence of shock (Grade I: positive tourniquet test as only sign of leakage, Grade II: positive tourniquet test plus spontaneous bleeding) and Grades III and IV are cases of DHF with shock (Grade III: signs of circulatory failure, Grade IV: profound shock), also known as dengue shock syndrome (DSS) (Nimmannitya, Halstead, Cohen, & Margiotta, 1969). If untreated, DHF may quickly progress from Grade I/II to DSS to death. Treatment involves volume replacement and, in cases of severe bleeding, blood transfusion. It is important to note that the death rates due to dengue vary significantly by region, from as low as 0.3% in more developed regions such as Thailand to as high as 12% in regions that lack the expertise and resources for appropriate, early clinical intervention (Chareonsook, Foy, Teeraratkul, & Silarug, 1999; Kalayanarooj, Rimal, et al., 2007). Some atypical presentations of DENV infection may fail to meet criteria for the clinically-severe DHF but are severe nonetheless, such as dengue encephalitis, dengue myocarditis, and dengue hepatitis (Gulati & Maheshwari, 2007).

V. Mechanisms of DHF.

DHF and secondary infection.

As discussed above, the World Wars heralded a dramatic shift in the epidemiology of DENV, notably through the increasing co-circulation of DENV serotypes in endemic regions. In the 1950s and 1960s, Thailand and the Philippines observed dramatic increases in the occurrence of hemorrhagic disease with DENV infection (Hammon, Rudnick, & Sather, 1960). An important preliminary finding in the 1960s was that the vast majority of children hospitalized with DHF/DSS in Bangkok were experiencing secondary DENV

infections (Halstead, Nimmannitya, & Cohen, 1970). The increased occurrence of DHF with secondary as compared to primary DENV infections has been confirmed in other epidemics and locales (Burke, Nisalak, Johnson, & Scott, 1988; M.G. Guzman, et al., 1990; Sangkawibha, et al., 1984; Thein, et al., 1997).

Despite the clear association between secondary infection and DHF, many complexities and questions remain. First, DHF can be (albeit rarely) observed in primary dengue infections. This suggests that poorly understood host and/or viral factors are sufficient to drive the occurrence of DHF outside of the setting of pre-existing antibodies. Second, only a small fraction (about 3%) of secondary infections progress to DHF; the majority of secondary infections are in fact asymptomatic (T. P. Endy, Chunsuttiwat, et al., 2002; S. B. Halstead, 1980). DHF is a relatively rare outcome of secondary infection; the high relative risk for secondary versus primary can be explained by the observation that DHF in primary infection is very rare (63). The factors that predispose one individual to develop DHF over another with secondary infection are unclear. Last, tertiary and quaternary infections with dengue are typically thought to be mild or asymptomatic (R. V. Gibbons, et al., 2007). This suggests that, while cross-reactive antibodies are potentially deleterious when experiencing a secondary dengue infection, the protection afforded by cross-reactive antibodies can be sufficient by the third infection to protect against clinical illness.

Antibody-dependent enhancement.

The earliest hypothesis put forward for the association of DHF with secondary DENV infection was that of antibody-dependent enhancement (ADE). While ADE is most famous for its purported association with DHF, it has been demonstrated to occur in a variety of

human and veterinary viruses, such as feline infectious peritonitis virus and human immunodeficiency virus (HIV) (Hohdatsu, Yamada, Ishizuka, & Koyama, 1993; Mascola, et al., 1993). ADE occurs when preexisting antibody (usually from a previous infection with a related virus) recognizes and binds to a heterologous virus and, failing to neutralize the virus, facilitates infection of Fcγ receptor-bearing cells, such as monocytes and macrophages (Morens, 1994). The ability to neutralize heterologous viruses may vary by antibody specificity and antibody titer (Halstead, 1989).

In ADE leading to DHF, preexisting NAbs from a primary DENV infection are hypothesized to enhance a subsequent infection with a different DENV serotype, resulting in increased viral load, increased immune activation, and ultimately the plasma leakage characteristic of DHF/DSS (S.B. Halstead, 1980). Kou et al investigated the dose-dependence of ADE by mixing serial dilutions of pooled human serum (containing cross-reactive DENV antibody) with heterologous DENV and applying it to mononuclear cells in culture (Kou, et al., 2008). Peaks in both the percentage of infected cells and viral output per infected cell were observed at low-to-intermediate titers of antibody, suggesting that the influence of cross-reactive antibodies on heterologous infection could range from moderate protection (high titers of antibody), to enhancement (intermediate titers), to no effect (low titers). ADE has been linked to DENV disease in humans only *in vitro,* with enhancing antibodies identified in the pre-infection serum of future DHF cases (Kliks, Nisalak, Brandt, Wahl, & Burke, 1989). Several additional hypotheses for the pathophysiological basis of DHF have been proposed, which may occur in conjunction with ADE or as an alternate mechanism. These proposed mechanisms include original antigenic sin, viral factors, and host factors, which are described below.

Original antigenic sin.

With respect to the antibody response to infection, original antigenic sin occurs when the immune response to a secondary infection is dominated by a memory response to the primary infection. This has been demonstrated to occur during sequential infections with influenza, enteroviruses, and dengue (Halstead, Rojanasuphot, & Sangkawibha, 1983). For DENV infections, original antigenic sin may contribute to the development of DHF by generating a rapid and robust response to a secondary infection that is poorly specific and exhibits suboptimal neutralizing capability. Further, the explosive memory response expends significant immunological resources on a response that is inappropriate and inefficient in containing the infection. The combination of a high viral load from a poorly-controlled infection and a continued strong inflammatory response from a highly activated immune system can result in the cytokine storm and plasma leakage observed with DHF.

This mechanism for DHF may work in conjunction with ADE by increasing the production and circulation of poorly specific antibodies and T cells, which may interact with virus and further facilitate infection. Antigenic sin has also been observed to occur in T-cell populations. A cell-mediated component to the antigenic sin mechanism has been proposed that involves the rapid activation and apoptosis of specific (appropriate) CD8+ T-cells during secondary infection, in combination with antigenic sin driving the expansion of T-cells with high specificity to the prior infecting DENV but low affinity for the present infecting DENV (Mongkolsapaya, et al., 2003).

Viral factors.

As discussed above, DENV epidemics vary considerably in severity and the occurrence of DHF. Some contribution of virus virulence factors appears likely, as supported by the observation that Asian strains of DENV-2 are associated with the increased occurrence of DHF relative to American strains (Rico-Hesse, et al., 1997). It is also possible that some viral features of the primary infecting virus are critical in predisposing an individual to develop DHF with subsequent infection, though these factors have not been identified (Halstead, 1997).

Host factors.

A multitude of host factors has been linked with DHF. Among individuals with the same baseline DENV immunity and risk of infection, DHF and hospitalization rates have been shown to peak in children aged 10 and younger (G. P. Kouri, Guzman, Bravo, & Triana, 1989). It is noteworthy, however, that in some epidemics DHF in adults was not uncommon and that the average age of DHF appears to be increasing in areas such as Thailand (Cummings, et al., 2009; Ellis, et al., 2006; Ong, Sandar, Chen, & Sin, 2007). Malnutrition has been associated with a decreased predilection towards DHF, presumably through a suppression of immune function (Thisyakorn & Nimmannitya, 1993). Genetic factors such as blood type AB and various HLA-A and HLA-B alleles have also been associated with the increased occurrence of DHF (Kalayanarooj, Gibbons, et al., 2007; Stephens, et al., 2002). Dengue infection has been suggested to be milder in individuals of African descent (M.G. Guzman, et al., 1990).

VI. The antibody response to infection.

Neutralizing antibodies (NAbs) play an important role in protecting against viral infection as they can confer 'sterilizing immunity,' binding and neutralizing an infectious agent prior to infection and replication. In the case of DENVs, however, NAbs are also implicated in the enhancement of infection and the occurrence of DHF. Characterization of dengue immunity as protective or pathologic is complicated by the immunological cross-reactivity observed between serotypes and the inability of available serological assays to distinguish between specific and cross-reactive antibodies.

To illustrate the complex cross-reactivity observed with DENV infections, consider a dengue-naïve individual (Figure 7). Following primary infection with a DENV (say, DENV-1), the individual will develop specific NAbs to DENV-1, as well as cross-reactive NAbs to other dengue serotypes to which they have not yet been exposed. Specific antibodies are thought to afford lifelong immunity (Sabin, 1952). Cross-reactive antibodies, in contrast, are more temporary and wane over a period of months to years (M. G. Guzman, et al., 2007). The ability of the cross-reactive NAbs to interact with a new infecting DENV, and the nature of these interactions, appears to vary as a combination of time elapsed between infections and NAb titer, as discussed below.

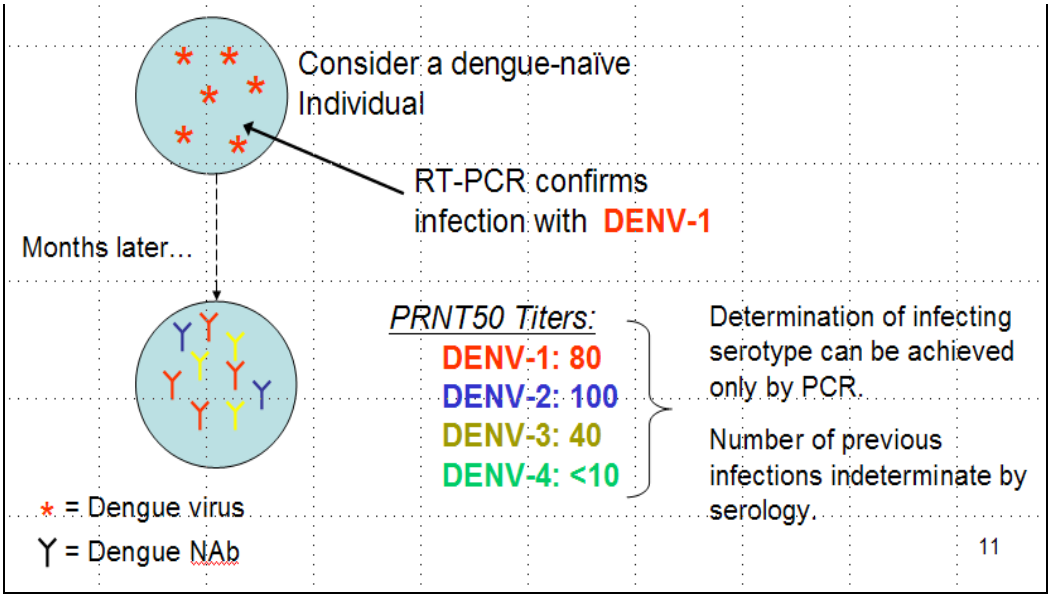


Figure 7. Example: generation of specific and cross-reactive antibodies following DENV infection.

Dose-dependence of cross-protection and enhancement.

In vitro assessments of the ability of cross-reactive antibodies to enhance a heterologous DENV infection have suggested that intermediate titers of antibodies, relative to high or very low, can result in DHF (Kou, et al., 2008). An analogy to this dose-dependent function of cross-reactive antibodies may exist in human infants, who are born with high levels of maternal antibody and are seemingly protected from DHF (Figure 8) (Halstead, et al., 2002). As maternal antibody wanes, infants pass into an 'intermediate phase' at approximately 6-8 months of age where they experience an elevated risk of DHF. As maternal antibody wanes further and disappears, infants pass into a phase where they again have a low risk of DHF. This suggests that at low-intermediate maternal antibody titers, cross-reactive antibodies can facilitate the occurrence of DHF but that at higher antibody titers, the dose of cross-reactive maternal antibodies is sufficient to prevent enhancement of infection.

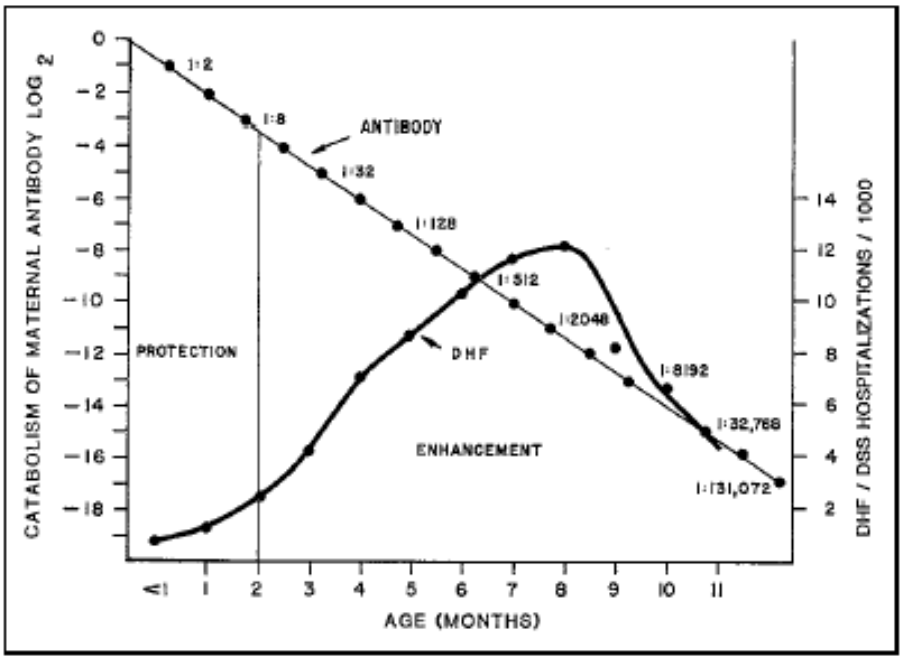


Figure 8. The waning of maternal antibodies and shifts in the risk of DHF in infants. The x-axis gives age of the infant in months. The left y-axis represents the catabolism (waning) of the titer of maternal antibodies. The right y-axis gives the incidence rate of DHF/DSS per 1000 infants.

* From: (Halstead, et al., 2002)

In a prospective study of school children by Endy et al in Kamphaeng Phet, Thailand, pre-infection sera were evaluated for the presence of preexisting heterologous antibodies against the subsequent infecting DENV serotype (T. P. Endy, et al., 2004). Since infection with a given DENV serotype is associated with lifelong immunity to that serotype, any preexisting antibodies to the infecting serotype were assumed to have arisen as a cross-reactive response to previous infection(s). Higher levels of preexisting cross-reactive antibodies were associated with milder illness and lower viremia (significant for DENV-3), while lower levels were associated with DHF. This provides additional evidence of a dose-dependent

relationship between the titers of preexisting heterologous antibodies and protection from illness versus enhancement of a secondary DENV infection.

Temporal shifts in cross-protection and risk of enhancement..

There is experimental evidence of a temporal window of cross-protection afforded by heterologous DENV antibodies. Albert Sabin administered sequential experimental inoculations of heterologous DENV to prisoner volunteers in New Jersey (Sabin, 1952). He concluded that modifications in the clinical severity and clinical presentation associated with a second heterologous DENV varied according to the amount of time elapsed between inoculations. Specifically, he found that up until two months post-primary inoculation, cross-protective immunity protected volunteers from infection and illness. Two to three months post-infection, exposure to a heterologous DENV serotype resulted in malaise and a slight fever of less than 24 hours, and the ability to transmit the virus to mosquitoes was reestablished. Nine months post-primary infection, heterologous exposure resulted in a febrile illness of two to three days duration and a mild rash. On the basis of this study, six months is generally quoted as the duration of cross-protection following a primary DENV infection.

Two important observations on temporal trends in disease risk have been made by Cuban scientists. A large DENV-1 epidemic passed through Cuba in 1977-8. Two epidemics of Asian-genotype DENV-2 followed, in 1981 and in 1997 (G. Kouri, et al., 1998; G. Kouri, et al., 1983). It was found that individuals that had experienced their primary DENV-1 infection in 1977-8 and their secondary DENV-2 infection in 1997 (i.e., had an interval of 20 years between infections) had a risk of DHF that was 3-4 times greater than those that

experienced their secondary DENV-2 infection in 1981 (an interval of four years). The authors concluded that a longer period of time between infections was associated with a greater risk of DHF, presumably because of the waning of cross-reactive/cross-protective antibodies(M. G. Guzman, et al., 2000). They later demonstrated an increase in the mean titer of the homologous antibody response in individuals who had been infected with DENV-1 over time, and a decline in heterologous antibodies (M. G. Guzman, et al., 2007).

Proposed model of trends in the risk of illness with secondary DENV infection.

These studies demonstrating a titer-dependent and time-dependent aspect to the influence of cross-reactive antibodies suggest the following model of the clinical outcome of secondary DENV infection (Figure 9):

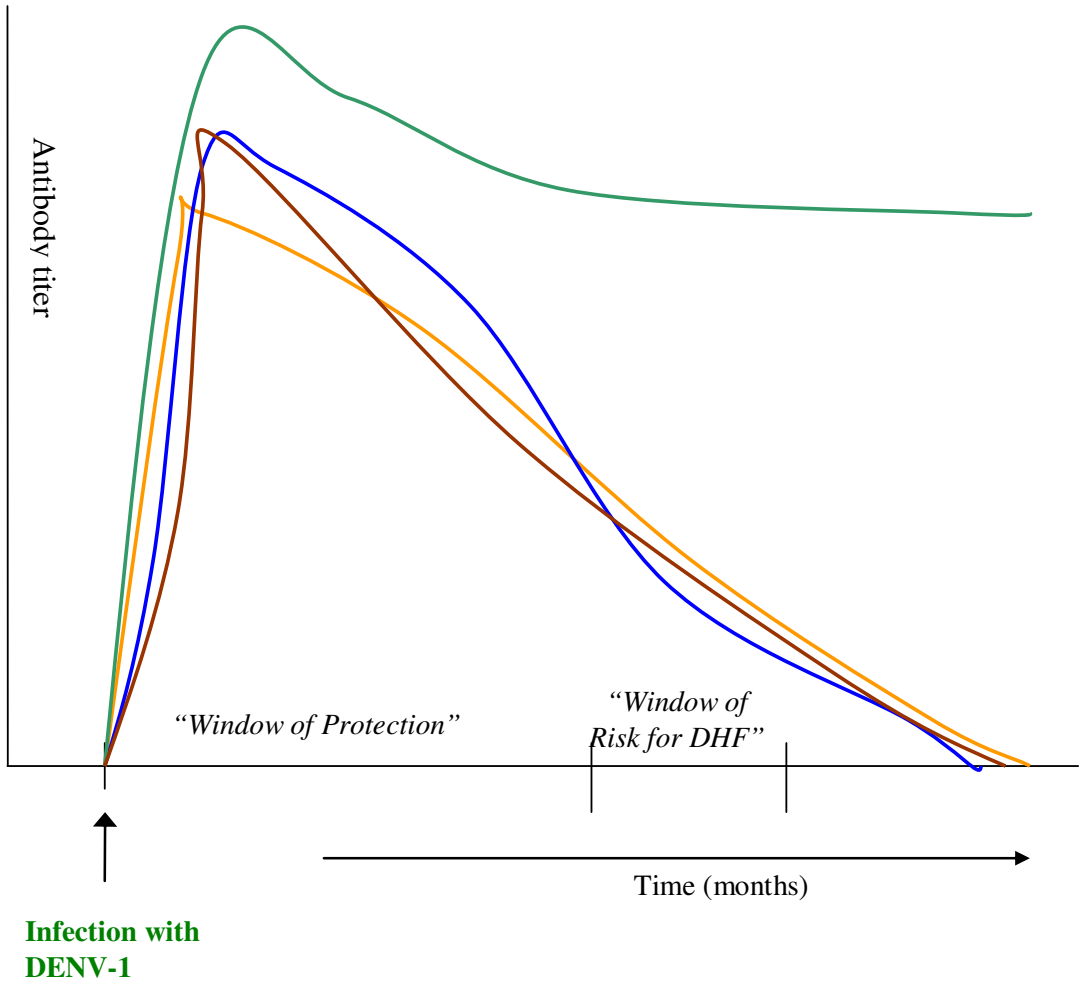


Figure 9. Temporal trends in the titers of specific and cross-reactive antibodies generated in response to a primary DENV infection (in this example, DENV-1) over time.

Following infection with a given serotype (say, with DENV-1, as in the figure above) an individual will experience a boost in antibody titers to the infecting DENV serotype but also antibodies that cross-react with heterologous (non-infecting) serotypes. These cross-reactive antibodies decay over time, more rapidly than antibodies against the homologous serotype. It is hypothesized that an individual will then pass through various 'windows' of risk as titers of cross-reactive antibodies decline. First, the cross-reactive antibodies will be of high enough titer to prevent illness upon exposure to a novel DENV serotype (the 'window of

protection'). Next, as these cross-reactive antibodies wane to low-to-intermediate titers, they may then serve as enhancing antibodies and be associated with an increased occurrence of DHF. Finally, cross-reactive antibodies will wane to the point where they are not present in amounts sufficient to drive enhancement.

In addition to these short-term trends with cross-reactive antibodies, there are likely longer-term trends in cross-protection. The paradigm is that as one accumulates specific immunity to different DENV serotypes with each infection, these specific antibodies can exert an increasingly cross-protective effect on other serotypes such that the third and fourth infections are silent. This could be due to the presence of higher titers of cross-protective antibodies with each infection or due to some refinement of the immune response. These long-term effects have not been clearly demonstrated in epidemiological studies, however.

VII. DENV serological assays – methods, capabilities, and limitations.

Despite the strong evidence that preexisting DENV antibodies can both protect from and enhance a subsequent heterologous DENV infection, the precise nature of these interactions remains poorly defined. One complicating factor in characterizing pre-existing immunity is the broad cross-reactive response elicited by a primary DENV infection. There are no means to clearly distinguish 'specific' antibodies (those mounted against the infecting serotype) from 'cross-reactive' antibodies using available DENV assays. Further, dengue is highly cross-reactive in serological assays with closely-related flaviviruses, which complicates assessments of DENV immunity in areas where DENV co-circulates with JEV or YFV (A, et al., 2008; K. J. Chang, 1997).

Plaque reduction neutralization titers (PRNTs).

The "gold standard" serologic assay for defining prior DENV exposure is the plaque reduction neutralization titer (PRNT) assay, which estimates the reciprocal of the serum dilution resulting in a given percent reduction in plaque formation (most commonly 50%, or $PRNT_{50}$) (P. K. Russell, Nisalak, Sukhavachana, & Vivona, 1967). The $PRNT_{50}$ titer can be calculated using a log probit model of the number of plaques at each serum dilution. A $PRNT_{50}$ >=10 is usually considered positive and <10 is negative. This assay is thought to represent the best available correlate of protection, as it measures neutralizing antibodies which are presumed to be important in limiting DENV infection.

Despite its designation as the current gold standard assay, PRNTs have several limitations. PRNTs are quite expensive and time-consuming to perform. As with all DENV serological assays, PRNTs cannot completely discriminate between cross-reactive immunity and specific immunity and PRNT data do not allow reliable identification of which serotypes a child has been previously exposed to (Kuno, Gubler, & Oliver, 1993). Even following primary infection, the highest $PRNT_{50}$ titer may be a cross-reactive response. Further, one cannot 'count' the number of previous DENV infections by looking at the number of serotypes with positive $PRNT_{50}$ titers, as some may be cross-reactive responses. Finally, subtle changes in assay conditions have been shown to have significant effects on the titers obtained using PRNT, making comparisons between laboratories and the identification of generalizable thresholds of protection a difficult task (Thomas, et al., 2009).

Some interpretations of $PRNT_{50}$ data have general acceptance. First, seronegativity to all four DENV serotypes is regarded as being 'dengue-naive' – that is, a subsequent infection

can somewhat reliably be called a primary DENV infection. Positivity to a single DENV serotype ('monotypic' immunity) is generally interpreted as the individual having had one DENV infection and being at risk for a secondary infection. It is also generally accepted that, in the case of monotypic immunity, one may interpret the serotype to which the child has the single, positive, $PRNT_{50}$ titer as the primary infecting virus.

Perhaps the greatest challenge in using $PRNT_{50}$ titers to infer protection from illness or risk of DHF is that the most interesting and most problematic group for these considerations is likely the group of individuals with 'multitypic immunity' – that is, those with pre-existing antibodies to two or more DENV serotypes. A child with a multitypic profile may have had multiple DENV infections (and their specific immunity to multiple serotypes may protect them from illness with subsequent infection) or they may have a broadly cross-reactive response which would place them at heightened risk of DHF.

Hemagglutination inhibition (HI).

The hemagglutination inhibition (HI) assay serves as the WHO standard for serological confirmation and serological classification of recent DENV infections (Vorndam & Kuno, 1997). The HI assay measures all manner of antibodies that recognized the DENV E protein, not simply neutralizing antibodies, and is less serotype specific than PRNT. The HI titer is defined as the final dilution of serum that prevents agglutination of red blood cells by a standard amount of viral antigen (Clarke & Casals, 1958). A recent DENV infection is defined as a four-fold rise in HI titers. HI antibody rises over a period of weeks following infection and persists for weeks to months. The convalescent titer is higher in secondary infections and HI may be used to classify infections as primary (titer ≤1:640) or secondary

(titer >1:1280) (Anonymous, 1997). An advantage of HI over the PRNT is that the assay is relatively quick, cheap, and easy to perform.

Enzyme-linked immunosorbent assays (ELISAs).

Immunoglobulin(Ig) G and IgM ELISAs are useful in characterizing acute infections as primary or secondary (Innis, et al., 1989). An IgM-to-IgG ratio of <1.8 has been used to identify primary infection, as secondary infections are associated with a rapid and heightened IgG antibody response; Additionally, ELISA can provide greater specificity than HI in discerning acute JEV infections from acute DENV infections in co-endemic locales.

VIII. The Future – Dengue Vaccines.

There is no treatment for dengue other than supportive care, and prevention efforts have necessarily relied upon mosquito control and the development of tetravalent dengue vaccines. The need for a dengue vaccine was evaluated in 2003 with a survey of policymakers and other professionals in four Southeast Asian countries (Cambodia, Indonesia, Philippines, and Vietnam) (DeRoeck, et al., 2003). This survey demonstrated a high-level of concern about dengue disease and a high perceived need for a dengue vaccine. Several dengue vaccines are currently at various stages of development, including live-attenuated tetravalent dengue vaccines, dengue virus deletion mutants, recombinant proteins of the dengue E protein, and a dengue chimera using a yellow fever virus backbone (Blaney, et al., 2007; Morrison, et al., 2010; Simasathien, et al., 2008; Whitehead, Blaney, Durbin, & Murphy, 2007). The most advanced candidates have entered human phase II and phase III trials.

Several important challenges pertaining to the development and safe implementation of dengue vaccines remain. A major issue is that defining protective versus pathological immunity for DENV is not possible with current serological assays and with an incomplete understanding of the complex nature of cross-reactivity. A second issue is that the association of antibody-dependent enhancement and DHF with secondary DENV infection suggests that an effective DENV vaccine must confer protective immunity against all four DENV serotypes (Halstead, et al., 1970). The worst-case scenario for DENV vaccine introduction would be a predisposition, or 'priming', for DHF by vaccination, rather than protection from illness. Historically, generating robust, balanced antibody responses to all components of live multivalent viral vaccines has proven a difficult task (Patriarca, et al., 1991).

REFERENCES

A, A. N., Panthuyosri, N., Anantapreecha, S., Chanama, S., Sa-Ngasang, A., Sawanpanyalert, P., et al. (2008). Cross-reactive IgM responses in patients with dengue or Japanese encephalitis. *J Clin Virol, 42*(1), 75-77.

Anderson, K. B., Chunsuttiwat, S., Nisalak, A., Mammen, M. P., Libraty, D. H., Rothman, A. L., et al. (2007). Burden of symptomatic dengue infection in children at primary school in Thailand: a prospective study. *Lancet, 369*(9571), 1452-1459.

Anonymous. (1997). *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control, 2nd edition*. Geneva: World Health Organization.

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., et al. (2006). Vascular Leakage in Severe Dengue Virus Infections: a Potential Role for the Nonstructural Viral Protein NS1 and Complement. *J Infect Dis, 193*(8), 1078-1088.

Blaney, J. E., Jr., Sathe, N. S., Hanson, C. T., Firestone, C. Y., Murphy, B. R., & Whitehead, S. S. (2007). Vaccine candidates for dengue virus type 1 (DEN1) generated by replacement of the structural genes of rDEN4 and rDEN4Delta30 with those of DEN1. *Virol J, 4*, 23.

Blok, J., McWilliam, S. M., Butler, H. C., Gibbs, A. J., Weiller, G., Herring, B. L., et al. (1992). Comparison of a dengue-2 virus and its candidate vaccine derivative: sequence relationships with the flaviviruses and other viruses. *Virology, 187*, 573-590.

Burke, D. S., & Monath, T. P. (2001). Flaviviruses. In D. M. Knipe & P. M. Mowley (Eds.), *Fields Virology* (4th edition ed., pp. 1043-1125). Philadelphia, PA: Lippincott, Williams, Wilkins.

Burke, D. S., Nisalak, A., Johnson, D. E., & Scott, R. M. (1988). A prospective study of dengue infections in Bangkok. *Am J Trop Med Hyg, 38*(1), 172-180.

Cardosa, J., Ooi, M. H., Tio, P. H., Perera, D., Holmes, E. C., Bibi, K., et al. (2009). Dengue virus serotype 2 from a sylvatic lineage isolated from a patient with dengue hemorrhagic fever. *PLoS Negl Trop Dis. , 3*(4), e423.

Case definitions. Dengue fever. (2000). *Epidemiol Bull, 21*(2), 14-15.

Chang, K. J. (1997). Studies on the serological cross-reaction between dengue and Japanese encephalitis. *J Microbiol Immunol Infect, 30*(4), 207-218.

Chareonsook, O., Foy, H. M., Teeraratkul, A., & Silarug, N. (1999). Changing epidemiology of dengue hemorrhagic fever in Thailand. *Epidemiol Infect, 122*(1), 161-166.

Clarke, D. H., & Casals, J. (1958). Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am J Trop Med Hyg, 7*, 561-573.

Cologna, R., & Rico-Hesse, R. (2003). American genotype structures decrease dengue virus output from human monocytes and dendritic cells. *J Virol, 77*(7), 3929-3938.

Cummings, D. A., Iamsirithaworn, S., Lessler, J. T., McDermott, A., Prasanthong, R., Nisalak, A., et al. (2009). The impact of the demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical modeling. *PLoS Med, 6*(9), e1000139.

de Thoisy, B., Lacoste, V., Germain, A., Munoz-Jordan, J., Colon, C., Mauffrey, J. F., et al. (2009). Dengue infection in neotropical forest mammals. *Vector Borne Zoonotic Dis, 9*(2), 157-170.

DeRoeck, D., Deen, J., & Clemens, J. D. (2003). Policymakers' views on dengue fever/dengue haemorrhagic fever and the need for dengue vaccines in four southeast Asian countries. *Vaccine, 22*(1), 121-129.

Division of Vector-Borne Infectious Diseases, N. C. f. I. D., Centers for Disease Control and Prevention. (1997, 1 June). Information on dengue fever and dengue hemorrhagic fever. Retrieved 3 September, 2000

dos Santos, L. U., & de Andrade, C. F. (1997). Survey of cyclopids (Crustacea, Copepoda) in Brazil and preliminary screening of their potential as dengue vector predators. *Rev Saude Publica, 31*(3), 221-226.

Ellis, R. D., Fukuda, M. M., McDaniel, P., Welch, K., Nisalak, A., Murray, C. K., et al. (2006). Causes of Fever in adults on the thai-myanmar border. *Am J Trop Med Hyg, 74*(1), 108-113.

Endy, T., Anderson, K., Nisalak, A., Yoon, I., Green, S., Rothman, A., et al. (2010). Determinants of Inapparent and Symptomatic Dengue Infection in a Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *Manuscript submitted for publication.*

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

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Endy, T. P., Nisalak, A., Chunsuttiwat, S., Libraty, D. H., Green, S., Rothman, A. L., et al. (2002). Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am J Epidemiol, 156*(1), 52-59.

Fried, J., Gibbons, R., Kalayanarooj, S., Thomas, S., Srikiatkhachorn, A., Yoon, I., et al. (2010). 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., 4*(3), e617.

From the Centers for Disease Control and Prevention. Dengue fever at the US-Mexico border, 1995-1996. (1996). *JAMA, 276*(18), 1464-1465.

Gambel, J. M., Drabick, J. J., Swalko, M. A., Henchal, E. A., Rossi, C. A., & Martinez-Lopez, L. (1999). Dengue among United Nations mission in Haiti personnel, 1995: implications for preventive medicine. *Mil Med, 164*(4), 300-302.

Gibbons, R. V., Kalanarooj, S., Jarman, R. G., Nisalak, A., Vaughn, D. W., Endy, T. P., et al. (2007). Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences. *Am J Trop Med Hyg, 77*(5), 910-913.

Gratz, N. G. (2004). Critical review of the vector status of Aedes albopictus. *Med Vet Entomol, 18*(3), 215-227.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever: Its history and resurgence as a global public health problem. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 1-22). New York: CAB International.

Gubler, D. J. (2002). The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res, 33*(4), 330-342.

Gubler, D. J., & Clark, G. G. (1995). Dengue/Dengue Hemorrhagic Fever: The Emergence of a Global Health Problem. *Emerg Infect Dis, 1*(2), 55-57.

Gulati, S., & Maheshwari, A. (2007). Atypical manifestations of dengue. *Trop Med Int Health, 12*(9), 1087-1095.

Guzman, M. G., Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Montes, T., Vazquez, S., et al. (2007). Neutralizing antibodies after infection with dengue 1 virus. *Emerg Infect Dis, 13*(2), 282-286.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazquez, S., et al. (2000). Epidemiologic studies on dengue in Santiago de Cuba, 1997. *Am J Epidemiol, 152*(9), 793-799.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Vazquez, S., & Halstead, S. B. (2002). Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica, 11*(4), 223-227.

Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S., & Morier, L. (1990). Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg, 42*, 179-184.

Halstead, S. B. (1980). Dengue haemorrhagic fever-a public health problem and a field for research. *Bull World Health Organ, 58*(1), 1-21.

Halstead, S. B. (1980). Immunological Parameters Of Togavirus Disease Syndromes. *The Togaviruses*, 107-173.

Halstead, S. B. (1989). Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev Infect Dis, 11*(Suppl 4), S830-S839.

Halstead, S. B. (1997). Epidemiology of dengue and dengue hemorrhagic fever. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 23-44). New York: CAB International.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., Nimmannitya, S., & Cohen, S. N. (1970). Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med, 42*, 311-328.

Halstead, S. B., Rojanasuphot, S., & Sangkawibha, N. (1983). Original antigenic sin in dengue. *Am J Trop Med Hyg, 32*(1), 154-156.

Hammon, W. M., Rudnick, A., & Sather, G. E. (1960). Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. *Science, 131*, 1102-1103.

Hawley, W. A., Reiter, P., Copeland, R. S., Pumpuni, C. B., & Craig, G. B., Jr. (1987). Aedes albopictus in North America: probable introduction in used tires from northern Asia. *Science, 236*(4805), 1114-1116.

Hohdatsu, T., Yamada, H., Ishizuka, Y., & Koyama, H. (1993). Enhancement and Neutralization of Feline Infectious Peritonitis Virus Infection in Feline Macrophages by Neutralizing Monoclonal Antibodies Recognizing Different Epitopes. *Microbiol Immunol, 37*, 499-504.

Innis, B. L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Suntayakorn, S., et al. (1989). An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg, 40*(4), 418-427.

Irvin, N., Hoddle, M. S., O'Brochta, D. A., Carey, B., & Atkinson, P. W. (2004). Assessing fitness costs for transgenic Aedes aegypti expressing the GFP marker and transposase genes. *Proc Natl Acad Sci U S A*.

Kalayanarooj, S., Gibbons, R. V., Vaughn, D., Green, S., Nisalak, A., Jarman, R. G., et al. (2007). Blood group AB is associated with increased risk for severe dengue disease in secondary infections. *J Infect Dis, 195*(7), 1014-1017.

Kalayanarooj, S., Rimal, H. S., Andjaparidze, A., Vatcharasaevee, V., Nisalak, A., Jarman, R. G., et al. (2007). Clinical Intervention and Molecular Characteristics of a Dengue Hemorrhagic Fever Outbreak in Timor Leste, 2005. *Am J Trop Med Hyg, 77*(3), 534-537.

Kimura, R., & Hotta, S. (1944). Studies on dengue: anti-dengue active immunization experiments in mice. *Japanese Journal of Bacteriology, 1*, 96-99.

Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L., & Burke, D. S. (1989). Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. *Am J Trop Med Hyg, 40*(4), 444-451.

Kou, Z., Quinn, M., Chen, H., Rodrigo, W., Rose, R., Schlesinger, J., et al. (2008). Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. *Journal of Medical Virology, 80*(1), 134-136.

Kouri, G., Guzman, M. G., Valdes, L., Carbonel, I., del Rosario, D., Vazquez, S., et al. (1998). Reemergence of dengue in Cuba: a 1997 epidemic in Santiago de Cuba. *Emerg Infect Dis, 4*(1), 89-92.

Kouri, G., Mas, P., Guzman, M. G., Soler, M., Goyenechea, A., & Morier, L. (1983). Dengue hemorrhagic fever in Cuba, 1981: rapid diagnosis of the etiologic agent. *Bull Pan Am Health Organ, 17*, 126-132.

Kouri, G. P., Guzman, M. G., Bravo, J. R., & Triana, C. (1989). Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. *Bull World Health Organ, 67*, 375-380.

Kuno, G., Chang, G. J., Tsuchiya, K. R., Karabatsos, N., & Cropp, C. B. (1998). Phylogeny of the genus Flavivirus. *J Virol, 72*(1), 73-83.

Kuno, G., Gubler, D. J., & Oliver, A. (1993). Use of Original Antigenic Sin Theory to Determine the Serotypes of Previous Dengue Infections. *Trans R Soc Trop Med Hyg, 87*, 103-105.

Lewis, J. A., Chang, G. J., Lanciotti, R. S., Kinney, R. M., Mayer, L. W., & Trent, D. W. (1993). Phylogenetic Relationships of Dengue-2 Viruses. *Virology, 197*, 216-224.

Locally Acquired Dengue --- Key West, Florida, 2009--2010. (2010). *MMWR Morb Mortal Wkly Rep, 59*(19), 577-581.

Mackenzie, J. S., Gubler, D. J., & Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med, 10*(12 Suppl), S98-S109.

Mascola, J. R., Mathieson, B. J., Zack, P. M., Walker, M. C., Halstead, S. B., & Burke, D. S. (1993). Summary Report - Workshop on the Potential Risks of Antibody- Dependent Enhancement in Human HIV Vaccine Trials. *AIDS Res Hum Retroviruses, 9*, 1175-1184.

Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., et al. (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med, 9*(7), 921-927.

Morens, D. M. (1994). Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin Infect Dis, 19*, 500-512.

Morrison, D., Legg, T., Billings, C., Forrat, R., Yoksan, S., & Lang, J. (2010). A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naive adults. *J Infect Dis, 201*(3), 370-377.

Mukhopadhyay, S., Kuhn, R. J., & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol, 3*(1), 13-22.

Nagao, Y., Thavara, U., Chitnumsup, P., Tawatsin, A., Chansang, C., & Campbell-Lendrum, D. (2003). Climatic and social risk factors for Aedes infestation in rural Thailand. *Trop Med Int Health, 8*(7), 650-659.

Nimmannitya, S. (1997). Dengue Hemorrhagic Fever: Diagnosis and Management. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 133-145). Cambridge: CAB International.

Nimmannitya, S., Halstead, S. B., Cohen, S., & Margiotta, M. R. (1969). Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *Am J Trop Med Hyg, 18*(6), 954-971.

Nisalak, A., Endy, T. P., Nimmannitya, S., Kalayanarooj, S., Thisayakorn, U., Scott, R. M., et al. (2003). Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am J Trop Med Hyg, 68*(2), 191-202.

Nishiura, H., & Halstead, S. B. (2007). Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. *J Infect Dis, 195*(7), 1007-1013.

Oliphant, T., Nybakken, G. E., Engle, M., Xu, Q., Nelson, C. A., Sukupolvi-Petty, S., et al. (2006). Antibody Recognition and Neutralization Determinants on Domains I and II of West Nile Virus Envelope Protein *J Virol. , 80*(24), 12149-12159.

Ong, A., Sandar, M., Chen, M. I., & Sin, L. Y. (2007). Fatal dengue hemorrhagic fever in adults during a dengue epidemic in Singapore. *Int J Infect Dis, 11*(3), 263-267.

Patriarca, P., Wright, P., & John, T. (1991). Factors affecting immunogenicity of oral poliovirus vaccine in developing countries: review. *Rev Infect Dis, 13*(926-939).

Ramos, M. M., Mohammed, H., Zielinski-Gutierrez, E., Hayden, M. H., Lopez, J. L., Fournier, M., et al. (2008). Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico border: results of a household-based seroepidemiologic survey, December 2005. *Am J Trop Med Hyg, 78*(3), 364-369.

Rico-Hesse, R. (1990). Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology, 174*, 479-493.

Rico-Hesse, R., Harrison, L. M., Salas, R. A., Tovar, D., Nisalak, A., Ramos, C., et al. (1997). Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology, 230*, 244-251.

Rodhain, F., & Rosen, L. (1997). Mosquito vectors and dengue virus-vector relationships. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 45-60). New York: CAB International.

Rush, B. (1789). An account of the bilious remitting fever, as it appeared in Philadelphia, in the summer and autumn of the year 1780 *Medical Inquires and Observations* (1 ed., pp. 89-100). Philadelphia: Prichard and Hall.

Russell, P. (1978, May 1978). *Dengue in the Caribbean, 1977*. Paper presented at the Dengue in the Caribean, 1977. Proceedings of a workshop held in Montego Bay, Jamaica., Montego Bay, Jamaica.

Russell, P. K., Nisalak, A., Sukhavachana, P., & Vivona, S. (1967). A plaque reduction test for dengue virus neutralization antibodies. *J Immunol, 99*(2), 285-290.

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., et al. (1984). Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol, 120*, 653-669.

Shope, R. E. (1997). Concepts of control of Japanese encephalitis and dengue. *Southeast Asian J Trop Med Public Health, 28*(Suppl 2), 131-134.

Siler, J. F., Hall, M. W., & Hitchens, A. P. (1926). Dengue: Its history, epidemilogy, mechanism of transmission, etiology, clinical manifestations, immunity, and prevention. *The Philippine Journal of Science, 29*, 1-304.

Simasathien, S., Thomas, S. J., Watanaveeradej, V., Nisalak, A., Barberousse, C., Innis, B. L., et al. (2008). Safety and Immunogenicity of a Tetravalent Live-attenuated Dengue Vaccine in Flavivirus Naive Children. *Am J Trop Med Hyg, 78*(3), 426-433.

Simmons, J. S., St John, J. H., & Reynolds, F. H. K. (1931). Experimental studies of dengue. *The Philippine Journal of Science, 44*, 1-252.

Smith, C. E. (1956). The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. *J Trop Med Hyg, 59*(10), 243-251.

Stephens, H. A., Klaythong, R., Sirikong, M., Vaughn, D. W., Green, S., Kalayanarooj, S., et al. (2002). HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. *Tissue Antigens, 60*(4), 309-318.

Strategic direction for dengue research: Disease burden and epidemiological trends. (Feb 2002.). *World Health Organization.* .

Thein, S., Aung, M. M., Shwe, T. N., Aye, M., Zaw, A., Aye, K., et al. (1997). Risk factors in dengue shock syndrome. *Am J Trop Med Hyg, 56*(5), 566-572.

Thisyakorn, U., & Nimmannitya, S. (1993). Nutritional Status of Children with Dengue Hemorrhagic Fever. *Clin Infect Dis, 16*, 295-297.

Thomas, S., Nisalak, A., Anderson, K., Libraty, D., Kalayanarooj, S., Vaughn, D., et al. (2009). 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., 81*(5), 825-833.

U.S. Department of Health and Human Services, N. I. o. H., National Institute of Allergy and Infectious Disease. NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report.

Vorndam, V., & Kuno, G. (1997). Laboratory diagnosis of dengue virus infections. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 313-333). Cambridge: CAB International.

Wahala, M. P. B., Wahala, E. F., Donaldson, R. A., Accavitti-Loper, M. A., Baric, R. S., & deSilva, A. M. (2010). Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses. *PLoS Pathog. , 6*(3), e1000821.

Watts, D. M., Harrison, B. A., Pantuwatana, S., Klein, T. A., & Burke, D. S. (1985). Failure to detect natural transovarial transmission of dengue viruses by Aedes aegypti and Aedes albopictus (Diptera: Culicidae). *J Med Entomol, 22*(3), 261-265.

Weaver, S. C., & Barrett, A. D. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nat Rev Microbiol, 2*(10), 789-801.

Weaver, S. C., & Vasilakis, N. (2009). Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infection, Genetics and Evolution 9*, 523-540.

Whitehead, S. S., Blaney, J. E., Durbin, A. P., & Murphy, B. R. (2007). Prospects for a dengue virus vaccine. *Nat Rev Microbiol, 5*(7), 518-528.

Yasuno, M., & Tonn, R. J. (1970). A study of biting habits of Aedes aegypti in Bangkok, Thailand. *Bull World Health Organ, 43*(2), 319-325.

STUDY ONE

DISCLAIMER: AT THE TIME OF THESIS PUBLICATION, THIS MANUSCRIPT WAS UNDER PREPARATION FOR SUBMISSION TO A SCIENTIFIC JOURNAL FOR REVIEW. WHERE POSSIBLE, THE READER IS ENCOURAGED TO READ THE FINAL, PUBLISHED MANUSCRIPT:

Anderson KB, Gibbons RV, Thomas SJ, Rothman AL, Nisalak A, Berkelman RL, Libraty DH, Endy TP. Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. PLoS Negl Trop Dis. 2011 Oct;5(10):e1311.

STUDY ONE

Title: Preexisting immunity to Japanese encephalitis virus and the increased occurrence of symptomatic dengue infection

Abstract

Dengue viruses (DENVs) and Japanese encephalitis virus (JEV) have significant cross-reactivity in serological assays; the clinical implications of this remain undefined. The association between preexisting JEV neutralizing antibodies (NAbs) and the clinical severity of DENV infection was evaluated in a prospective school-based cohort in Thailand that captured asymptomatic, non-hospitalized, and hospitalized DENV infections. JEV NAbs were associated with increased odds of symptomatic versus asymptomatic DENV infection (odds ratio [OR]=1.55, 95% CI: 1.08 to 2.23). The association didn't vary by antibody titer. The association was strongest in DENV-naïves (OR=2.75, 95% CI: 1.12 to 6.72), for whom JEV NAbs were also associated with longer illness. JEV NAbs were associated with increased dengue hemorrhagic fever in younger children with multitypic DENV NAb profiles (OR=4.05, 95% CI: 1.18 to 13.87). The observed association between JEV NAbs and DENV illness contrasts with studies suggesting an attenuating effect of heterologous flavivirus immunity on DENV disease severity.

Introduction

The dengue viruses (DENV) and Japanese encephalitis virus (JEV) are closely-related members of the virus family *Flaviviridae*. DENV and JEV co-circulate in the Indian subcontinent and in Southeast Asia, where they are important causes of human disease and mortality. While inactivated and live-attenuated JEV vaccines are licensed for use in humans, vaccination does not interrupt the primary JEV transmission cycle involving pigs, waterfowl, and *Culicine* mosquitoes (1). There is no licensed DENV vaccine and vector control efforts have been largely ineffective in containing transmission.

The co-occurrence of JEV and DENV has been documented in Thailand since 1969, when severe epidemics were observed in the Chiang Mai valley region (2). A large Phase III trial of JEVAX, a formaldehyde-inactivated, mouse brain-derived JEV vaccine, was conducted in 1984 in Kamphaeng Phet, Thailand (3). JEVAX became a part of the Expanded Program for Immunization (EPI) in Kamphaeng Phet in 1992 and efforts were made for "catch-up" vaccination of older children. Despite reported high levels of JEV vaccination (estimated to be 84% in 1998 and 98% in 2008), infections continue to be detected in Thailand each year (4).

JEV and DENV exhibit significant serological cross-reactivity, which can complicate assessment of the relative burdens of each in co-endemic areas and their possible interactions (5-6). There exists limited, inconclusive evidence regarding the clinical implications of prior JEV exposure or JEV vaccination and the severity of subsequent DENV infection. If the interactions observed between different DENV serotypes can serve as an analogy, we might expect that JEV/DENV cross-reactive immunity may be protective

(7), detrimental (8), or inconsequential. Hoke *et al.* reported that recipients of JEVAX experienced a non-significant decrease in the occurrence of DHF relative to placebo during the first two years after vaccination and that, among DHF patients, vaccinees experienced milder disease (3). There was no evidence of an association between JEV vaccination and the occurrence of DHF in a study of hospitalized DENV patients in Bangkok (9). There has been no reported increase in adverse events following live-attenuated DENV vaccination of JEV-immune volunteers (10-11). However, DENV vaccine recipients have demonstrated heightened and broadened DENV antibody responses in the setting of preexisting heterologous flavivirus immunity (12-13).

Potential interactions between flaviviruses are important for public health because wild-type JEV continues to co-circulate with DENV in Southeast Asia, the area with the highest burden of DENV illness, and JEV vaccination coverage in this region is high. As DENV vaccines advance toward licensure and implementation in co-endemic regions, an improved understanding of what constitutes protective immunity with DENV exposure is necessary. Given the conflicting data from prior studies, we examined how preexisting JEV immunity influenced the clinical severity of subsequent DENV infection using data from a prospective school-based cohort study in Thailand (14-15). The availability of information on asymptomatic DENV infections as well as outpatient and hospitalized dengue cases provided a unique opportunity to assess these interactions.

Methods

Study Population:

Data were collected during a five-year, school-based, prospective cohort study for DENV infections in children in Northern Thailand. The study design and methods have been described previously (14-15). Briefly, the study was conducted in Kamphaeng Phet Province from 1998-2002. In January 1998, 2,214 children were recruited from grades 1 through 5 at twelve primary schools. New participants were enrolled from the 1st grade class in January of each year. At enrollment, the children or their parents were asked about prior JEV vaccination and age of vaccination. A total of 3,687 children were enrolled for at least one year during the study period, with an average of 2.9 years of follow-up per child.

Study Methods

Serum samples were collected for dengue serology four times each year (January, June, August, and November). Active case surveillance of the participants was conducted from June 1 to November 1, with potential illnesses identified based on absence from school, visit to a school nurse or public health clinic, or admission to the hospital. Absent students were visited by village health workers and evaluated with a symptom questionnaire and an oral temperature. Acute blood samples were obtained for students with a history of fever within 7 days of fever onset, as were 14-day convalescent samples.

Characterization of Symptomatic Illnesses

Acute and convalescent blood specimens from incident febrile illnesses were tested using immunoglobulin M (IgM) and G (IgG) enzyme immunoassays for DENV and JEV. Acute

DENV infections were defined serologically as a DENV-specific IgM level ≥ 40 units and with DENV-IgM > JEV-IgM. The infecting DENV serotype was identified from acute blood specimens using serotype-specific reverse-transcriptase polymerase chain reaction (RT-PCR) or virus isolation in C6/36 cells or *Toxorhynchites splendens* mosquitoes. Symptomatic infections were defined as a documented history of febrile illness with virologic or serologic evidence of acute DENV infection. Charts of hospitalized children were independently reviewed and classified as DF or DHF and assigned a severity grade following WHO criteria (16). If a child experienced a febrile DENV illness but did not meet the criteria for DHF, they were characterized as having DF.

The duration of illness was derived from home visit data and hospitalization records. If a child was hospitalized, the duration of illness was the pre-hospitalization time (from date of fever onset) plus the time in the hospital. If a child was not hospitalized, the duration of illness was the length of time from the date of school absence or clinic visit to the date of the last home visit at which the child had a fever, muscle or joint pain, headache, nausea, vomiting, diarrhea, or any signs of bleeding or hemorrhage.

Characterization of Asymptomatic Infections

Asymptomatic infections were defined as a four-fold or greater rise in hemagglutination inhibition (HI) titers for any of the four DENV serotypes between two consecutive routine serum samples in the absence of a concurrent four-fold rise in JEV HI titers, or in the presence of a four-fold rise in JEV HI titers but with higher HI titers for any DENV serotype than for JEV, and in the absence of a confirmed acute symptomatic DENV infection in that individual for that year.

Characterization of DENV and JEV Antibody Status

Plaque reduction neutralization titers (PRNTs) were obtained for pre-infection samples to quantify neutralizing antibodies using standard methods (17). Briefly, LLC-MK2 cell monolayers were infected with DENV1 – DENV4 and JEV in the presence of serial dilutions of heat-inactivated patient plasma. The concentration of patient plasma that resulted in a 50% reduction in plaque formation was calculated using log probit regression. The reciprocal titer of this dilution was defined as the $PRNT_{50}$. A $PRNT_{50}$ <10 was defined as undetectable or 'negative' and a titer ≥10 was defined as 'positive.'

Children were grouped into three categories of DENV immunity based upon their pre-infection DENV antibody profiles: DENV-naïve (pre-infection $PRNT_{50}$<10 for all DENV serotypes), DENV-monotypic (pre-infection $PRNT_{50}$ ≥10 for a single DENV serotype), and DENV-multitypic (pre-infection $PRNT_{50}$ ≥10 for two or more DENV serotypes). PRNT data were used throughout this manuscript to describe pre-infection DENV immunity.

As a supplemental analysis, it was desirable to investigate the public health impact of JEV immunity on DENV illness by calculating the relative risk. Hemagglutination inhibition antibodies were used for this analysis because these were routinely run for all individuals in the cohort study, therefore allowing calculation of risk (probability of symptomatic infection given enrollment into the cohort study) instead of a conditional probability (probability of being symptomatic given infection). Using JEV HI antibody status at enrollment (negative: <10 negative, positive: ≥10) and stratifying on DENV HI status (positive: ≥10 for one or

more DENV serotype, negative: <10 for all serotypes), the relative risk of symptomatic illness given JEV Ab positivity was calculated.

Inferring the Infecting Serotype:

The infecting DENV serotype could not be determined for asymptomatically infected children and for 26.8% of symptomatically infected individuals. Given that severity may vary by infecting serotype, we imputed the infecting serotype for these individuals for some analyses. We assumed that the DENV serotypes detected among the RT-PCR positive cases were representative of the serotypes causing asymptomatic and RT-PCR negative symptomatic infections. This assumption is supported by evidence that DENV transmission is highly clustered in space and time in this community (18-19). Children missing serotype data were assigned the serotype that caused the majority of RT-PCR positive infections at their school for the period during which they were infected. If two or more serotypes 'tied' in causing the majority of PCR-positive infections (which occurred for 9% of infections missing serotype data), one of them was randomly selected for each DENV-infected child missing serotype data at that school.

Statistical Analyses:

Bivariate analyses were performed using chi-square testing for categorical variables and ANOVA or nonparametric testing for continuous variables. Logistic regression models were constructed using SAS' LOGISTIC procedure. The best model was then chosen by backward selection. Analyses were performed using SAS software, version 8 (SAS Institute, Cary, NC), SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL), and R version 2.10.1 (R Foundation for Statistical Computing, Vienna, Austria).

Human Subjects Research Approval

The study protocol was approved by the Human Use Review and Regulatory Agency of the Office of the Army Surgeon General, the Institutional Review Board of the University of the Massachusetts Medical School, and the Ethical Review Board of the Ministry of Public Health, Thailand. Secondary data analysis was additionally approved by the Institutional Review Board of Emory University.

Results

Descriptive statistics

A total of 942 children experienced at least one DENV infection between 1998 and 2002, out of 3,687 children who were enrolled for at least one full year. While some experienced multiple DENV infections during their enrollment, analyses are restricted to the first detected infection for each child. Further characterization of infections as symptomatic or asymptomatic was limited to those 569 cases that occurred within the active surveillance period (June 1 – November 1). Three-hundred-sixteen (56%) of these infections were asymptomatic and 253 (44%) were symptomatic. Of the symptomatic cases, 217 (86%) were DF and 36 (14%) were DHF. No deaths were attributed to DENV infection.

Factors associated with symptomatic illness

There were no differences by age, gender, or school in the proportions of infections that were symptomatic (Table 1). The proportion of symptomatic infections did vary by year, from 26% in 2000 to 51% in 2001 ($p=0.03$, by χ^2 testing across all strata of epidemic year). There were no differences in the proportion of infections that were symptomatic by pre-infection DENV antibody status. Children with JEV NAbs were more likely to experience symptomatic infection than children without JEV NAbs (57% versus 46%, $p=0.021$ by χ^2 testing). There was no difference in the occurrence of symptomatic illness by reported JEV vaccination history and, among those reporting a history of vaccination, no difference in the time between vaccination and the first detected infection (mean±SD 5.01±2.22 years for asymptomatics, 4.93±2.11 for symptomatics).

Factors associated with JEV positivity:

Of the 569 children with first-detected DENV infections during the active surveillance period, 479 (84%) had JEV and DENV NAb titer information available. Children developing DF were most likely to be JEV NAb positive, those experiencing asymptomatic infection the least (50% versus 39%, p=0.017, by χ^2 testing across all strata of infection severities) (Table 2). There were no differences in the proportions of children that were JEV NAb positive by age or gender. The proportion JEV NAb positive varied by school and year. DENV-naives were most likely to be JEV NAb positive (54%), then DENV-multitypics (47%), then DENV-monotypics (26%) (p=0.001 by χ^2 testing). Those reporting a history of JEV vaccination were more likely to be JEV NAb positive (51% versus 36% p=0.012 by χ^2 testing across all strata of reported vaccination histories). Among those reporting a history of vaccination, there was no difference in time since vaccination (mean±SD 5.01±2.27 years for JEV NAb positives, 4.96±2.19 for JEV NAb negatives).

Associations of JEV NAb positivity with DENV illness:

JEV NAb positivity was associated with an increase in the odds of symptomatic infection among those who experienced a DENV infection (OR=1.55, 95% CI: 1.08 to 2.23) (Table 3). There were no statistically significant differences in the occurrence of hospitalized illness or the occurrence of DHF.

Individuals with JEV NAbs were more likely to experience symptomatic DENV infection for all strata of preexisting DENV immunity (Figure 1a). In subgroup analysis, the association between JEV NAbs and symptomatic infection was significant and was strongest for those lacking DENV immunity prior to infection (DENV-naives) (OR=2.75, 95% CI:

1.12 to 6.72). The association was non-significant and weakest for children with DENV-monotypic immunity prior to infection. To attempt to discern cross-reactive immunity from serotype-specific immunity, we stratified DENV-multitypics according to mean age of observed infections in the cohort study (mean: 10 years of age), based upon the logic that older children would have had more time to experience multiple DENV infections and generate multiple serotype-specific responses, while younger children with multitypic profiles would, on average, reflect more cross-reactivity. Both younger and older children with DENV-immunity had approximately the same odds of symptomatic infection with JEV NAbs; neither association was significant. The direction of the associations between JEV NAbs and hospitalized illness was not consistent across strata of preexisting DENV immunity (Figure 1b).

JEV NAbs were associated with a decreased risk of hospitalized illness for DENV-monotypics and older DENV-multitypics and associated with an increased risk of hospitalized illness for DENV-naives and younger DENV-multitypics; none of these associations were significant. The directions of the associations for DHF across strata of DENV immunity were the same as for hospitalized illness (Figure 1c). The association was significant only for younger DENV-multitypics, who had increased odds of DHF with JEV NAbs (OR=4.05, 95% CI: 1.18 to 13.87).

Stratifying on reported JEV vaccination status ('yes' or 'no'), JEV NAbs were associated with the increased occurrence of symptomatic illness for both groups (significant only for those reporting a history of vaccination) (Supplemental table 1). Notably, the probability of symptomatic infection for those who reported a history of vaccination (collapsing over JEV

antibody status) was not higher than the probability of symptomatic infection for those who denied a history of vaccination.

Duration of illness

The presence of JEV NAbs was associated with an increased duration of DENV illness in DENV-naives (5.70 versus 2.69 days, p=0.045) (Table 4). For DENV-monotypics and multitypics, no difference in the duration of illness was observed.

Titer of JEV NAbs

Among those with JEV NAbs prior to infection, there was no significant difference in the geometric mean titer between asymptomatically and symptomatically infected individuals (Figure 2). The distributions of the titers were similar.

Role of infecting DENV serotype

The probability of symptomatic infection was increased for all four DENV serotypes in the presence of JEV NAbs (significant for DENV-3 in subgroup analysis, p=0.026) (Figure 3a). Hospitalized illness was (non-significantly) increased with JEV NAbs upon infection with DENV-1 (p=0.302) and DENV-2 (p=0.424), and decreased with DENV-3 (p=0.090) (Figure 3b). This indicates that the increase in symptomatic infections with DENV-3 was attributable to an increase in non-hospitalized illnesses. Limiting the comparisons to symptomatic, RT-PCR positive infections (with no imputation of infecting serotype), DENV-3 infection in the setting of preexisting JEV NAbs was associated with decreased hospitalized illness (p=0.001) (Figure 3c).

Multivariate model of JEV NAb positivity and symptomatic DENV infection

The positive, significant association between the presence of JEV NAbs and symptomatic infection remained after controlling for age, pre-infection DENV immunity, epidemic year, and infecting (imputed) DENV serotype in multivariate analysis (data not shown). The adjusted odds ratio was 1.61 (95% CI 1.10 to 2.35). No interaction effect was observed between JEV NAbs and the infecting serotype.

Serological cross-reactivity between JEV and DENV

Among individuals seronegative to JEV prior to DENV infection, 60.2% seroconverted to JEV in the post-season sample. JEV seroconversion did not differ significantly by baseline DENV immunity; multitypics were most likely to seroconvert (63.6%), then monotypics (56.0%), then naives (50.0%).

Relative risk of symptomatic infection with JEV antibodies.

Notably, in contrast to the NAb analyses, JEV HI Abs were associated with a decreased occurrence of symptomatic illness given infection (OR = 0.55, $p<0.01$) (Supplemental table 2). JEV HI Abs were also associated with a decreased risk of symptomatic infection (RR = 0.68, $p<0.01$). However, stratifying on enrollment DENV HI Ab status (positive or negative), the risk of symptomatic infection was increased for DENV-naives with JEV Abs (RR=1.72, $p=0.05$). For DENV-multitypics, the risk of symptomatic infection was decreased with JEV Abs (RR = 0.66, $p = 0.015$).

Discussion

The serological cross-reactivity between DENV serotypes is well-documented and has been linked to both cross-protection and enhanced disease. In contrast, the clinical implications of the significant serological cross-reactivity observed between DENV and other non-DENV flaviviruses remain unclear. In this study, we characterized the association between preexisting JEV antibodies and the clinical severity of DENV infection and found that JEV NAbs were associated with an increased occurrence of symptomatic infection.

The increased symptomatic illness with JEV NAbs was observed for all levels of preexisting DENV immunity. The strongest association was observed in DENV-naives, for whom JEV NAbs were also associated with a longer duration of illness. This is notable because in this group JEV NAbs are unlikely to reflect cross-reactive antibodies generated in response to a prior DENV infection. The association was not as strong for DENV-monotypics and DENV-multitypics, which could be due to cross-protection from DENV illness with increasing DENV immunity and/or a confounding effect with the inclusion of JEV NAbs as a result of cross-reactivity.

Preexisting JEV NAbs were not associated with the occurrence of hospitalized DENV illness or DHF in most groups; rather, JEV NAbs were associated with an increase in non-hospitalized illness. Given the sensitive method of identifying febrile illnesses in this cohort, non-hospitalized DENV illnesses included a wide range of clinical severities, from a single day of fever to a prolonged and debilitating illness. It is notable that we observed an increase in the duration of illness in those with JEV NAbs, suggesting that their influence was not to simply increase the occurrence of transient fever but rather to increase the occurrence and

severity of clinically-meaningful DF. A significant association between JEV NAbs and DHF was observed only among younger children who had multitypic DENV NAb profiles; the group likely representing the most immunologically cross-reactive state prior to infection. In these children it is possible that JEV NAbs served as a marker of a highly cross-reactive profile that was itself associated with the increased occurrence of DHF.

The prevalence of JEV NAbs in children experiencing DENV infection in the cohort was 45%, remarkably low given that vaccine coverage was estimated to exceed 80% at the time of the study. This low seropositivity could be due to a waning of the antibody response. A third dose of JEVAX was added to the EPI in 2000 to boost seroconversion rates and the durability of the immune response. The majority of children in the cohort would have been vaccinated the 1990s and the low seroprevalence may therefore be a true reflection of the immunogenicity of the two-dose regimen. While the self-reported JEVAX coverage was lower than would be expected (57%), those reporting a history of vaccination were significantly more likely to be JEV NAb positive.

Some challenges exist in interpreting the JEV antibody data and it is possible that some children were misclassified with respect to pre-infection serostatus. First, 76% of DENV-naïve/JEV-negatives exhibited a secondary-type response during acute infection. This suggests that negative JEV and DENV antibody titers may not preclude the possibility of prior JEV or DENV exposure and the possibility of an anamnestic response with DENV infection. Second, 60% of children who lacked JEV NAbs 'seroconverted' to become JEV NAb positive in the post-season sample following a DENV infection. Given the cross-reactivity between JEV and DENV in serological assays, it is possible that JEV NAbs in

some cases were present as a cross-reactive response to a prior DENV infection. Lastly, while JEV vaccination was widespread during the study period, wild-type JEV continued to circulate. In summary, the JEV antibodies detected in the cohort may have arisen as a result of JEV vaccination, JEV infection, cross-reactivity from DENV infection, or combinations of these. It may be that these different sources of detectable JEV titers may modulate the severity of DENV infection in different ways. Future studies should seek to distinguish between vaccine-derived JEV immunity and immunity derived from natural exposure, perhaps by analysis of NS1-specific antibodies (20).

Additional studies are needed to confirm and explore possible mechanisms for the observed association between preexisting JEV NAbs and DENV illness. Two observations from our analysis are relevant to this end. First, the titer of JEV NAbs did not appear to modulate the risk of disease, but rather the presence or absence of JEV NAbs. This suggests that preexisting JEV antibodies may interact with DENV in a manner that is distinct from the dose-dependent interactions of preexisting heterologous DENV antibodies (21-23). Alternately, JEV NAbs may be a marker of another underlying immune function that was not considered in this analysis, such as cell-mediated or innate immunity. Second, JEV NAbs were generally associated with increases in the occurrence and duration of non-hospitalized illness, but not DHF. This may suggest that antibody-dependent enhancement is less likely to be the mechanism of increased DENV illness.

An additional possibility is that of an immuno-pathological response to DENV infection associated with receipt of the inactivated JEV vaccine. Some inactivated virus vaccines have been associated with increased disease, perhaps due to the generation of a lower titer

immune response directed against a limited repertoire of epitopes. Early efforts to develop inactivated vaccines against measles and respiratory syncytial virus were abandoned after they were linked with the occurrence of atypical, occasionally severe disease (Kim, et al., 1973; Rauh & Schmidt, 1965). A recent study found an association between the inactivated trivalent seasonal influenza vaccine and increased pandemic influenza A (H1N1) illness (Skowronski, et al., 2010). There have been no reports to date of immuno-pathological responses following inactivated flavivirus vaccination in humans. However, a recent study in mice reported that low doses of JEVAX were associated with increased viral load and death following subsequent Murray Valley Encephalitis Virus challenge, relative to placebo, high doses of JEVAX, and the live Chimerivax-JEV vaccine (Lobigs, Larena, Alsharifi, Lee, & Pavy, 2009).

This first report of an association between preexisting JEV NAbs and DENV illness warrants further study. Because this study was conducted on a relatively limited temporal and spatial scale, similar analyses should be repeated in other cohorts. It would be of particular interest to compare DENV-endemic regions where live-attenuated JEV vaccines and inactivated JEV vaccines are in use. Possible associations between other flaviviruses, such as yellow fever and West Nile, and DENV illness should be evaluated. Our analysis only considered NAbs as an indicator of preexisting JEV immunity. Future studies should consider the role of vaccine-induced and wild-type JEV-derived cell-mediated immunity and the occurrence of DENV illness. Importantly, the findings indicate that DENV vaccine developers should include preexisting flavivirus immunity and vaccination histories in assessments of vaccine safety and efficacy. The results of these studies may be important for shaping DENV vaccine implementation strategies.

In summary, we report that the prior existence of JEV NAbs was associated with an increased probability of symptomatic DENV illness in a cohort of school-children in Thailand. These findings have public health importance in that DENVs co-circulate with other flaviviruses in much of their geographic range (e.g., JEV in Asia, yellow fever virus in Africa and South America, and West Nile virus in various locations in both hemispheres) and JEV vaccination is common throughout South and Southeast Asia. We suggest that the issue of heterologous flavivirus immunity and DENV, usually considered to be inconsequential or perhaps protective, merits renewed interest and investigation.

Acknowledgements.

We thank John McGowan, Derek Cummings, and Dana Flanders for their helpful comments on the analysis and the manuscript. We thank the staff at the Department of Virology, Armed Forces Research Institute of Medical Science (Bangkok, Thailand) for their careful diagnostic testing and data collection and entry. We acknowledge the support of the Office of the Provincial Public Health, Kamphaeng Phet province and the clinical research nurses at AFRIMS and the support staff at the Kamphaeng Phet Field Station for all their efforts. This project and publication was made possible by Dissertation Grant 1R36CK00104 from the CDC, NIH Grant P01 AI34533, and the United States Army Medical Research and Materiel Command, Ft Detrick, MD, USA. The opinions expressed in this manuscript do not necessarily represent the official views of the US National Institutes of Health, the US Department of Defense, or the US Department of the Army.

Financial Disclosure. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Competing Interests. The authors state that there are no competing interests with the information presented in this manuscript.

References

Burke, D. S., Nisalak, A., Johnson, D. E., & Scott, R. M. (1988). A prospective study of dengue infections in Bangkok. *Am J Trop Med Hyg, 38*(1), 172-180.

Clarke, D. H., & Casals, J. (1958). Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am J Trop Med Hyg, 7*, 561-573.

Endy, T., Anderson, K., Nisalak, A., Yoon, I., Green, S., Rothman, A., et al. (2010). Determinants of Inapparent and Symptomatic Dengue Infection in a Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *Manuscript submitted for publication.*

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

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Gibbons, R. V., Kalanarooj, S., Jarman, R. G., Nisalak, A., Vaughn, D. W., Endy, T. P., et al. (2007). Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences. *Am J Trop Med Hyg, 77*(5), 910-913.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever: Its history and resurgence as a global public health problem. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 1-22). New York: CAB International.

Gubler, D. J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol, 10*(2), 100-103.

Guzman, M. G., Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Montes, T., Vazquez, S., et al. (2007). Neutralizing antibodies after infection with dengue 1 virus. *Emerg Infect Dis, 13*(2), 282-286.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazquez, S., et al. (2000). Epidemiologic studies on dengue in Santiago de Cuba, 1997. *Am J Epidemiol, 152*(9), 793-799.

Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S., & Morier, L. (1990). Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg, 42*, 179-184.

Halstead, S. B. (1980). Dengue haemorrhagic fever-a public health problem and a field for research. *Bull World Health Organ, 58*(1), 1-21.

Halstead, S. B. (1980). Immunological Parameters Of Togavirus Disease Syndromes. *The Togaviruses*, 107-173.

Halstead, S. B. (1989). Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev Infect Dis, 11*(Suppl 4), S830-S839.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., Nimmannitya, S., & Cohen, S. N. (1970). Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med, 42*, 311-328.

Hammon, W. M., Rudnick, A., & Sather, G. E. (1960). Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. *Science, 131*, 1102-1103.

Jarman, R. G., Holmes, E. C., Rodpradit, P., Klungthong, C., Gibbons, R. V., Nisalak, A., et al. (2008). Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. *J Virol, 82*(11), 5494-5500.

Kou, Z., Quinn, M., Chen, H., Rodrigo, W., Rose, R., Schlesinger, J., et al. (2008). Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells.. *Journal of Medical Virology, 80*(1), 134-136.

Kouri, G., Guzman, M. G., Valdes, L., Carbonel, I., del Rosario, D., Vazquez, S., et al. (1998). Reemergence of dengue in Cuba: a 1997 epidemic in Santiago de Cuba. *Emerg Infect Dis, 4*(1), 89-92.

Kouri, G., Mas, P., Guzman, M. G., Soler, M., Goyenechea, A., & Morier, L. (1983). Dengue hemorrhagic fever in Cuba, 1981: rapid diagnosis of the etiologic agent. *Bull Pan Am Health Organ, 17*, 126-132.

Mammen, M. P., Pimgate, C., Koenraadt, C. J., Rothman, A. L., Aldstadt, J., Nisalak, A., et al. (2008). Spatial and temporal clustering of dengue virus transmission in Thai villages. *PLoS Med, 5*(11), e205.

Nimmannitya, S. (1997). Dengue Hemorrhagic Fever: Diagnosis and Management. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 133-145). Cambridge: CAB International.

Nisalak, A., Endy, T. P., Nimmannitya, S., Kalayanarooj, S., Thisayakorn, U., Scott, R. M., et al. (2003). Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am J Trop Med Hyg, 68*(2), 191-202.

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., et al. (1984). Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol, 120*, 653-669.

Thein, S., Aung, M. M., Shwe, T. N., Aye, M., Zaw, A., Aye, K., et al. (1997). Risk factors in dengue shock syndrome. *Am J Trop Med Hyg, 56*(5), 566-572.

U.S. Department of Health and Human Services, N. I. o. H., National Institute of Allergy and Infectious Disease. NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report.

Zhang, C., Mammen, M. P., Jr., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Monkongdee, P., et al. (2005). Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. *J Virol, 79*(24), 15123-15130.

Table 1. Cohort characteristics associated with symptomatic DENV illness.

	# Infections * (% of Total)	% Symptomatic	p-value **
Total infections	569	44.5%	
Infection Severity			
Asymptomatic	316 (55.5%)	---	
DF	217 (38.1%)	---	---
DHF	36 (6.3%)	---	
Age (years)			
7-9	216 (38.1 %)	47.7%	
10-12	341 (60.1%)	42.2%	0.423
13-15	10 (1.8%)	50.0%	
Gender			
Male	275 (48.5%)	42.5%	0.699
Female	292 (51.5%)	46.2%	
School (12 total)			
Range (Min – Max)	5 – 139	32.3 – 80.0%	0.618
Epidemic Year			
1998	168 (29.5%)	42.3%	0.032
1999	133 (23.4%)	42.9%	
2000	46 (8.1%)	26.1%	
2001	173 (30.4%)	51.4%	

2002	49 (8.6%)	49.0%	
Pre-Infection DENV NAb Profile			
DENV-Naïve	83 (17.3%)	55.4%	
Monotypic	74 (15.4%)	59.5%	0.131
Multitypic	322 (67.2%)	47.8%	
Reported JEV Vaccination			
Yes	311 (54.8%)	40.8%	
Can't recall	108 (19.0%)	51.9%	0..119
No	149 (26.2%)	36.3%	

* Refers to first-detected DENV infections in the cohort that occurred during the active surveillance period each year (June 1 – November 1) and had neutralizing antibody data available

** Statistical tests considered the association between variables of interest and the occurrence of symptomatic versus asymptomatic infection. p-values were obtained using the Pearson chi-square test for categorical variables, with $\alpha = 0.05$ as the level of significance.

Table 2. Factors associated with JEV seropositivity prior to DENV infection

	# Infections *	% JEV positive	p-value **
Total infections	479	44.7%	
Infection Severity			
Asymptomatic	235	39.1%	
DF	211	50.2%	0.017
DHF	33	48.5%	
Age (years)			
7-9	185	43.2%	
10-12	285	44.9%	0.209
13-15	8	75.0%	
Gender			
Male	232	44.8%	0.667
Female	246	44.7%	
School (12 total)			
Range (Min – Max)	4 - 127	13.3% - 75.0%	0.004
Epidemic Year			
1998	145	36.6%	
1999	104	57.7%	
2000	32	50.0%	0.021
2001	159	42.1%	
2002	39	46.2%	

Pre-Infection DENV NAb Profile			
DENV-Naïve	83	54.2%	
Monotypic	74	25.7%	0.001
Multitypic	322	46.6%	
Reported JEV Vaccination			
Yes	259	51.0%	
Can't recall	95	38.9%	0.012
No	124	36.3%	

* Refers to first-detected DENV infections in the cohort that occurred during the active surveillance period each year (June 1 – November 1) and had neutralizing antibody data available

** Statistical tests considered the association between variables of interest and the presence or absence of JEV NAbs in the pre-infection sample. p-values were obtained using the Pearson chi-square test for categorical variables, with $\alpha = 0.05$ as the level of significance. Two-way analysis of variance tests (ANOVA) were used for mean time since vaccination.

Table 3. Crude associations of DENV illness severity and JEV antibody status.

	# Infections	% JEV Positive	OR (95% CI) †
Symptomatic	244	50.0%	
			1.55 (1.08 – 2.23)
Asymptomatic	235	39.1%	
Hospitalized	48	43.8%	
			0.96 (0.53 – 1.75)
Non-hospitalized *	431	44.8%	
DHF	33	48.5%	
			1.18 (0.58 – 2.39)
Non-DHF **	446	44.4%	

* Non-hospitalized infections incorporate both non-hospitalized DF and asymptomatic seroconversions.

** Non-DHF infections incorporate both non-hospitalized and hospitalized DF and asymptomatic seroconversions.

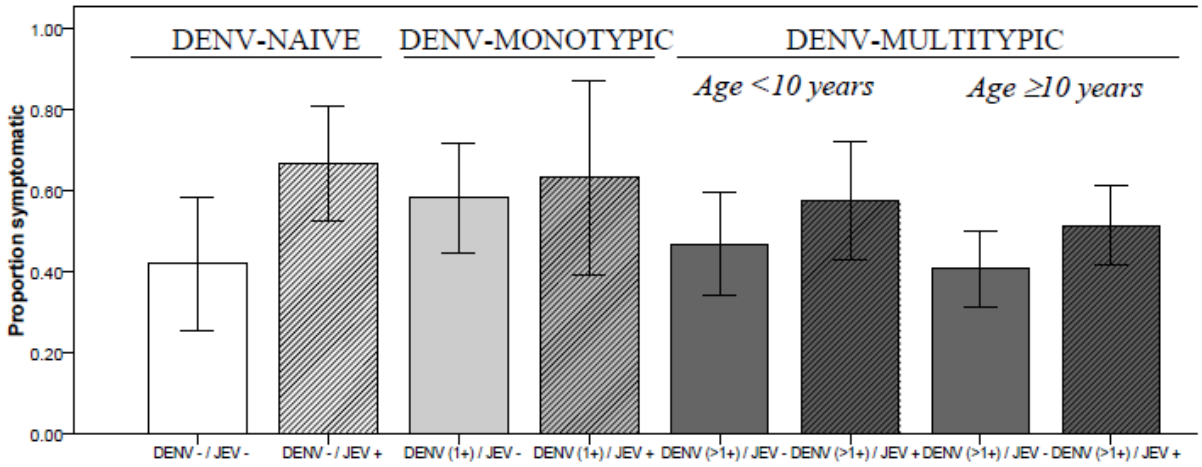
† P-values were calculated using the Mantel-Haenzel chi-square statistic.

Table 4. Duration of DENV illness (days) by JEV NAb status

Mean duration of illness in days (SD) *	JEV NAb -	JEV NAb+	p-value **
All symptomatic infections	4.51 (5.28)	4.89 (5.66)	0.591
DENV-Naives	2.69 (1.74)	5.70 (5.68)	0.045
DENV-Monotypic	5.52 (7.62)	4.75 (5.79)	0.756
DENV-Multitypic	4.51 (4.58)	4.60 (5.68)	0.915

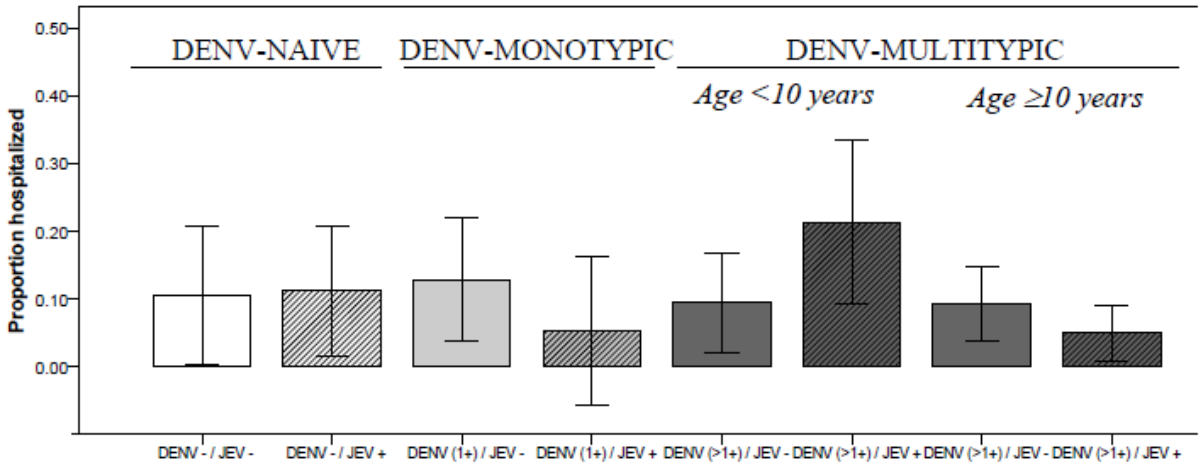
* Duration of illness was calculated as the number of days elapsed from first day of febrile illness to the last day that a child reported any fever, muscle or joint pain, headache, nauseas, vomiting, diarrhea, or any signs of bleeding or hemorrhage.

** P-values were calculated using 2-way analysis of variance testing (ANOVA), with $\alpha = 0.05$ as the level of significance.



$OR_{(Sx \mid JEV\ pos)} = 2.75$ (1.12 to 6.72) | $OR_{(Sx \mid JEV\ pos)} = 1.23$ (0.55 to 3.61) | $OR_{(Sx \mid JEV\ pos)} = 1.53$ (0.72 to 3.27 | $OR_{(Sx \mid JEV\ pos)} = 1.54$ (0.89 – 2.66)

Figure 1a.



$OR_{(Hosp \mid JEV\ pos)} = 1.06$ (0.26 to 4.27) | $OR_{(Hosp \mid JEV\ pos)} =$ 0.38 (0.04 to 3.32) | $OR_{(Hosp \mid JEV\ pos)} =$ 2.61 (0.88 to 7.79) | $OR_{(Hosp \mid JEV\ pos)} =$ 0.50 (0.16 – 1.52)

Figure 1b.

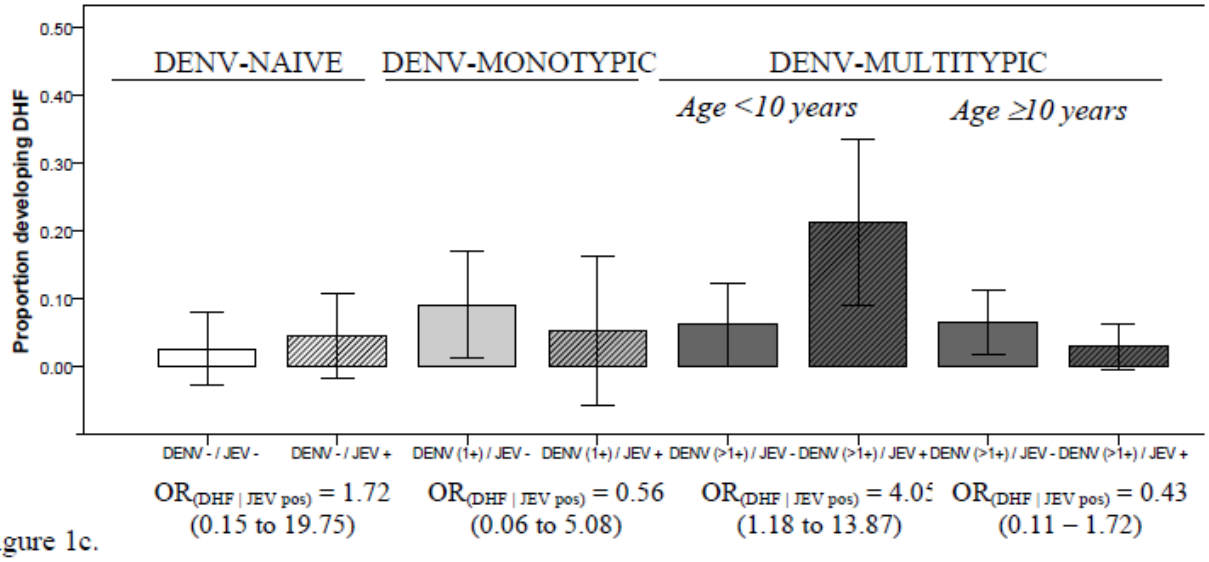


Figure 1c.

Figures 1a-1c. The proportions of dengue (DENV) infections developing symptomatic illness (1a), hospitalized illness (1b), and dengue hemorrhagic fever (1c). Data are stratified by preexisting DENV immunity (naïve [DENV -], monotypic [DENV 1+], multitypic older and younger than 10 years of age [DENV >1+]) and preexisting Japanese encephalitis virus (JEV) neutralizing antibodies (+ or -). Odds ratios (ORs) estimate the odds of being experiencing the disease severity of interest (dengue hemorrhagic fever [DHF], hospitalized illness [Hosp], or symptomatic illness [Sx]) in the presence of JEV NAbs over the odds of experiencing the disease severity of interest in the absence of JEV NAbs. Values in parentheses indicate the 95% confidence intervals for the ORs. Error bars indicate the 95% confidence intervals for proportions.

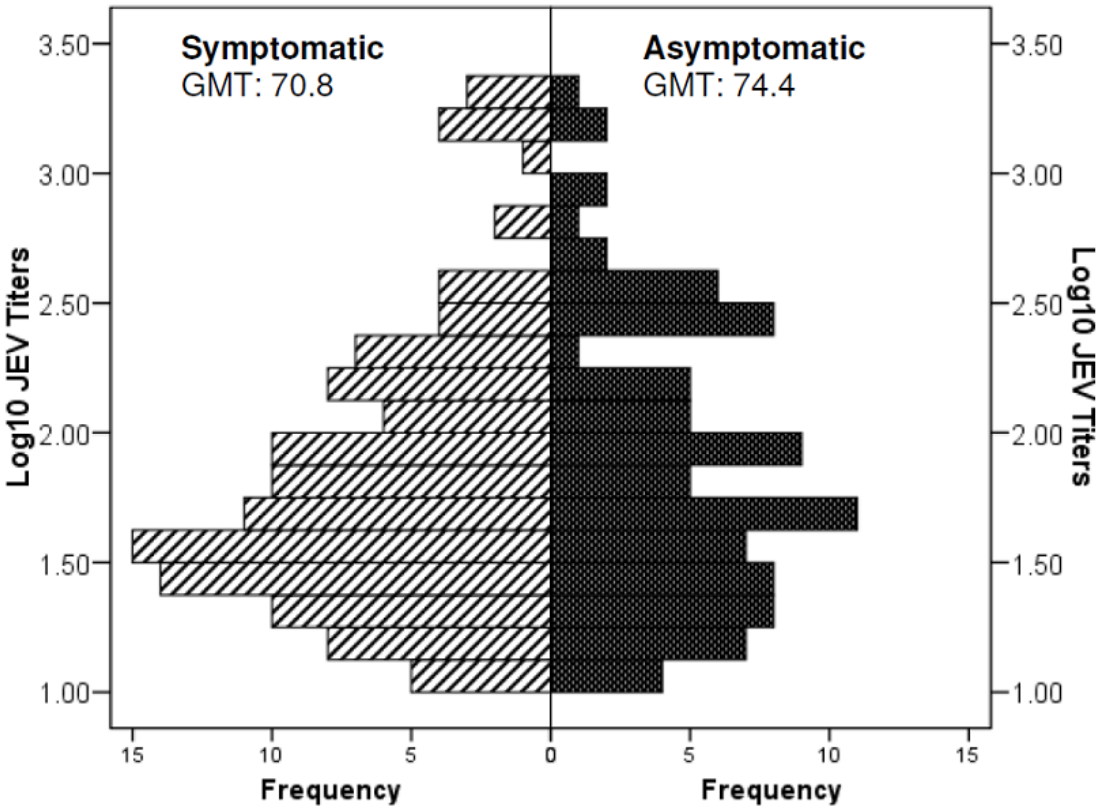
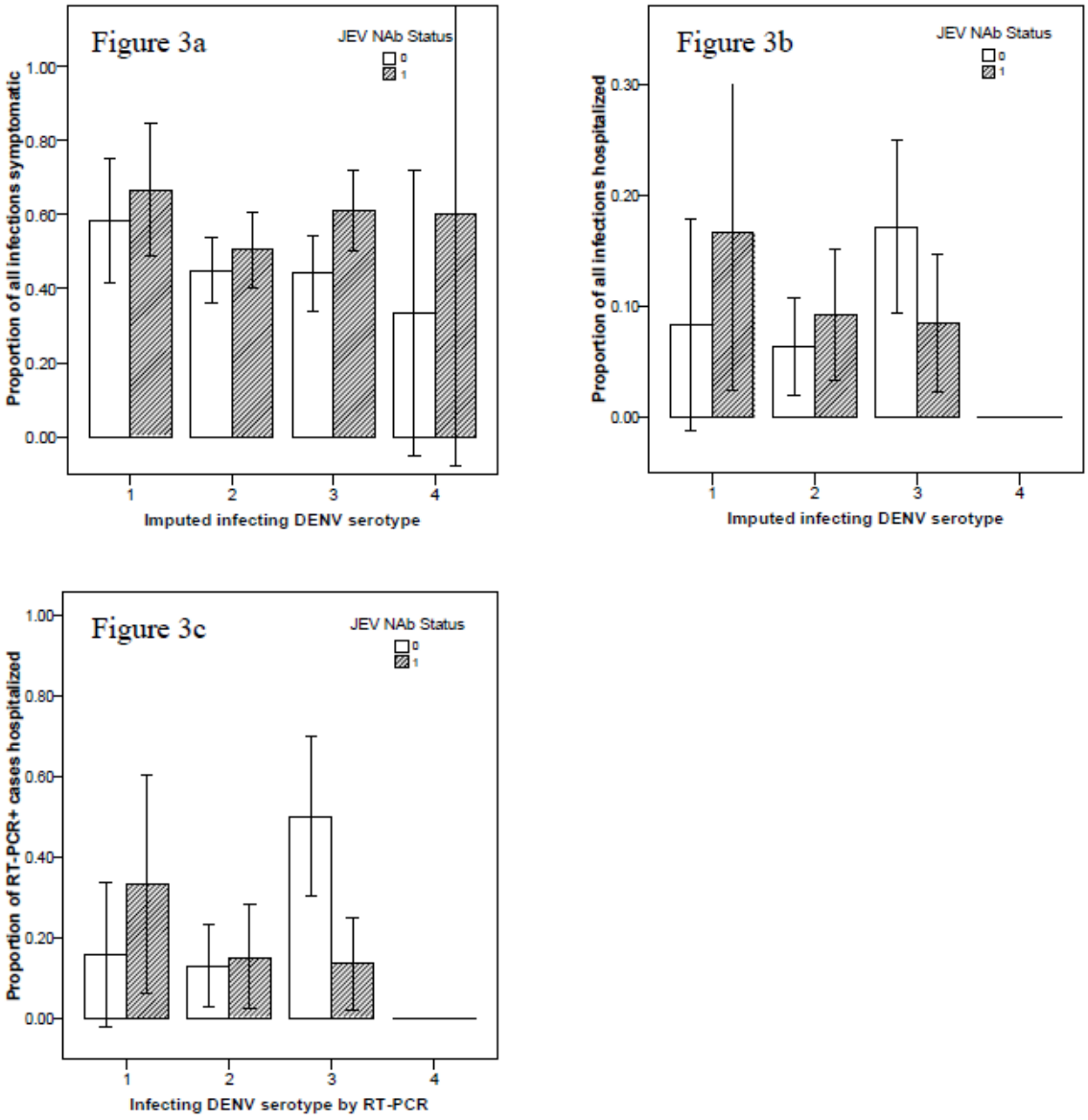


Figure 2. Distribution of JEV neutralizing antibody titers among those with detectable JEV antibodies, by DENV illness severity. Geometric mean titers (GMT) are shown for asymptomatic (dark gray) and symptomatic (striped) infections.



Figures 3a-3c. Proportions of infections developing symptomatic illness (3a) and hospitalized illness (3b and 3c) by infecting DENV serotype. The infecting DENV serotype was imputed to allow consideration of all DENV infections (asymptomatic and RT-PCR-negative symptomatic infections) in 3a and 3b, using information on the predominate DENV serotype in circulation at a child's school for the time interval during which they were

infected. Analysis was restricted to RT-PCR-positive infections in 3c. Error bars indicate the 95% confidence interval for the proportion developing hospitalized illness and symptomatic illness out of all infections (3a and 3b) and all PCR-positive infections (3c).

Supplemental Table 1. Association between JEV NAb status and asymptomatic (Asx) DENV infection, stratified on reported receipt of the JEV vaccine.

		Total N	% Asx (N)	p-value *
Reported Receipt of the JEVAX Vaccine				
	Yes	320	60.3% (193)	0.272
	No	152	54.6% (83)	
(Stratifying on Reported Receipt of the JEVAX Vaccine)				
Yes				
	JEV NAb Pos	136	47.8% (65)	0.049
	JEV NAb Neg	128	60.2% (77)	
No				
	JEV NAb Pos	45	40.0% (18)	0.27
	JEV NAb Neg	82	51.2% (42)	

* p-value calculated used Chi-square statistics

Supplemental Table 2. Relative risk of symptomatic DENV infection with JEV hemagglutination inhibition (HI) antibodies* time of enrollment into the cohort study

	Probability of symptomatic infection, given that a DENV infection occurred *						Risk of symptomatic infection based upon enrollment into the cohort **					
	JEV HI Ab+		JEV HI Ab-		OR	p †	JEV HI Ab+		JEV HI Ab-		RR	p
	(N)	% Sx	(N)	% Sx			(N)	% Sx	(N)	% Sx		
Total	624	18.8%	523	29.6%	0.55	<0.001	1926	6.6%	1523	9.7%	0.68	0.001
Stratifying on enrollment DENV HI Ab profile												
	JEV HI Ab+		JEV HI Ab-		OR	P	JEV HI Ab+		JEV HI Ab-		RR	p
	(N)	% Sx	(N)	% Sx					(N)	% Sx		
Naïve	38	44.7%	221	33.5%	1.61	0.182	101	15.8%	818	9.2%	1.72	0.05
Monotypic	18	33.3%	66	37.9%	0.82	0.723	45	6.7%	207	13.0%	0.52	0.313
Multitypic	567	16.6%	236	23.7%	0.64	0.018	1778	6.1%	498	9.2%	0.66	0.015

* Analogous to the JEV NAb analyses in Table 3 and Figure 1, using pre-infection HI Ab data. HI Abs were used to compare the ORs observed based upon JEV NAb positivity (Figure 1) and JEV HI Ab positivity (above).

** Considers the risk of symptomatic out of all individuals at risk of infection, regardless of infection status, using JEV HI Ab status at enrollment into the cohort study. HI Abs were used because they were available for all children enrolled in the cohort study, allowing calculation of the relative risk (RR.) of illness with JEV HI Abs.

† p- values calculated using chi-square statistics.

STUDY TWO

DISCLAIMER: AT THE TIME OF THESIS PUBLICATION, THIS MANUSCRIPT WAS UNDER PREPARATION FOR SUBMISSION TO A SCIENTIFIC JOURNAL FOR REVIEW. WHERE POSSIBLE, THE READER IS ENCOURAGED TO READ THE FINAL, PUBLISHED MANUSCRIPT.

STUDY TWO

Title: A shorter time interval between first and second dengue infections is associated with protection from clinical illness in a prospective school-based cohort in Thailand

Abstract:

Background. Despite the strong association between secondary dengue (DENV) infections and dengue hemorrhagic fever (DHF), the majority of secondary infections are in fact asymptomatic or dengue fever (DF). The determinants of the clinical severity of secondary infections remain unclear, though some studies have suggested a possible titer-dependent and time-dependent role of cross-protective dengue DENV antibodies. Here, we investigate the association between the time interval separating sequential DENV infections and clinical severity and whether, among individuals with the same interval between infections, there were immunological differences that were associated with disease severity.

Methods. To assess this, we used data from two phases of a prospective cohort study to detect asymptomatic and symptomatic DENV infections in school-children in Kamphaeng Phet, Thailand, conducted from 1998 to 2002 and 2004 to 2007. Children who experienced at least one DENV infection during their enrollment were selected as the population for analysis.

Results. 1696 children had at least one DENV infection detected during their enrollment and 268 of these children had two DENV infections detected. A shorter time interval between the first and second DENV infections detected in the cohort was associated with an increased probability of asymptomatic infection. The association was strongest in children who were seronegative for DENV-1 – DENV-4 by hemagglutination inhibition

(HI) assay at enrollment (average interval separating sequential infections of 2.6 years for DHF, 1.9 years for DF, and 1.6 years for asymptomatic infections, p=0.01 by exact Wilcoxon rank statistic). In the final model combining time since first observed infection and the magnitude of the antibody response to first infection, the highest probability of being asymptomatic was observed in individuals who experienced their second infection at shorter intervals after the first infection and with a higher titer HI antibody response generated to the first infection.

Conclusions. These findings are consistent with a temporal/immunological model of disease risk where cross-reactive antibodies wane from higher-titer, protective levels to lower-titer, detrimental levels. This is the first time that a temporary window of cross-protection following DENV infection has been demonstrated using human infection data since early observations from human challenge studies in the 1940s.

Introduction

Infection with dengue viruses (DENV) is a major cause of disease and death throughout tropical and subtropical regions of the globe. Dengue viruses are presently classified as priority emerging pathogens due to remarkable increases in geographic range, incidence of infection, and clinical severity in recent decades (U.S. Department of Health and Human Services). Increased international travel and commerce have facilitated the introduction of DENV into novel regions and increased the co-circulation of DENV serotypes in endemic regions (Gubler, 2002a). Southeast Asia, the region with the greatest burden of DENV infection, has all four DENV serotypes (DENV-1 – DENV-4) in circulation..

The increasing co-circulation of DENV serotypes in recent decades is notable because it is associated with the emergence of the clinically-severe dengue hemorrhagic fever (DHF), an outcome of DENV infection that was thought to be rare prior to this century (Gubler, 1997). In the 1960s, studies of the dramatic increases in hemorrhagic disease with DENV in Southeast Asia established an early link with between DHF and secondary DENV infections (Halstead, et al., 1970; Hammon, et al., 1960). This association has since been confirmed in other epidemics and other locales (Burke, et al., 1988; M.G. Guzman, et al., 1990; Sangkawibha, et al., 1984; Thein, et al., 1997). Secondary DENV infection remains the strongest known risk factor for DHF, with a relative risk that been estimated to be as high as 50-100 compared to primary DENV infection (S. B. Halstead, 1980; Thein, et al., 1997).

Despite the strong association between DHF and secondary infection, secondary infection appears to be neither sufficient nor necessary for causing DHF. DHF can be (albeit rarely) observed in primary dengue infections (M. G. Guzman, et al., 2000). This suggests that

unknown host and/or viral factors are sufficient to drive the occurrence of DHF outside of the setting of pre-existing antibodies. Second, only a small fraction (about 3%) of secondary infections progress to DHF; the majority of secondary infections are in fact asymptomatic (T. P. Endy, Chunsuttiwat, et al., 2002; S. B. Halstead, 1980). The factors that predispose one individual to develop DHF over another with secondary infection are unclear. Last, tertiary and quaternary infections with dengue are typically thought to be mild or asymptomatic (R. V. Gibbons, et al., 2007). This suggests that, while cross-reactive memory immune responses are potentially deleterious when experiencing a secondary dengue infection, cross-protection can be sufficient by the third infection to protect against clinical illness.

The earliest and most prominent hypothesis put forward for the pathogenesis of DHF was that of antibody-dependent enhancement (ADE) (Halstead, et al., 1970). In ADE leading to DHF, preexisting neutralizing antibodies from a primary DENV infection are hypothesized to enhance a subsequent infection with a different DENV serotype, resulting in increased viral load, increased immune activation, and ultimately the plasma leakage characteristic of DHF/DSS (S.B. Halstead, 1980). Several observations have suggested that the ability to neutralize or enhance heterologous viruses may vary by antibody specificity and antibody titer (Halstead, 1989). *In vitro* studies have demonstrated a titer-dependence of ADE by mixing serial dilutions of pooled human serum with DENV and applying to mononuclear cells in culture (Kou, et al., 2008). Peaks in both the percentage of infected cells and viral output per infected cell were observed at low-intermediate titers of antibody, suggesting that the influence of cross-reactive antibodies with heterologous infection could range from moderate protection (high titers of antibody), to enhancement (intermediate titers), to no effect (low titers).

An analogy to this titer-dependent function of cross-reactive antibodies may be found in human infants, who are born with high levels of maternal antibody and are seemingly protected from DHF (Halstead, et al., 2002). As maternal antibody wanes, infants are postulated to pass into an 'intermediate phase' at approximately 6-8 months where they experience an elevated risk of DHF. As maternal antibody wanes further and disappears, infants again pass into a phase with a low risk of DHF. This suggests that at high maternal antibody titers, the quantity of antibodies is sufficient to provide some protection from DENV infection but that as antibodies wane to low-intermediate levels, cross-reactive antibodies may facilitate the occurrence of DHF. The same prospective study of school children in Thailand (1998-2002) that wass used for the present analysis has provided epidemiological evidence of a possible titer-dependent role of cross-reactive antibodies (T. P. Endy, et al., 2004). An earlier analysis of PCR-positive DENV-3 infections in the cohort found that higher levels of pre-existing (heterologous) DENV-3 antibodies were associated with milder illness and lower viremia (significant for DENV-3 only) (T. P. Endy, et al., 2004).

There is experimental evidence of a temporal window of cross-protection afforded by heterologous DENV antibodies. Albert Sabin administered serial experimental inoculations of DENV to human volunteers and concluded that modifications in the clinical severity and clinical presentation associated with a second heterologous DENV infection varied according to the amount of time elapsed between inoculations (Sabin, 1952). Specifically, he found that up until two months post-primary inoculation, volunteers were protected from illness and were unable to infect mosquitoes (i.e., were protected from infection). Two to three months post-infection, exposure to a heterologous DENV serotype resulted in

transient fever and mild malaise and the ability to infect mosquitoes was reestablished. Nine months post-primary infection, heterologous exposure resulted in two to three days of fever and a rash.

Two important epidemiological observations on temporal trends in disease risk were made based upon epidemics in Cuba. A large DENV-1 epidemic passed through Cuba in 1977-8 followed by two epidemics of DENV-2, in 1981 and in 1997 (G. Kouri, et al., 1998; G. Kouri, et al., 1983). It was found that individuals that had experienced their primary DENV-1 infection in 1977-8 and their secondary DENV-2 infection in 1997 (twenty years between infections) had a risk of DHF that was 3-4 times greater than individuals that had their secondary DENV-2 infection in 1981 (with four years between infections). The authors concluded that a longer period of time between infections was associated with a greater risk of DHF (M. G. Guzman, et al., 2000). They later demonstrated a decline in the proportion of the immune response that was heterotypic over time, in a subset of individuals infected during 1977 epidemic and this waning of cross-reactive antibodies over time was proposed as a possible mechanism for the increased risk of DHF with increased time between infections (M. G. Guzman, et al., 2007).

The present study sought to identify temporal and immunological factors associated with the significant variability observed in the clinical outcome of secondary DENV infections. We hypothesize that shorter time intervals between infections would be associated with decreased risk of symptomatic infection and that longer time intervals would be associated with the increased occurrence of DHF. We further hypothesize that, among children with the same amount of time between infections, immunological factors such as the immune

response to a prior infection, the decay in antibodies over time, and antibody titers prior to a subsequent infection may further influence the occurrence of asymptomatic versus symptomatic infection.

Methods

Study population

Data were derived from two phases of a school-based, prospective cohort study for DENV infections in children in Northern Thailand. The studies were conducted from 1998-2002 (Kamphaeng Phet study #1, or 'KPS1') and from 2004-2007 (Kamphaeng Phet study #2, or 'KPS2'). The study designs and methods for the two studies were similar and have been described previously (T. P. Endy, Chunsuttiwat, et al., 2002; Mammen, et al., 2008). In January of 1998 and January of 2004, approximately 2000 children were recruited from primary schools in the community; 12 schools participated for KPS1 and 11 schools for KPS2. With the exception of one school which was involved in both study phases, KPS1 and KPS2 took place at different schools and in different villages. No child was enrolled in both studies. Children aged 5-16 were eligible for enrollment in KPS1 and children aged 4-16 were eligible for KPS2.

Acute illness specimens and active fever surveillance

Active, fever-based surveillance for dengue illnesses was conducted from June 1 to November 1 each year for both study phases. During the active surveillance period, potential illnesses in enrolled children were identified based on absence from school, visit to a school nurse or public health clinic, or admission to the hospital; the occurrence of any of these events triggered notification of the study staff and prompt evaluation of the child by a village health worker with a symptom questionnaire and oral thermometer. Acute blood samples were obtained from children experiencing potential illnesses if they had a reported history of fever within 7 days of school absence or an oral temperature $\geq 38°C$. 14-day convalescent blood samples were also collected. Children with severe disease were referred

for further evaluation at the Kamphaeng Phet provincial hospital. A student who was absent from school was evaluated on each day of school absence until a fever or history of fever was documented or the student returned to school.

Acute and convalescent blood specimens from incident febrile illnesses were tested using immunoglobulin M (IgM) and G (IgG) enzyme immunoassays for DENV and Japanese encephalitis virus (JEV). Acute DENV infections were defined serologically as a DENV-specific IgM level ≥ 40 units and with DENV-IgM > JEV-IgM. The infecting DENV serotype was identified from acute blood specimens using serotype-specific reverse transcriptase-polymerase chain reaction (RT-PCR) or virus isolation in C6/36 cells or *Toxorhynchites splendens* mosquitoes.

Symptomatic infections were defined as a documented history of febrile illness with virologic or serologic evidence of acute DENV infection. Charts of hospitalized children were independently reviewed and classified as dengue fever (DF) or DHF and assigned a severity grade following WHO criteria (Nimmannitya, 1997). If a child experienced a febrile DENV illness but did not meet the criteria for DHF, they were characterized as having DF. The sensitive nature of the active fever surveillance system meant that the symptomatic infections captured in the cohort studies covered a wide range of clinical severities, from a single day of transient fever, to a prolonged and debilitating DF illness, to DHF and dengue shock syndrome (DSS).

Routine blood specimens and detection of seroconversions

Routine blood specimens were drawn from all enrollees four times a year for KPS1 (January 1, June 1, August 16, and November 1) and two times a year for KPS2 (January 1 and June 1). These specimens were tested for the presence of hemagglutination inhibiting antibodies against DENV-1,2,3 and 4 and JEV using the standard method of Clark and Casals (Clarke & Casals, 1958). Asymptomatic seroconversions were defined according to WHO criteria as a four-fold or greater rise in hemagglutination inhibition (HI) titers for any of the four DENV serotypes between two consecutive routine serum samples in the absence of a concurrent four-fold rise in JEV HI titers, or in the presence of a four-fold rise in JEV HI titers but with higher HI titers for any DENV serotype than for JEV, and an absence of a confirmed acute symptomatic DENV infection in that individual for that year.

Characterization of first-detected and second-detected infections

In this analysis, DENV infections are designated 'first detected' and 'second detected' infections, regardless of preexisting antibody profile. The ability to characterize the clinical severity of these infections was dependent upon when an infection occurred. Infections occurring during the active surveillance period (June 1 to October 31) could be classified as asymptomatic, DF, or DHF, as active fever surveillance allowed detection of non-hospitalized DENV illnesses and distinction of these mild illnesses from asymptomatic seroconversions. Outside of the active surveillance period (November 1 to May 31), DENV illnesses were reported to study staff only if a child became hospitalized with a serologically or virologically-confirmed DENV infection; asymptomatic and non-hospitalized illnesses were detected solely as seroconversions during this period and could not be distinguished.

Time to second infection was calculated as one-year intervals from the first infection; the date of infection was not known for asymptomatic infections and therefore a finer temporal resolution was not possible. As an example, sequential infections occurring in two consecutive active surveillance periods (June through October) were coded as separated by one year. The cohort study was not designed to capture multiple DENV infections in the same individual in the same year; therefore, the possible time intervals between a first and a second infection were one, two, three, or four years. In reality, a one-year interval may represent an actual interval as short as seven months and as long as 16 months.

Assumptions applied to the analysis were (1): All infections with a novel serotype were identified for each child in the cohort. (2) A rise in HI titers is indicative of a new infection even among individuals with previously high HI titers to 2 serotypes or 3 serotypes (i.e. third and fourth infections are possible) (3) Re-exposures to a previous infecting serotype would not have been misclassified as new infections since serotype-specific immunity is lifelong and sterilizing. (4) There were no changes in the probability of detecting asymptomatic or symptomatic infections over time that could cause an artificial increase or decrease of one relative to the other in the years following a first infection. As noted above, a longer period of time elapsed between routine blood collections in KPS2; therefore, some asymptomatic infections might have been missed if antibodies had risen and then waned to undetectable levels between routine blood draws. Sensitivity analyses were conducted to evaluate the degree to which this possible detection bias could influence time interval estimates for symptomatic and asymptomatic infections, by varying the proportion of asymptomatic infections that were detected over time and observing how the mean time to infection was altered Estimates were found to be insensitive to changes in the detection of asymptomatic

cases across a broad range of missed cases (from 0% to 50% of asymptomatic cases missed); suggesting that assumption 4 is likely valid despite the protocol differences between the studies.

Appendix 1 shows the serial HI titers for DENV-1, 2, 3 and 4 from just prior to the first infection to just after the second infection in six representation cases (3 from KPS1 and 3 from KPS2). HI titers in post-infection specimens in KPS2 (2004-2007) tended to be lower than those observed in KPS1; again, this is may be a reflection of the longer interval between routine blood draws in KPS2.

Defining baseline DENV immunity and other immunological parameters

A unique aspect of these studies is the availability of serotype-specific HI data for all routine samples collected from all children during enrollment, allowing insight into both short-term and long-term trends in the immunological response to DENV infections. Using these HI data, children were classified upon entry into the cohort as HI-negative (enrollment HI antibody titers ≤ 10 for all four DENV serotypes), HI-monotypic (enrollment HI >10 for a single DENV serotype), and HI-multitypic (enrollment HIs >10 for two or more DENV serotypes).

Immunological parameters

The changing immunological states of children experiencing DENV infections were further characterized by considering HI profiles prior to their first infection, following their first infection, and prior to their second detected infection. Specifically, four immunological parameters were evaluated: (1) the log of the rise in HI titers following the first infection,

summed across serotypes, (2) the proportion of the total (summed) rise in HI titers that was directed against serotypes other than the infecting serotype (the heterotypic response) for the first infection, (3) the annual percentage change in the proportion of the summed antibody response that was heterotypic, from the routine blood draw collected after the first infection to just prior to the second infection, and (4) the log of the preexisting antibody titer against the second infecting serotype, in the specimen collected prior to the second infection.

Inferring the infecting serotype

The infecting DENV serotype could not be determined for asymptomatically infected children and for 25.2% of symptomatically infected individuals. Based upon evidence that DENV transmission in this community is highly clustered, we assumed that the serotypes detected among RT-PCR positive cases were representative of the serotypes causing asymptomatic and RT-PCR negative symptomatic infections at a given school for a given year (Jarman, et al., 2008; Mammen, et al., 2008). Children missing serotype data were assigned the serotype that caused the majority of RT-PCR positive infections at their school for the period during which they were infected. 69% of epidemics at the schools had a clear 'majority' serotype, defined as a serotype representing ≥70% of the PCR-positive cases.

Statistical analyses

Exact Wilcoxon rank (non-parametric) tests were used to compare the time to infection between groups, using SAS' NPAR1WAY. Immunological parameters (detailed above) were also evaluated using Wilcoxon tests in bivariate analyses.

Two different modeling methods were used to explore possible associations between time and the occurrence of symptomatic infection while controlling for possible confounding due to baseline immunity and conditioning on factors presumably related to exposure risk (study period and sub-district of residence) and age at first infection. Age at first infection was divided into tertiles (5 to 8 years of age, 9 to 10 years of age, and 11 to 15 years of age). The first approach was a logistic survival analysis, which calculated the probability of symptomatic infection for each year since the first infection, given that a child was still at risk (i.e., had not already been infected or withdrawn from the study) for that interval. The study population for this analysis was therefore the children that had experienced at least a first infection during their enrollment. Figure 1 illustrates the numbers of children 'at risk' for infection each year, the numbers of cases that were lost to follow-up or infected outside of the active surveillance window, and the numbers and severities of cases observed. DENV immunity at enrollment and the time interval between infections, and a term for their interaction, were incorporated into the model.

The second approach was a conditional logistic regression that estimated the probability of symptomatic infection, given that a second infection was observed in an individual. This model conditioned on the sub-district of residence, age at first infection, and the study period (i.e., KPS1 or KPS2). Enrollment DENV immunity, the time interval between infections, and an interaction term for immunity and interval were incorporated into the model. Immunological factors were dichotomized as being higher or lower than the mean for each variable. All immunological factors were initially incorporated into the model and were removed using backward elimination until only significant variables remained in the

model. Both models used the EXACT statement in proc logistic and conditional regression was specific using the STRATA statement.

Analyses were performed using SAS software, version 8 (SAS Institute, Cary, NC), SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL), and R version 2.10.1 (R Foundation for Statistical Computing, Vienna, Austria).

Human subjects research approval

The study protocol was approved by the Human Use Review and Regulatory Agency of the Office of the Army Surgeon General, the Institutional Review Board of the University of the Massachusetts Medical School, and the Ethical Review Board of the Ministry of Public Health, Thailand. Secondary data analysis was additionally approved by the Institutional Review Board of Emory University.

Results

A total of 1696 individuals experienced at least one dengue infection during the two cohort studies combined; 268 of these individuals experienced a second infection during their enrollment. Figure 2 illustrates the total number of infections detected during the active surveillance period each year, combining first and second infections (Figure 2a), and the numbers of second infections detected during active surveillance each year (Figure 2b). The proportion of all infections detected during active surveillance that were asymptomatic varied by year, from a minimum of 33.1% in 2006 to a maximum of 76.7% in 2000 ($p<0.01$ by $\chi 2$ testing across all strata of year). The proportion of second infections that were asymptomatic did not vary by year ($p>0.50$). First detected infections were more likely to be asymptomatic in KPS1 (1998-2002, 56.6%) than in KPS2 (2004-2007, 48.9%) ($p = 0.031$ by $\chi 2$ testing). Second detected infections were more likely to be asymptomatic than first detected infections for both KPS1 (76.2 % versus 56.6%, $p<0.01$ by $\chi 2$ testing) and KPS2 (58.6% versus 48.9%, $p=0.33$ by $\chi 2$ testing).

Time from first to second detected infection and clinical severity.

Second infections detected during active surveillance for all nine years of the studies consisted of a total of 89 asymptomatic infections, 27 cases of DF, and 7 cases of DHF. In unstratified analysis, there was no significant difference in time between infections by clinical severity (table 1). Stratifying on HI antibody profiles at enrollment, there was a significant trend observed among children that were HI-negative at enrollment: asymptomatic individuals had the shortest mean time between infections (1.41 years), then DF cases (1.92 years), and DHF cases had the longest mean time between infections (2.60 years) ($p = 0.010$). This trend was not observed among those with some DENV-HI immunity at enrollment

and there was no significant difference in time to infection by clinical severity for HI-monotypics and HI-multitypics.

HI antibody data were available for all children in the cohort, regardless of whether they had a DENV infection during their enrollment; plaque reduction neutralization titer (PRNT) antibody data were available only for a subset of children experiencing laboratory confirmed infections during the active surveillance period. PRNT were available for these children's serum samples collected prior to their infection (a pre-season sample). 47% of children who were negative by HI at enrollment had detectable antibodies by PRNT. Therefore, we evaluated whether the same association between time interval and disease risk observed for HI-negative children was observed after stratifying these individuals on the basis of PRNT positivity (Appendix 2). The association between a shorter interval between infections and asymptomatic infection appeared to hold for PRNT-positive children ($p<0.01$). This association was not significant for PRNT-negative children but the same trend was observed.

The probability of asymptomatic infection decreased each year for children that were HI-negative at enrollment (versus symptomatic infection, conditional on experiencing a second infection), decreasing from 79% one year post-first infection, to 38% two years post-infection, to 33% three years post-infection ($p = 0.042$ by $\chi 2$ testing across all strata of time, Figure 3). There was no significant change in the probability of asymptomatic infection over time in those children with some HI-immunity at enrollment; in fact, the probability increased slightly over time.

Predictors of the time to infection and the severity of the second-detected infection

Age at first detected infection was not significantly associated with the clinical severity of the second detected infection but younger children experienced a longer time interval between first and second detected infections ($p<0.01$, table 2). This is likely an artifact of the study design; there was an upper age limit for eligibility and older children would have had less time to experience a second detected infection during their enrollment. Enrollment HI profile was associated with clinical severity; children that were HI-negative at enrollment were most likely to be symptomatic with their second infection (43.6% symptomatic) and children with HI-monotypic profiles were the least likely to be symptomatic (10% symptomatic, $p = 0.020$ comparing across all three strata of immunity). Enrollment HI profile was not associated with time to second infection. The (inferred) serotype from the first detected infection and the (inferred) serotype for the second detected infection did not significantly influence the clinical severity of the second infection. The study phase was weakly associated with the probability of a symptomatic second infection; second infections were more likely to be symptomatic in KPS2 than KPS1 (41.4% versus 23.4%, $p = 0.058$). Lastly, the severity of the first infection was not associated with the severity of the second infection or time to infection.

Immunological correlates of infection severity.

The associations between each of the immunological factors considered and the occurrence of symptomatic or asymptomatic infection are illustrated in the figure in Appendix 3. None of these associations was significant in bivariate analysis.

Modeling the probability of asymptomatic infection over time

Appendix 4 provides details regarding the construction of the final models and relevant model output.

The logistic survival analysis evaluated the risk of symptomatic infection given that an individual was still at risk for infection (i.e., had not withdrawn or already been infected). For children that were HI-negative at enrollment, the time interval between the first and second DENV infection was associated with an increased risk of symptomatic infection, comparing years two, three, and four to year one post-infection (Figure 4a, Appendix 4). While there was a slight increase in the risk of asymptomatic and symptomatic infections (combined) in intervals two and three, there was a disproportionate increase in the risk of symptomatic infections. This suggests a true increase in severity, rather than an increase in the incidence of both symptomatic and asymptomatic infections and no proportional change in severity. The risk of symptomatic infections did not increase appreciably with time since first infection for children with some DENV-immunity by HI at enrollment (Figure 4b). At one year post-first infection, the odds of symptomatic infection in children who were HI-negative at enrollment were 5.0 times the odds in children with some HI-immunity. This increased to an OR of 7.36 two years post-infection, and an OR of 9.78 three years post-infection (Appendix 4).

For the model incorporating time interval and immunological factors, the summed rise in titers following the first infection was a significant predictor of symptomatic second infection, given that a second infection occurred (Figure 5, Appendix 4). An interaction term for time interval and enrollment DENV HI immunity was also significant. The highest

probability of symptomatic infection for every time point was observed in children lacking HI antibodies at enrollment and who demonstrated a low HI response to their first infection. Conversely, the lowest probability of symptomatic infection was observed in those with some HI antibodies at enrollment and who demonstrated a high HI response to their first infection.

Discussion

The strong association between the occurrence of DHF and secondary compared to primary DENV infection has been well characterized, whereas the factors involved in determining whether a secondary infection is asymptomatic, DF, or DHF have not. In this study, we used unique longitudinal data on the occurrence of asymptomatic, mild, and severe DENV infections in school-children in Thailand to evaluate whether the time interval between infections is associated with the clinical severity of subsequent DENV infections.

The time interval between infections was an important predictor of the severity of the second detected DENV infection in children that were HI antibody-negative at enrollment, with asymptomatic infections occurring at shorter time intervals (mean 1.61 years) and DF and DHF occurring at longer intervals (mean 1.81 and 2.14 years, respectively). The presence of this trend in HI-negative children is noteworthy because for some of these children, their second detected infection may reflect their true second DENV infection.

This study found evidence of both short-term (temporary cross-protection) and long-term effects of cross-reactive immunity. That is, while the effects of temporary cross-protection were evident for HI-negative children, no temporal trends in disease risk were observed in children with some DENV immunity, as measured by HI antibody prior to their first observed infection. This is consistent with suggestions that the cross-protection afforded by the accumulation of serotype-specific responses to multiple DENV infections can attenuate the clinical severity of a third or fourth infection (R. V. Gibbons, et al., 2007). These short-term and long-term effects may lend insight into the complicated dynamics of DENV

epidemics, as population-level shifts in cross-protection may underlie the observed, as yet unpredictable fluctuations in epidemic incidence and severity.

Controlling for time to infection, the rise in HI antibodies following the first infection was a significant predictor of experiencing a symptomatic infection, with higher antibody responses to the first infection being associated with asymptomatic second infections. This is consistent with a model of disease risk where antibodies decay over time; a higher peak in the response to infection may allow antibodies to persist longer at high titers and presumably increase the duration of cross-protection. It is interesting that time to second infection was significant in the multivariate model even after controlling for antibody titers. This suggests that time to infection itself may be associated with some shifts in disease risk, perhaps through qualitative shifts in antibody specificity

This is the first study since Sabin's 1952 paper to present evidence of a possible window of cross-protection following infection (Sabin, 1952), using data from a unique longitudinal study that spanned years and captured both symptomatic and asymptomatic infections. The presumed protective capability of high-titer cross-reactive antibodies is an important finding. As DENV vaccine development efforts intensify, there has been concern that some antibody responses to vaccination may be a reflection of cross-reactivity and that these antibodies may have unintended disease-enhancing effects. The finding that cross-reactive antibodies could also provide some protection against DENV illness is encouraging. However, this analysis also found that cross-protection appeared to decline over time; it may be that boosting by intermittent virus exposure (or vaccination) is critical to maintaining the high-titer responses necessary to effect a protective antibody profile. Future studies should

investigate the importance of natural boosting in contributing to individual cross-protection and population-level herd immunity. This is an important question in that DENV vaccines, if effective, would serve to decrease DENV transmission and therefore the frequency of boosting.

We would expect a vaccination program to on average lengthen the interval between sequential infections for those that are not immunized due to reducing the force of infection. Our findings indicate that some vaccine programs might increase the proportion of unvaccinated individuals experiencing symptomatic infections due to lengthening the interval between sequential infections. Vaccine trials should consider the occurrence of this phenomenon in their evaluations of vaccine candidates and programs.

There were several limitations to this analysis. The first is the relatively limited timescale of the studies, from 1998-2002 (five years) and 2004-2007 (four years). There is a great deal of variability in the incidence of DENV infection; large epidemics have historically occurred in Thailand approximately every three to five years (Nisalak, et al., 2003). The clinical severity of DENV epidemics, as defined by the proportion of infections that are symptomatic, also varies from year to year (T. Endy, et al., 2010) and there is evidence of longer-term shifts in the dominance of different serotypes (Zhang, et al., 2005). Year of infection may have been an important confounder in these data; we attempted to control for these annual peaks and valleys in disease occurrence by incorporating study period into the models but it is possible that there was residual confounding. Last, these findings may not be translatable to other regions where transmission rates are not as high as in Thailand and where this temporary cross-protection may not be a significant aspect of the epidemiology of DENV.

It is a unique feature of these cohort studies that asymptomatic infections were detected; however, there are important gaps in the information available for these cases. Since the cases did not present with an acute illness, acute blood specimens were not collected and it was not possible to identify the infecting serotype with certainty. Attempts were made to impute the infecting serotype for asymptomatic infections, based upon what serotypes were detected among RT-PCR positive cases at their school during the year of their infection. It is possible that the serotype distribution among acute, PCR-positive cases may not be representative of the distribution of serotypes among the asymptomatically-infected individuals. Second, since asymptomatic infections were identified based upon seroconversion between two routine blood specimens, it was not possible to determine precisely when cases were infected; only that they seroconverted within a window of time. The timescale for this analysis was therefore rather crude, permitting the evaluation of only one-year intervals between infections. Given that Sabin estimated the "window of protection" to be six months following infection (Sabin, 1952), it is possible that there were larger effects of cross-protection in the months following infection that were missed.

The use of HI antibody data is both a strength and a limitation of this study. While plaque reduction neutralization titer (PRNT) data are considered the best method for characterizing protective immunity with DENV infection, these are very labor-intensive and it was therefore not possible to obtain longitudinal PRNTs for all routine specimens for all children. Approximately 50% of children who were HI-negative at enrollment were DENV seropositive by PRNT. This suggests that use of HI antibody data to infer DENV infection histories in these children resulted in the misclassification of some children with a history of

DENV infection as DENV-naïve. On the other hand, the availability of these complete, extensive HI data for all children in the cohort provided a unique opportunity to evaluate long-term trends in the antibody response to infection. Further, while HI antibodies are not specific to neutralizing antibodies (as with PRNT) but rather measure all varieties of antibodies, it may be that non-neutralizing antibodies are also important in protection from illness and the pathophysiology of disease.

These analyses of temporal trends in DENV disease severity are strengthened by the longitudinal nature of the cohort studies. It is a unique strength of these studies to have pre-infection antibody data available for all four DENV serotypes. A second strength of these longitudinal studies is that they captured the entire range of clinical severities with DENV infection. The inclusion of asymptomatic and non-hospitalized DENV infections, in addition to severe hospitalized cases, provided a unique opportunity to assess factors associated with protection from illness.

The characterization of individuals as protected from DENV illness or at risk of enhanced disease with DENV infection remains an unsolved problem and one that looms large as DENV vaccines approach licensure and implementation. In this analysis we provide evidence that for secondary DENV infections, individuals are more likely to be asymptomatically infected with a shorter interval between infections and with a more robust antibody response to the previous infection. This study suggests that there may exist a temporary period of cross-protection following DENV infection, the first time this has been demonstrated using data on natural human infections.

References.

Burke, D. S., Nisalak, A., Johnson, D. E., & Scott, R. M. (1988). A prospective study of dengue infections in Bangkok. *Am J Trop Med Hyg, 38*(1), 172-180.

Clarke, D. H., & Casals, J. (1958). Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am J Trop Med Hyg, 7*, 561-573.

Endy, T., Anderson, K., Nisalak, A., Yoon, I., Green, S., Rothman, A., et al. (2010). Determinants of Inapparent and Symptomatic Dengue Infection in a Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *Manuscript submitted for publication.*

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

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Gibbons, R. V., Kalanarooj, S., Jarman, R. G., Nisalak, A., Vaughn, D. W., Endy, T. P., et al. (2007). Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences. *Am J Trop Med Hyg, 77*(5), 910-913.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever: Its history and resurgence as a global public health problem. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 1-22). New York: CAB International.

Gubler, D. J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol, 10*(2), 100-103.

Guzman, M. G., Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Montes, T., Vazquez, S., et al. (2007). Neutralizing antibodies after infection with dengue 1 virus. *Emerg Infect Dis, 13*(2), 282-286.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazquez, S., et al. (2000). Epidemiologic studies on dengue in Santiago de Cuba, 1997. *Am J Epidemiol, 152*(9), 793-799.

Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S., & Morier, L. (1990). Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg, 42*, 179-184.

Halstead, S. B. (1980). Dengue haemorrhagic fever-a public health problem and a field for research. *Bull World Health Organ, 58*(1), 1-21.

Halstead, S. B. (1980). Immunological Parameters Of Togavirus Disease Syndromes. *The Togaviruses*, 107-173.

Halstead, S. B. (1989). Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev Infect Dis, 11*(Suppl 4), S830-S839.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., Nimmannitya, S., & Cohen, S. N. (1970). Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med, 42*, 311-328.

Hammon, W. M., Rudnick, A., & Sather, G. E. (1960). Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. *Science, 131*, 1102-1103.

Jarman, R. G., Holmes, E. C., Rodpradit, P., Klungthong, C., Gibbons, R. V., Nisalak, A., et al. (2008). Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. *J Virol, 82*(11), 5494-5500.

Kou, Z., Quinn, M., Chen, H., Rodrigo, W., Rose, R., Schlesinger, J., et al. (2008). Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells.

. *Journal of Medical Virology, 80*(1), 134-136.

Kouri, G., Guzman, M. G., Valdes, L., Carbonel, I., del Rosario, D., Vazquez, S., et al. (1998). Reemergence of dengue in Cuba: a 1997 epidemic in Santiago de Cuba. *Emerg Infect Dis, 4*(1), 89-92.

Kouri, G., Mas, P., Guzman, M. G., Soler, M., Goyenechea, A., & Morier, L. (1983). Dengue hemorrhagic fever in Cuba, 1981: rapid diagnosis of the etiologic agent. *Bull Pan Am Health Organ, 17*, 126-132.

Mammen, M. P., Pimgate, C., Koenraadt, C. J., Rothman, A. L., Aldstadt, J., Nisalak, A., et al. (2008). Spatial and temporal clustering of dengue virus transmission in Thai villages. *PLoS Med, 5*(11), e205.

Nimmannitya, S. (1997). Dengue Hemorrhagic Fever: Diagnosis and Management. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 133-145). Cambridge: CAB International.

Nisalak, A., Endy, T. P., Nimmannitya, S., Kalayanarooj, S., Thisayakorn, U., Scott, R. M., et al. (2003). Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am J Trop Med Hyg, 68*(2), 191-202.

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., et al. (1984). Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol, 120*, 653-669.

Thein, S., Aung, M. M., Shwe, T. N., Aye, M., Zaw, A., Aye, K., et al. (1997). Risk factors in dengue shock syndrome. *Am J Trop Med Hyg, 56*(5), 566-572.

U.S. Department of Health and Human Services, N. I. o. H., National Institute of Allergy and Infectious Disease. NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report.

Zhang, C., Mammen, M. P., Jr., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Monkongdee, P., et al. (2005). Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. *J Virol, 79*(24), 15123-15130.

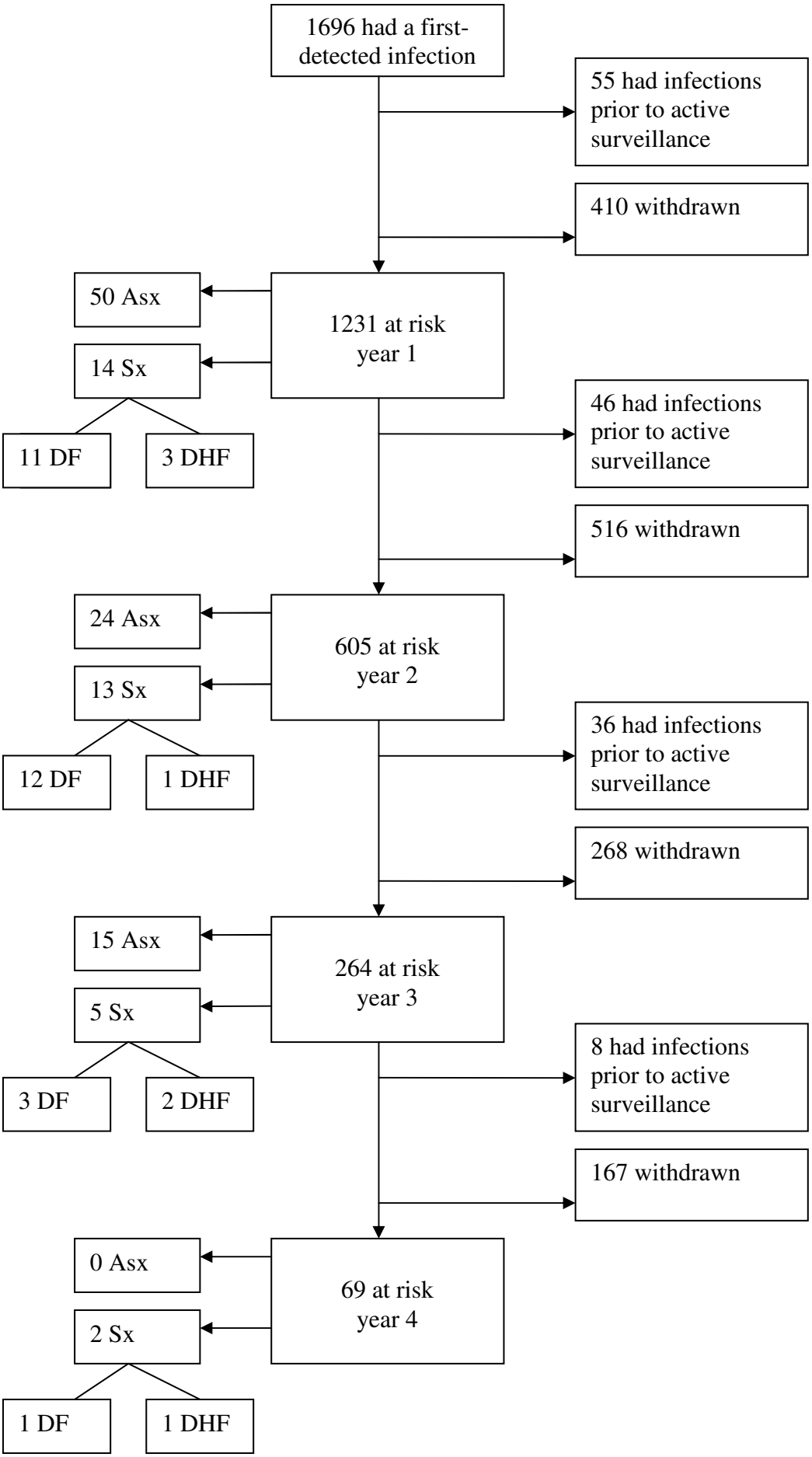
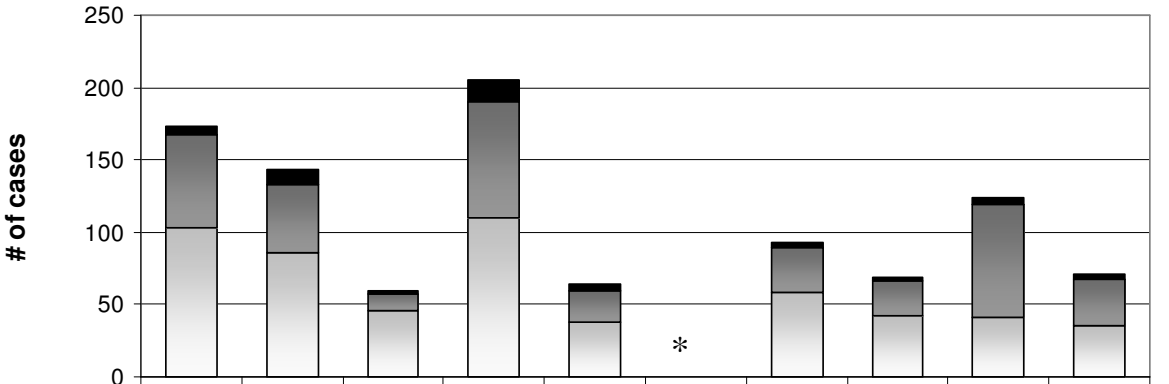

1696 had a first-detected infection
55 had infections prior to active surveillance
410 withdrawn
50 Asx
14 Sx
1231 at risk year 1
11 DF
3 DHF
46 had infections prior to active surveillance
516 withdrawn
24 Asx
13 Sx
605 at risk year 2
12 DF
1 DHF
36 had infections prior to active surveillance
268 withdrawn
15 Asx
5 Sx
264 at risk year 3
3 DF
2 DHF
8 had infections prior to active surveillance
167 withdrawn
0 Asx
2 Sx
69 at risk year 4
1 DF
1 DHF

Figure 1. Flowchart of the total starting population for this study (children that experienced at least one DENV infection), detailing the numbers remaining at risk for a second infection each year, the numbers of cases, and their severities.

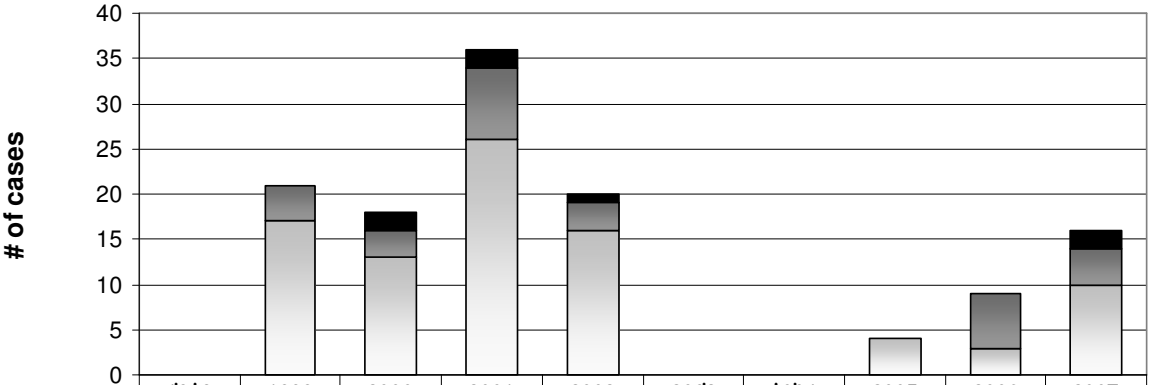
Figure 2a. Total number of infections, by year



	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
% Asx	59.5%	60.1%	76.7%	53.7%	59.4%		63.4%	62.3%	33.1%	49.3%
N	173	143	60	205	64		93	69	124	71
DHF	6	10	3	15	4		4	2	5	3
DF	64	47	11	80	22		30	24	78	33
Asx	103	86	46	110	38		59	43	41	35

Year

Figure 2b. Second-detected infections, by year



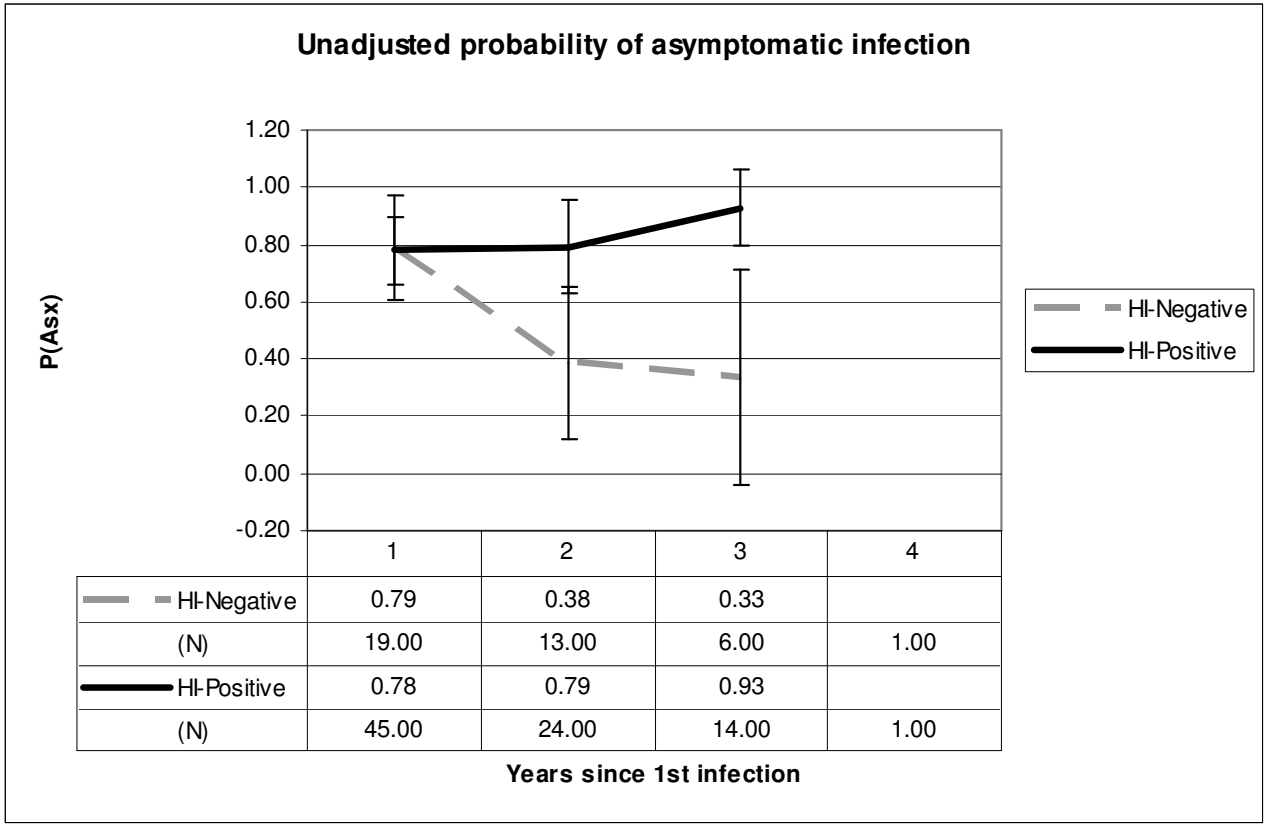
	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
% Asx		81.0%	72.2%	72.2%	80.0%			100.0%	33.3%	62.5%
N		21	18	36	20			4	9	16
DHF		0	2	2	1			0	0	2
DF		4	3	8	3			0	6	4
Asx		17	13	26	16			4	3	10

Year

* The cohort study was not conducted during 2003.

** It was not possible to have two infections detected during the same epidemic year in the same individual, therefore the first years that children were 'eligible' to have a second infection were 1999 and 2005.

Figure 2. Total numbers of infections, and the severity of infections, detected each year of the cohort studies. Severity is categorized as asymptomatic (Asx), dengue fever (DF), and dengue hemorrhagic fever (DHF). Cases are limited to those detected during the active surveillance period (June 1 – November 1) each year. Figure 2a shows the total number of cases detected (combining first and second infections), Figure 2b shows the total number of second infections detected each year.



	1	2	3	4
HI-Negative	0.79	0.38	0.33	
(N)	19.00	13.00	6.00	1.00
HI-Positive	0.78	0.79	0.93	
(N)	45.00	24.00	14.00	1.00

Figure 3. Probability of asymptomatic infection by year since the first-detected infection in the cohort studies, by whether a child had detectable HI antibodies at enrollment (HI-positive) or was negative by HI at enrollment (HI-negative). Error bars indicate the 95% confidence intervals for the proportions.

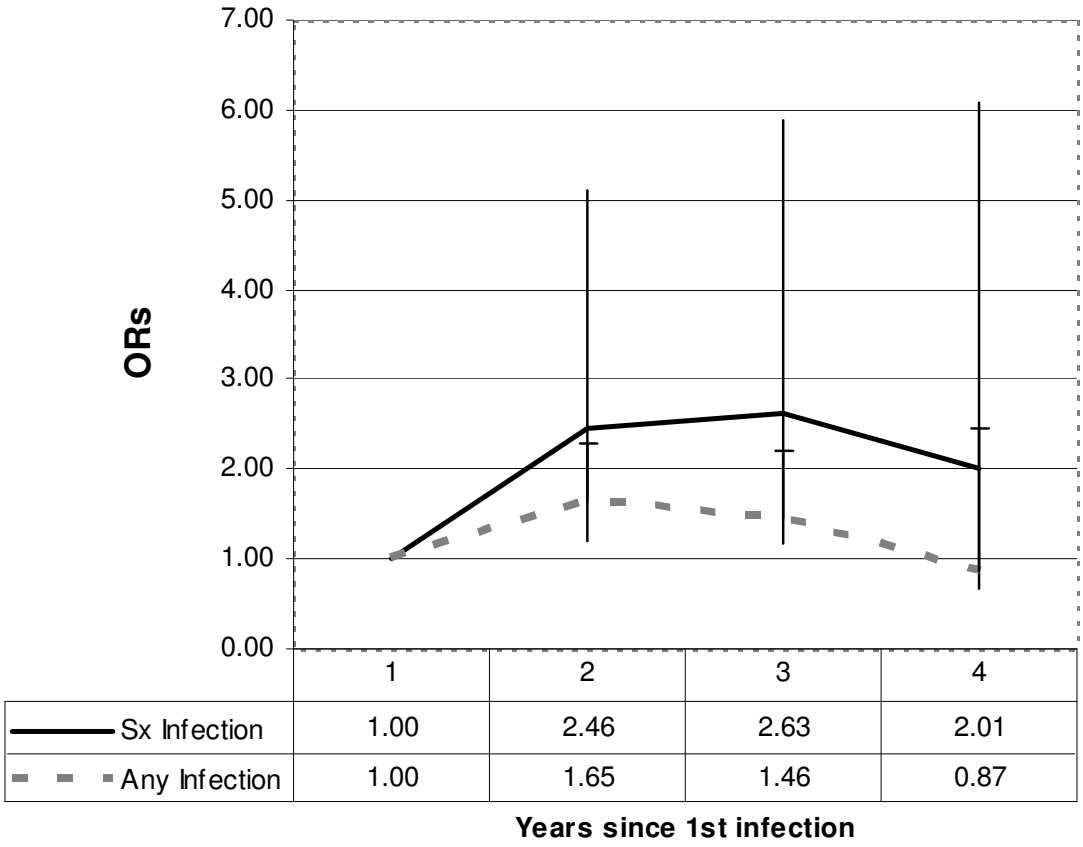


Figure 4. Odds ratios (ORs) for the risk of symptomatic infection in HI-negatives given that a child was still at risk for a second infection during an interval. ORs provide the odds of symptomatic infection for a given interval as compared to year one. Upper and lower 95% confidence intervals (CIs) are shown for the odds of symptomatic infection; for the odds of any infections, only upper 95% CIs are shown as a horizontal line. Estimates are derived from output from logistic survival models controlling for enrollment HI profile, time interval since infection, their interaction, and conditioning on sub-district of residence, age at first infection, and study phase of infection (KPS1 or KPS2)

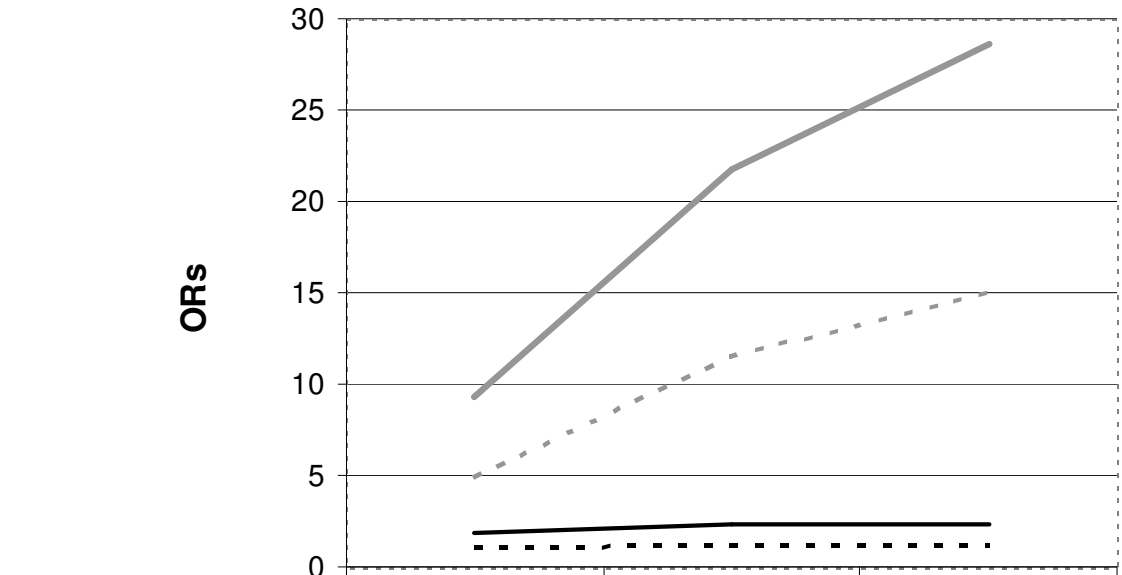


Figure 5. Odds ratios (ORs) for experiencing a symptomatic versus an asymptomatic second infection, given that a second infection occurred. Results are stratified by HI antibody profile at enrollment (HI positive [Pos] or HI Negative [Neg]) and whether the total HI antibody response to the first infection was higher than the mean (High rise) or lower than the mean (Low rise). ORs compare the odds of symptomatic infection for years two and three in each stratum to the odds of symptomatic infection in year one for the reference stratum (children who were HI-positive and demonstrated a high antibody response to infection).

Appendix 1. Time-course of HII titers, from 1st infection to 2nd infection (6 examples from individuals).

KPS1: 4 blood draws per year (January, June, mid-August, November)

Note:

- "pre" refers to the HI draw prior to the 1st infection.
- "int" refers to the surveillance interval during which a child was infected:
  - 1: January – May
  - 2: June – mid-August
  - 3: mid-August – October
  - 4: November - December

ID	yr_1st(int)	yr_2nd(int)	Dx	Time_to_2nd	Date_Ill(2nd)
1971	1999 (4)	2001(2)	DF	2 yrs	20-JUN-2001

Dates of routine specimen collection:

	Pre	11/99	1/00	6/00	8/00	11/00	1/01	6/01	8/01	11/01
D1	10	80	40	20	20	20	20	10	20	160
D2	10	80	40	20	20	20	20	10	10	160.
D3	10	80	40	20	20	20	20	20	20	320.
D4	10	320	80	80	80	80	40	20	40	640.

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 734

Dates of routine specimen collection:

ID	yr_1st(int)	yr_2nd(int)	Dx	Time_to_2nd	Date_Ill(2nd)
1975	1998 (4)	2001(2)	DHF	3 yrs	04-JUL-2001

	Pre	1/99	6/99	8/99	11/99	1/00	6/00	8/00	11/00	1/01	6/01	8/01
D1	10	80	40	40	20	20	20	20	20	40	20	640
D2	10	80	40	40	20	20	20	20	10	10	10	320
D3	10	160	80	80	40	40	40	40	40	80	40	1280
D4	10	320	160	80	80	80	80	80	40	40	160	640

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 924

3566	2001 (2)	2002 (2)	Asx	1 yr	N/A

Dates of routine specimen collection:

	Pre	8/01	11/01	1/02	6/02	8/02
D1	20	80	40	40	80	640
D2	10	20	20	20	20	320
D3	20	40	40	40	40	1280
D4	40	40	40	40	80	640

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 366

KPS2: 2 blood draws per year (January and June)

Note:

- "pre" refers to the HI draw prior to the 1st infection.
- "int" refers to the surveillance interval during which a child was infected:
  - 1: January – May
  - 2: June – December

ID	yr_1st(int)	yr_2nd(int)	Dx	Time_to_2nd	Date_Ill(2nd)
5516	2004 (2)	2007(2)	DF	3 yrs	22-JUN-2007

Dates of routine specimen collection:

	Pre	1/05	6/05	1/06	6/06	1/07	6/07	1/08
D1	10	10	10	10	10	10	10	160
D2	10	40	40	20	40	20	20	80
D3	10	10	10	10	10	10	10	160
D4	10	10	20	10	10	10	10	320

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 1087

ID	yr_1st(int)	yr_2nd(int)	Dx	Time_to_2nd	Date_Ill(2nd)
6088	2006 (2)	2007(2)	Asx	1 yrs	N/A

Dates of routine specimen collection:

	Pre	1/06	6/06	1/07
D1	10	20	10	40
D2	10	10	10	20
D3	10	20	20	40
D4	10	80	20	80

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 364

ID	yr_1st(int)	yr_2nd(int)	Dx	Time_to_2nd	Date_Ill(2nd)
7056	2004 (2)	2005 (2)	Asx	1 yr	N/A

Dates of routine specimen collection:

	Pre	1/04	6/04	1/05	6/05

D1	10	10	10	20	20
D2	10	10	10	10	10
D3	10	20	20	80	80
D4	10	20	10	40	40

Days between 1st and 2nd (based upon days blood draw post-1st and pre-2nd): 357

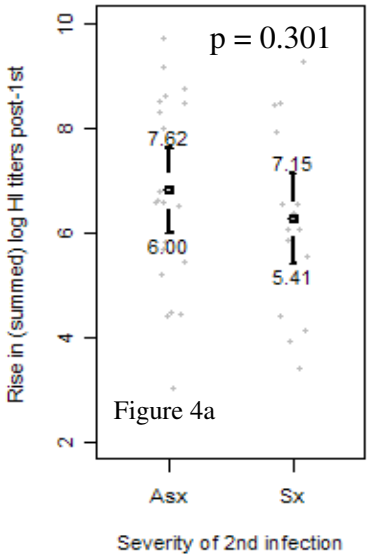
Appendix 2. Time to infection – restricting to HI-negatives

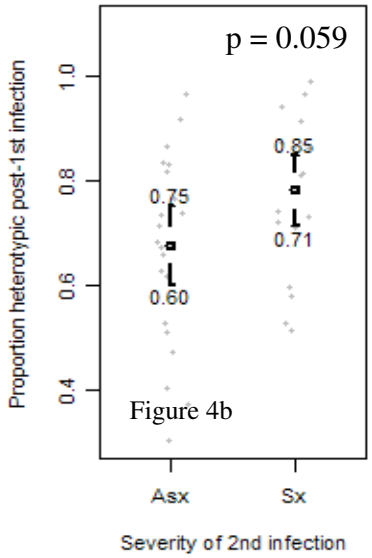
2nd-detected Infection	N	Mean time to 2nd infection (SD)	Median time to 2nd infection (range)	P*
PRNT-negative				
Asymptomatic	5	1.8 (0.837)	2 (1 – 3)	0.121
DF	4	1.75 (0.5)	2 (1 – 2)	
DHF	2	3.5 (0.707)	3.5 (3 – 4)	
PRNT-positive				
Asymptomatic	12	1.25	1 (1 – 3)	0.004
DF	4	2.5 (0.577)	2.5 (2 – 3)	
DHF	1	2 *	2 *	

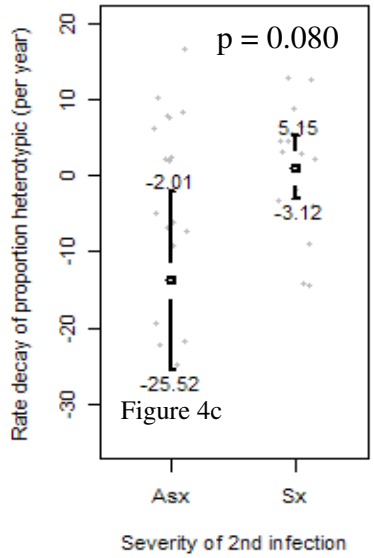
* p-values were obtained using exact non parametric Wilcoxon tests with SAS' npar1way procedure.

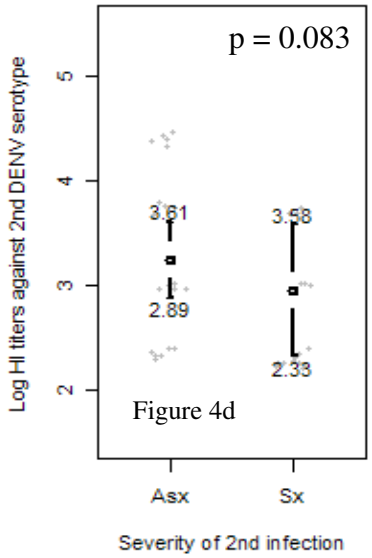
Appendix 3.

HI-Negative on enrollment

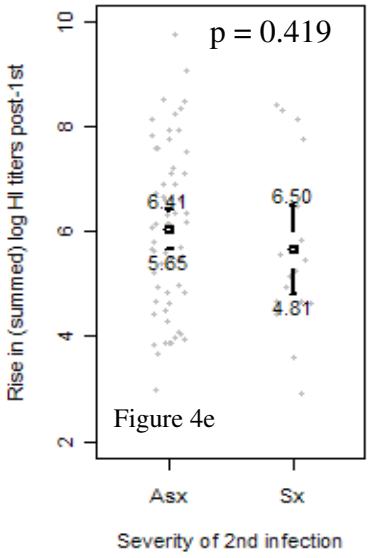


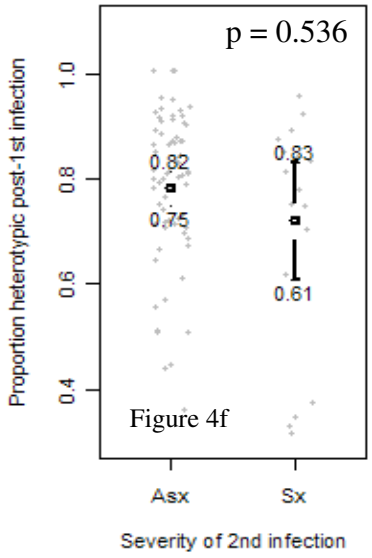


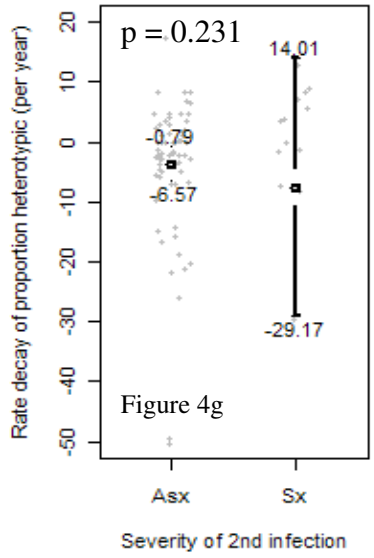


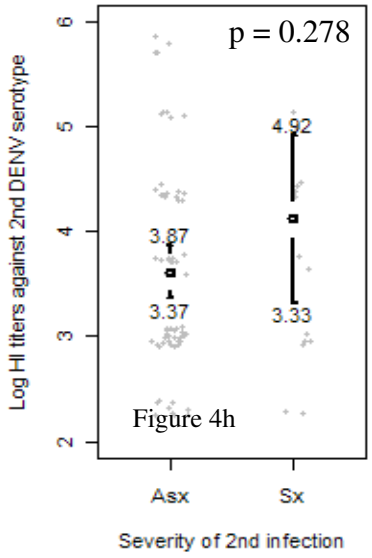


HI-Positive on enrollment









Appendix 3. Associations of immune parameters with clinical severity of 2nd-detected DENV infections, stratified by whether a child was hemagglutination inhibition (HI) antibody positive or HI-negative for DENV serotypes on enrollment. The four immunological factors considered were: (1) the rise in HI titers following the first infection, summed across serotypes (HI negatives: Figure 4a; HI positives: Figure 4e), (2) the proportion of the total (summed) rise in HI titers that was directed against serotypes other than the infecting serotype (the heterotypic response) for the first infection, (HI negatives: Figure 4b; HI positives: Figure 4f), (3) the annual percentage change in the proportion of the summed antibody response that was heterotypic, from the routine blood draw collected after the first infection to just prior to the second infection (HI negatives: Figure 4c; HI positives: Figure 4g), and (4) the preexisting antibody titer against the second infecting serotype, in the specimen collected prior to the second infection (HI negatives: Figure 4d; HI positives: Figure 4h).

Appendix 4. Multivariate logistic model output.

Method 1 – Logistic survival analyses:

Model 1a. Risk of symptomatic infection. *

Parameter	Coding	Coefficient	Standard Error	p-value **
Outcome: Symptomatic infection	1 = symptomatically infected (n = 34) 0 = not symptomatically infected and still at risk for a second infection (n = 1951)	---	---	---
Enrollment antibody profile	1 = HI-positive 0 = HI-negative	1.434	0.497	0.019
Two years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn, already infected, asymptomatically infected that interval, or infected outside of the active surveillance period	0.388	0.238	0.139
Three years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn, already infected, asymptomatically infected that interval, or infected outside of the active surveillance period	0.329	0.329	0.361
Four years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn, already infected, asymptomatically infected that interval, or infected outside of the active surveillance period	0.893	0.382	0.076
Immunity*(Year 2)	Interaction term for two years post-1st infection and immunity	0.485	0.241	0.074
Immunity*(Year 3)	Interaction term for three years post-1st infection and immunity	0.614	0.320	0.087
Immunity*(Year 4)	Interaction term for four years post-1st infection and immunity	0.356	0.387	0.422

* Conditioned on sub-district of residence, age at first infection, and study period of enrollment (KPS1 or KPS2).

** Exact p-values were calculated.

Model 1b. Risk of symptomatic or asymptomatic infection. *

Parameter	Coding	Coefficient	Standard Error	p-value **
Outcome: Infection	1 = infected (n = 213) 0 = not infected and still at risk for a second infection (n = 1951)	---	---	---
Enrollment antibody profile	1 = HI-positive 0 = HI-negative	0.666	0.390	0.088
Two years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn or already infected	0.283	0.097	0.003
Three years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn or already infected	0.123	0.127	0.333
Four years post-1st infection	1 = At risk two years post-1st infection 0 = Withdrawn or already infected	-0.572	0.369	0.122
Immunity*(Year 2)	Interaction term for two years post-1st infection and immunity	0.217	0.097	0.025
Immunity*(Year 3)	Interaction term for three years post-1st infection and immunity	0.257	0.125	0.040
Immunity*(Year 4)	Interaction term for four years post-1st infection and immunity	0.430	0.368	0.245

* Conditioned on sub-district of residence, age at first infection, and study period of enrollment (KPS1 or KPS2)

** Exact p-values were calculated

Method 2 – Logistic regression conditional on experiencing a 2nd DENV infection *

Parameter	Coding	Coefficient	Standard Error	p-value **
Outcome: Symptomatic Infection	1 = Symptomatic infection in active surveillance window (n = 34) 0 = Asymptomatic infection in active surveillance window (n = 89)	---	---	---
Enrollment antibody profile	1 = HI-negative 0 = HI-negative	1.593	0.467	<0.001
Two years post-1st infection †	1 = Infected in year two 0 = Infected in year one or three	0.193	0.305	0.379
Three years post-1st infection	1 = Infected in year three 0 = Infected in year one or two	0.195	0.391	0.734
Some_imm*Time2	Interaction term for two years post-1st infection and immunity	0.651	0.317	0.065
Some_imm*Time3	Interaction term for three years post-1st infection and immunity	0.921	0.385	0.011
Rise in summed HI titers following the 1st infection	1 = Less than or equal to the mean 0 = Greater than the mean	0.643	0.317	0.040

* Conditioned on sub-district of residence and study period of enrollment (KPS1 or KPS2)

** Exact p-values were calculated

† Year four was not assessed in this model since no asymptomatic infections occurred four years after the first.

STUDY THREE

DISCLAIMER: AT THE TIME OF THESIS PUBLICATION, THIS MANUSCRIPT WAS UNDER PREPARATION FOR SUBMISSION TO A SCIENTIFIC JOURNAL FOR REVIEW. WHERE POSSIBLE, THE READER IS ENCOURAGED TO READ THE FINAL, PUBLISHED MANUSCRIPT:

Anderson KB, Gibbons RV, Edelman R, Eckels KH, Putnak RJ, Innis BL, Sun W. Interference and facilitation between dengue serotypes in a tetravalent live dengue virus vaccine candidate. J Infect Dis. 2011 Aug 1;204(3):442-50.

STUDY THREE

Title: Immunological interference and facilitation between dengue serotypes in tetravalent formulations

Abstract

Background: Live, multivalent vaccines have historically exhibited interference in humans; dengue (DENV) vaccines have proven no exception.

Methods: To characterize immunological interactions between DENV serotypes in tetravalent formulations, we analyzed data from a factorial clinical trial that evaluated all combinations of high and low dose DENV serotypes (16 formulations, n=64). Logistic and linear regression models controlled for all serotype doses simultaneously. Additionally, results were compared against monovalent formulations.

Results: DENV-1 was strongly dominant in both monovalent and tetravalent formulations. For DENV-2 and DENV-4, the shift to tetravalent formulations significantly decreased seroconversion. In tetravalent formulations, DENV-1 and DENV-2 appeared to antagonize each other, with a high dose of one decreasing seroconversion to the other. However, high dose DENV-1 significantly increased the odds that a vaccine elicited seroconversion against three or more serotypes, increasing seroconversion to DENV-1, DENV-3, and DENV-4. The greatest reactogenicity occurred when DENV-1 was at high dose and all others were low; reactogenicity decreased with the incorporation of other high-dose serotypes.

Conclusions: Both interference and facilitation occur between DENV serotypes in tetravalent vaccines. These analyses suggest that it may be possible to exploit facilitation to increase overall seroconversion and that increasing the dose of subdominant serotypes may decrease reactogenicity.

Introduction.

There is a recognized need for safe and effective dengue vaccines (Almond, et al., 2002; DeRoeck, et al., 2003) but development efforts have been hampered by unique aspects of dengue epidemiology and biology. The co-circulation of serotypes in DENV-endemic regions, and the ability of all four dengue serotypes to cause disease in humans, suggests that an effective vaccine should confer immunity to multiple serotypes (Halstead, 1997). A second, perhaps more critical, rationale for tetravalent vaccination arises from the phenomenon of antibody-dependent enhancement (ADE) (Halstead & Simasathien, 1970). In ADE, preexisting immunity from a DENV infection is thought to facilitate infection with a second, heterologous dengue serotype, resulting in higher viral loads, an intensified immune response, and ultimately the plasma leakage characteristic of dengue hemorrhagic fever (DHF) (Halstead, Chow, & Marchette, 1973). There is concern that a dengue vaccine that would confer immunity to a single dengue serotype, or suboptimal immunity to multiple serotypes, could prime an individual for DHF upon natural exposure to DENVs (Stephenson, 2005).

Historically, the generation of a balanced immune response with multitypic viral vaccines has been a challenging task. Suboptimal seroconversion rates were reported for combination yellow fever / vaccinia vaccines (Dick & Horgan, 1952), yellow fever / vaccinia / measles vaccines (Meyer, Hopps, Bernheim, & Douglas, 1967), the measles-mumps-rubella (MMR) vaccine (Berger, Just, & Glück, 1998 Oct), and the MMR vaccine in combination with vaccinia (Berger & Just, 1988 Oct). In the case of MMR, dose adjustments were initially necessary to increase seroconversion to the subdominant measles and mumps components; dose adjustments were again necessary with the concurrent administration of the varicella vaccine.

The significant challenges in obtaining a balanced immune response with the trivalent oral polio vaccine (OPV) may be most analogous to DENV, in that the OPV contains three different serotypes of live polio virus. It was found that seroconversion rates which exceeded 90% for monovalent polio vaccines were reduced to varying degrees (68% for type 1, 82% for type 2, and 43% for type 3) in trivalent formulations (Payne, 1960). Decreasing the dose of the type 2 virus, which consistently dominated in viremia, seroconversion, and geometric mean titer (GMTs), tended to increase seroconversion to the subdominant serotypes. However, seroconversion to type 3 remained unacceptably low and the imbalance in seroconversion was finally overcome with three doses of vaccine (Patriarca, et al., 1991).

The development of safe and immunogenic tetravalent dengue vaccines has proven no less challenging. The four live-attenuated vaccine strains developed by Aventis (now Sanofi) Pasteur and Mahidol University elicited high rates of seroconversion and high neutralization titers when administered as mono-, bi-, and trivalent formulations (Bhamarapravati & Yoksan, 2000). Notably, none of the bi- and trivalent formulations contained DENV-3, which was later shown to dominate the antibody response in tetravalent formulations (Kanesa-thasan, et al., 2001). DENV-3 also dominated in causing viremia post-tetravalent vaccination, suggesting that inter-serotypic competition was occurring and that DENV-3 had a replicative advantage (Sabchareon, et al., 2002). As OPV, it was concluded that multiple doses of vaccine were necessary to achieve seroconversion to three or more DENV serotypes. Evaluation of these tetravalent formulations was terminated after several adult volunteers developed mild dengue syndrome post-vaccination, the cause of which was hypothesized to be the under-attenuated DENV-3 component (Kitchener, et al., 2006).

Similar observations of interference in the serological response to vaccination were made for ChimeriVax DENV vaccines, live vaccines that use the yellow fever 17D virus strain backbone with the envelope and premembrane genes of DENVs inserted (F. Guirakhoo, et al., 2002). Monovalent Chimerivax DENV vaccines had higher seroconversion rates and GMTs than when the strains were combined in a tetravalent formulation; ultimately, three doses were required to drive seroconversion rates for subdominant strains above 80% (Morrison, et al., 2010). A recent study examined interference between the four Chimerivax DENV vaccines in a primate model and concluded that decreasing the dose of the dominant serotype (in this case, DENV-4) resulted in increased seroconversion to other serotypes (Guy, et al., 2009).

Live tetravalent dengue vaccines were also developed by GlaxoSmithKline and the Walter Reed Army Institute of Research (WRAIR) and are the focus of this analysis. Four live-attenuated monovalent candidates were found to be safe and acceptably immunogenic (Sun, et al., 2003). These were later combined in a factorial trial of sixteen different tetravalent vaccine formulations which evaluated all possible combinations of high- and low-dose for each serotype (Edelman, et al., 2003). In the present study, we evaluate whether there is evidence of interactions between serotypes with respect to the serological response to vaccination and the occurrence of adverse events. An improved understanding of whether and how DENV serotypes interact to influence the immune response to vaccination could inform the development of safe and immunogenic tetravalent vaccines.

Methods

Data sources: Monovalent and tetravalent clinical trials

The four DENV serotype strains were isolated from clinically-ill patients and attenuated by passaging multiple times in primary dog kidney (PDK) cells and fetal rhesus lung (FRhL) cells (Sun, et al., 2003). The DENV-1 – DENV-4 candidate strains were initially evaluated as monovalent formulations in 54 flavivirus-naïve adults in the United States (Table 1). The methods of this clinical trial have been detailed previously (Sun, et al., 2003). Freeze-dried monovalent vaccines were reconstituted in sterile water and administered subcutaneously in doses of 10^6, 10^6, 10^5, and 10^5 plaque forming units (PFUs) for the four DENV serotypes, respectively. Multiple dosing schedules were evaluated in the clinical trial; this analysis considers only the response to the first dose of vaccine. Adverse reactions to vaccination were assessed by symptom diaries which were recorded daily for three weeks post-vaccination.

The four monovalent candidate vaccines were later combined into 16 different tetravalent formulations, representing all possible combinations of high and low dose for each monovalent vaccine strain. The 'high doses' for DENV-1, DENV-2, DENV-3, and DENV-4 corresponded to the doses administered as a monovalent formulation (10^6, 10^6, 10^5, and 10^5, respectively). The 'low dose' for each serotype was 1.5 log PFUs lower than the high dose for each serotype. The details of this factorial-design clinical trial have been described previously (Edelman, et al., 2003). Fifteen formulations were administered to groups of 3-4 flavivirus-naïve adults in the United States; one formulation (with all four serotypes administered at high doses) was administered to 10 individuals. Formulations are denoted by 'L' or 'H' for the dose of each serotype in order from DENV-1 through DENV-4 (e.g., formulation LHHL corresponds to low dose DENV-1, high DENV-2, high DENV-3, low DENV-4). Adverse reactions to vaccination were assessed by a symptom diary

which volunteers completed daily. The diary was maintained for three weeks after each vaccination for formulations 1-15 and for two weeks after vaccination with formulation 16.

Quantification of Immunogenicity - Serological Methods

The neutralizing antibody response to vaccination was assessed by measuring the maximum dilution of sera that yielded a 50% reduction in DENV plaque formation (plaque reduction neutralization titer 50%, $PRNT_{50}$). The assay was conducted using standard procedures described by Russell et al (P. K. Russell, et al., 1967) and the DENV strains used in the PRNT were the parent strains of the vaccine viruses. The $PRNT_{50}$ for all four serotypes were assessed on day 30 for monovalent candidates and day 28 post-vaccination for tetravalent candidates. A $PRNT_{50}$ greater than 1:10 was defined as a seroconversion. Notably, complement was added to the neutralization mixture when assessing the response to monovalent vaccination which was later omitted when assessing the tetravalent formulations. Complement has been shown to increase $PRNT_{50}$ titers, in a manner and to a degree that varies by DENV serotype, and therefore antibody titers may not be strictly comparable between the monovalent and tetravalent vaccine recipients (Thomas, et al., 2009).

Quantification of Reactogenicity

Volunteers were instructed to keep a daily symptom diary for three weeks following vaccination. Symptom severity was graded as 0 (no disability), 1 (mild – no medication or alteration of daily activity required), 2 (moderate – required medication or changing of activity), or 3 (severe – required bed rest or symptoms not relieved by medication). The occurrence, severity, and duration of adverse reactions to vaccination each day were summarized using the reactogenicity index (RI), a standard metric for assessing vaccine safety at WRAIR which was calculated as follows:

$$RI = \sum_{day\,1}^{day\,21} (Fever\ or\ Chills) + \left(Nausea\ or\ Vomiting\ or\ Abdominal\ Pain\right) + \left(Headache\ or\ Eye\ Pain\right) + \left(Myalgia\ or\ Arthralgia\right)$$

For each day, the maximum severity was selected from the symptoms in each category (e.g., for a given day, if a volunteer listed a grade 2 headache and grade 1 eye pain, the value for the category [Headache or Eye Pain] for that day would be 2). The severity of adverse events for each category were summed for each day, and the total severity for days 1–21 post-vaccination were summed to obtain the RI. Notably, the symptom data elicited in the diaries and the duration of reporting differed between formulations 1-15 and formulation 16, as the clinical trials occurred at different times and with slightly different methodologies. As the RIs for these two sets may not be comparable, the RI data for formulation 16 are excluded from analysis.

Statistical Analyses.

The effect of changing the dose of a serotype from low to high was evaluated with respect to the vaccine-induced immune response to that serotype and other serotypes. In bivariate analysis, t-tests were used to assess whether the geometric mean titer (GMT) of the antibody response to a given serotype was significantly different by whether each of the four serotypes were high or low dose. Pearson chi-square tests evaluated whether the proportion of individuals seroconverting to each serotype was significantly different by high or low serotype dose.

Four multivariate logistic regression models considered the associations of low and high dose for each serotype with the outcomes of seroconversion to DENV1-4. A logistic regression model considered the associations of low and high dose for each serotype with the outcome of seroconversion to three or more versus less than three serotypes. Lastly, a multivariate linear regression model considered the associations of low and high dose and the RI. For all multivariate

models, all possible two-way interaction terms were entered and then removed by backward elimination using the Wald test.

Analyses were conducted using SAS software, version 9.2 (SAS Institute, Cary, NC), SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL).

Human Subjects Clearance.

Clinical protocols were reviewed and approved by the Institutional Review Boards of the University of Maryland at Baltimore, the University of Maryland at College Park, and the Human Use Review and Regulatory Agency of the Office of the Army Surgeon General.

Results

Descriptive Results

Following tetravalent vaccination, the mean (±SD) number of serotypes an individual had detectable neutralizing antibodies against at day 28 ranged from 0.33± 0.57 for formulation LHLL to 2.5± 1.29 SD serotypes for HLHH (top, Figure 1). The wide error bars are an indication of the low numbers of individuals that received each of the 16 formulations. A possible negative influence of increasing the dose of DENV-2 was evident in the total number of serotypes an individual seroconverted to vaccination. As illustrated in the figure, there was a trend that a high doses of DENV-2 were associated with lower seroconversion rates to DENV serotypes; low doses of DENV-2 were associated with higher seroconversion rates..

The greatest number and duration of signs and symptoms following the first dose of vaccination (indicated by a high reactogenicity index) was observed with formulation HLLL (RI = 33.25, ± 18.10 SD) (bottom, Figure 1). The lowest reactogenicity was observed with HHHL (RI = 2.33, ± 3.23 SD). As illustrated in the figure, there was a trend that the highest reactogenicity was observed for tetravalent formulations that included a high dose of DENV-1 but the incorporation of other serotypes at high dose decreased the reactogenicity. A positive correlation was observed between the number of serotypes an individual seroconverted to at day 28 and the reactogenicity index (RI = 0.491, $p<0.001$).

Comparison of Monovalent and Tetravalent Formulations

There was no significant difference in seroconversion to DENV-1 between the 8 individuals receiving the monovalent DENV-1 formulation (corresponding to 'high dose') and the 36 individuals that received a tetravalent formulation containing a high dose DENV-1 (100% and

80.6% seroconverted to DENV-1, respectively, p=0.17) (Figure 2a). The monovalent DENV-2 formulation resulted in significantly higher seroconversion to DENV-2 compared to high dose DENV-2 tetravalent formulations (100% versus 41.2%, p<0.01). Seroconversion to DENV-3 was low for both the monovalent and high-dose DENV-3 tetravalent formulations, and not significantly different between the two groups (25.0% versus 35.1%, p=0.58). Seroconversion to DENV-4 was significantly higher following monovalent vaccination versus high dose DENV-4 tetravalent formulations (71.4% versus 22.2%, p=0.02). Among monovalent formulations, the geometric mean titers (GMTs) for DENV-1 were the highest (GMT=787), DENV-2 and DENV-4 were the lowest (GMT=42 and GMT=80, respectively) (Figure 2b).

Bivariate Analysis: Effects of Dose on Homologous and Heterologous Serotypes in Tetravalent Formulations

Among tetravalent formulations in bivariate analysis, high doses of DENV-1 resulted in significantly higher seroconversion to DENV-1 compared to low dose DENV-1 (figure 3a). High dose DENV-1 also resulted in a higher probability of seroconversion to DENV-3 and DENV-4. No significant effect of DENV-2 dose was observed for seroconversion to DENV-2 (Figure 3b). However, high doses of DENV-2 were associated with significantly lower seroconversion to DENV-1. High doses of DENV-3 were associated with a significantly increased probability of seroconversion to DENV-4; no effect was observed on seroconversion to DENV-3 (Figure 3c). High dose DENV-4 had no significant effect on seroconversion to any serotype (Figure 3d).

Multivariate Analysis: Effects of Dose on Homologous and Heterologous Serotypes in Tetravalent Formulations

Four multivariate logistic regression models were constructed for the outcomes of seroconversion to DENV1 – DENV4 (Table 2). High doses of DENV-1 resulted in significantly increased seroconversion to DENV-1 (model 1). In contrast, high doses of DENV-2 and DENV-4 resulted

in significantly decreased seroconversion to DENV-1. For model 2, a high dose of DENV-1 resulted in significantly decreased seroconversion to DENV-2, while a high dose of DENV-3 increased seroconversion to DENV-2. For model 3, only a high dose of DENV-1 was associated with increased seroconversion to DENV-3. For model 4, high doses of DENV-1 and DENV-3 significantly increased seroconversion to DENV-4.

Effects of Dose on Multitypic Immunogenicity

As a primary concern in implementing tetravalent DENV vaccines is maximizing seroconversion to multiple serotypes, a logistic regression model was constructed to evaluate the association of serotype dose with seroconversion to 0-2 versus 3-4 DENV serotypes (table 3). High dose DENV-1 was significantly associated with an increased odds of seroconversion to 3-4 serotypes, as compared to low dose DENV-1 (OR = 6.37, 95% CI: 1.13 – 25.86). High dose DENV-2 was borderline associated with decreased seroconversion to 3-4 DENV serotypes (OR = 0.22, 95% CI: 0.05 – 1.01). The doses of DENV-3 and DENV-4 were not associated with overall immunogenicity.

Effect of Dose on Vaccine Reactogenicity

A multivariate linear regression model was constructed to evaluate the effect of serotype dose on the overall reactogenicity associated with the first dose of tetravalent vaccine (table 4). DENV-1 was significantly associated with an increased occurrence of signs and symptoms following vaccination (OR = 23.6, $p<0.01$). However, including high-doses of DENV-2, DENV-3, or DENV-4 in formulations containing high dose DENV-1 resulted in a significant dampening of the association between DENV-1 and increased reactogenicity.

Discussion

The generation of strong antibody responses to all four DENV serotypes in tetravalent formulations has proven a challenging task, due to immunological and/or viral interference between strains. In this study, we characterized interactions between DENV serotypes using data from a factorial-design clinical trial of tetravalent vaccine formulations that evaluated all combinations of high and low dose for each serotype. Multivariate logistic and linear regression models were used to evaluate the effects of the dose of each serotype on serotype-specific seroconversion, overall immunogenicity, and reactogenicity. We found evidence of both interference and facilitation between DENV serotypes with respect to the immune response to tetravalent vaccination and the occurrence and severity of adverse events.

The shift from monovalent to tetravalent vaccination had affected seroconversion rates differently for DENV serotypes. The serotype demonstrating the strongest immunogenicity in monovalent formulations (DENV-1) did not experience notable decreases in immunogenicity after incorporation into tetravalent vaccines. In contrast, DENV-2 and DENV-4 demonstrated modest immunogenicity as monovalent formulations and the immunogenicity of each was further decreased in tetravalent formulations. Evaluations of other live tetravalent dengue vaccines have similarly reported that the serotype eliciting the strongest immune response with monovalent administration tends to dominate when incorporated into tetravalent formulations and that weaker monovalent strains tend to suffer from the greatest interference (Bhamarapravati & Yoksan, 2000; Blaney, Matro, Murphy, & Whitehead, 2005; Guy, et al., 2009).

We found evidence of interference as well as facilitation in the overall immune response to vaccination. DENV-1 and DENV-2 appeared to antagonize each other in tetravalent formulations,

with high doses of one decreasing seroconversion to the other. This was surprising because DENV-2 appeared to be a relatively weak immunogen in both monovalent and tetravalent formulations. In contrast, increasing the dose of the dominant serotype (DENV-1) appeared to facilitate seroconversion to DENV-3 and DENV-4. It is unclear if this is a reflection of increased cross-reactivity or if a specific response to the subdominant components was elicited. Increased seroconversion due to cross-reactivity would seem more probable, since increasing the dose of DENV-1 would likely not serve to decrease competition and facilitate the replication of other serotypes.

It is a well-known limitation of current serological assays that cross-reactive antibodies cannot be discerned from serotype-specific antibodies, which complicates the interpretation of immunogenicity data from multivalent DENV vaccine trials. The protective antibody threshold for DENV infection is therefore unknown and the optimal immune response to vaccination remains to be defined. While there is evidence of an association between cross-reactive antibodies and DHF [6], there is also evidence that higher titers of cross-reactive antibodies can be associated with protection from illness (T. P. Endy, et al., 2004; Halstead, et al., 2002; Halstead & Simasathien, 1970; Sabin, 1952). Therefore, whether cross-reactive or specific, one may define the optimal immune response to tetravalent vaccination to be one that is broad (against all four serotypes) and high-titer. The finding that high doses of the dominant serotype (DENV-1) increased overall seroconversion rates suggests that facilitation between serotypes, once identified, could be exploited to enhance the overall immunogenicity of tetravalent vaccines. Field efficacy studies of tetravalent vaccine candidates are needed to provide insight into the degree of protection afforded against each serotype.

These findings suggest that the total dose of virus administered in a multivalent vaccine and the reactogenicity of a formulation are not necessarily correlated. The highest reactogenicity was observed with a high dose of DENV-1 in tetravalent formulations; increasing the dose of other serotypes dampened the overall reactogenicity of the formulations. This suggests that increasing the dose of sub-dominant strains may paradoxically serve to dampen the overall reactogenicity associated with tetravalent formulations, perhaps due to an improved competitive advantage of the sub-dominant strains. These findings may inform future efforts to optimize vaccine doses for multivalent vaccines by suggesting a means to increase overall seroconversion rates without associated increases in the occurrence of vaccine-associated adverse events.

This analysis was subject to limitations. The numbers of subjects receiving each vaccine formulation was quite small and it was not possible to assess higher-order statistical interactions between serotypes. The clinical trials of monovalent and tetravalent vaccine candidates were conducted at different centers and at different points in time, and it is possible that results are not strictly comparable. A second dose of tetravalent vaccine was administered 30 days after the first; it has since been concluded that cross-reactivity and persistent immune activation following an initial dose of vaccine interferes with subsequent doses for up to 60 days (Guy, et al., 2009). Subsequently, the immunogenicity data following the second dose for this clinical trial were not informative. Perhaps the greatest limitation of this study is that the nature of interactions between DENV serotypes will depend upon passage histories, strains used, and the nature of the vaccine (e.g., live-attenuated or chimeric) and the serotype-specific interactions observed may not be translatable to other vaccine candidates (Price & Thind, 1972).

In addition to dose adjustments, other methods of overcoming interference in DENV vaccines have been demonstrated. It is unlikely that DENV vaccines will be developed that can confer sufficiently broad immunity following a single dose and a multiple dose immunization schedule will likely be necessary. Studies have demonstrated the importance of allowing sufficient time to elapse between doses to minimize the effects of persistent immune activation (Edelman, et al., 2003; Sabin, 1952). Multisite vaccination has been suggested to decrease interference by allowing DENV serotypes to replicate in different sites of the body (Guy, et al., 2009; Zhou & Deem, 2006) . Finally, previous studies have shown that the antibody response to tetravalent vaccination is stronger and broader in the setting of pre-existing DENV, Japanese encephalitis virus (JEV), or yellow fever virus immunity (YFV) (F Guirakhoo, et al., 2006; Kanesa-thasan, et al., 2002). It may be that immunological interference may be lessened in regions where DENV is coendemic with other flaviviruses or where JEV or YFV vaccination is common.

The unique clinical trial design allowed an unprecedented opportunity to look at how changes in the dose of one serotype influenced the serological response to another; other tetravalent candidates have evaluated only a small number of formulations with select dosing changes (F. Guirakhoo, et al., 2002; Sabchareon, et al., 2002), but none have evaluated the influence of serotype dose using a full factorial design. In this study, we present evidence of immunological interference as well as facilitation between serotypes and evidence that increasing the doses of non-dominant serotypes can serve to decrease reactogenicity. These findings are of public health importance in that they contribute to an improved understanding of how DENV serotypes may interact in multivalent vaccines and further suggest that these interactions, once identified, could possibly be exploited to increase overall seroconversion rates while minimizing reactogenicity.

Acknowledgements.

We thank the study volunteers at the University of Maryland (College Park, MD) and at WRAIR (Silver Spring, MD) for their participation. We thank Dana Flanders (Emory University), Alan Rothman (University of Massachusetts), and Derek Cummings (Johns Hopkins University) for their helpful comments on the analysis and the manuscript.

References.

Almond, J., Clemens, J., Engers, H., Halstead, S., Khiem, H., Pablos-Mendez, A., et al. (2002). Accelerating the development and introduction of a dengue vaccine for poor children, 5-8 December 2001, Ho Chi Minh City, VietNam. *Vaccine, 20*(25-26), 3043.

Berger, R., & Just, M. (1988 Oct). Interference between strains in live virus vaccines. II: Combined vaccination with varicella and measles-mumps-rubella vaccine. *J Biol Stand, 16*(4), 275-279.

Berger, R., Just, M., & Glück, R. (1998 Oct). Interference between strains in live virus vaccines, I: Combined vaccination with measles, mumps and rubella vaccine. *J Biol Stand, 16*(4), 269-273.

Bhamarapravati, N., & Yoksan, S. (2000). Live attenuated tetravalent dengue vaccine. *Vaccine, 18 Suppl 2*, 44-47.

Blaney, J. E., Jr., Matro, J. M., Murphy, B. R., & Whitehead, S. S. (2005). 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, 79*(9), 5516-5528.

DeRoeck, D., Deen, J., & Clemens, J. D. (2003). Policymakers' views on dengue fever/dengue haemorrhagic fever and the need for dengue vaccines in four southeast Asian countries. *Vaccine, 22*(1), 121-129.

Dick, G., & Horgan, E. (1952). Vaccination by scarification with combined 17D yellow fever and vaccinia vaccine. *J Hyg (Camb), 50*, 376-383.

Edelman, R., Wasserman, S. S., Bodison, S. A., Putnak, R. J., Eckels, K. H., Tang, D., et al. (2003). Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. *Am J Trop Med Hyg, 69*(6 Suppl), 48-60.

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Guirakhoo, F., Kitchner, S., Morrison, D., Forrat, R., McCarthy, K., Nichols, R., et al. (2006). Live attenuated chimeric yellow fever dengue type 2 (Chimerivax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity. *Human Vaccines, 2*(2), 60-67.

Guirakhoo, F., Pugachev, K., Arroyo, J., Miller, C., Zhang, Z. X., Weltzin, R., et al. (2002). Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. *Virology, 298*(1), 146-159.

Guy, B., Barban, V., Mantel, N., Aguirre, M., Gulia, S., Pontvianne, J., et al. (2009). Evaluation of interferences between dengue vaccine serotypes in a monkey model. *Am J Trop Med Hyg, 80*(2), 302-311.

Halstead, S. B. (1997). Epidemiology of dengue and dengue hemorrhagic fever. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 23-44). New York: CAB International.

Halstead, S. B., Chow, J., & Marchette, N. J. (1973). Immunologic enhancement of dengue virus replication. *Nature New Biology, 243*(122), 24-26.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., & Simasathien, P. (1970). Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. *Yale J Biol Med, 42*, 276-292.

Kanesa-thasan, N., Hernandez , L., Lyons, A., Putnak, R., Sun, W., McKinney, D., et al. (2002, 6-8 May 2002). *A phase I study of the WRAIR tetravalent live attenuated dengue vaccine in flavivirus-immune adult volunteers.* Paper presented at the 5th Annual Conference on Vaccine Research, Baltimore MD.

Kanesa-thasan, N., Sun, W., Kim-Ahn, G., Van Albert, S., Putnak, J. R., King, A., et al. (2001). Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. *Vaccine, 19*(23-24), 3179-3188.

Kitchener, S., Nissen, M., Nasveld, P., Forrat, R., Yoksan, S., Lang, J., et al. (2006). Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. *Vaccine, 24*(9), 1238-1241.

Meyer, H., Hopps, H., Bernheim, B., & Douglas, R. (1967). Combined measles-smallpox and other vaccines. First International Conference on Vaccines Against Viral and Rickettsial Diseases in Man. Pan American Health Organization. *Scientific Publ. No. 147, Washington, DC, Pan American Health Organization*, 336-342.

Morrison, D., Legg, T., Billings, C., Forrat, R., Yoksan, S., & Lang, J. (2010). A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naive adults. *J Infect Dis, 201*(3), 370-377.

Patriarca, P., Wright, P., & John, T. (1991). Factors affecting immunogenicity of oral poliovirus vaccine in developing countries: review. *Rev Infect Dis, 13*(926-939).

Payne, A. (1960). Oral immunization against poliomyelitis. *Bull WHO, 23*, 695-703.

Price, W. H., & Thind, I. S. (1972). The mechanism of cross-protection afforded by dengue virus against West Nile virus in hamsters. *J Hyg Camb, 70*, 611-617.

Russell, P. K., Nisalak, A., Sukhavachana, P., & Vivona, S. (1967). A plaque reduction test for dengue virus neutralization antibodies. *J Immunol, 99*(2), 285-290.

Sabchareon, A., Lang, J., Chanthavanich, P., Yoksan, S., Forrat, R., Attanath, P., et al. (2002). Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. *Am J Trop Med Hyg, 66*(3), 264-272.

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Stephenson, J. R. (2005). Understanding dengue pathogenesis: implications for vaccine design. *Bull World Health Organ, 83*(4), 308-314.

Sun, W., Edelman, R., Kanesa-Thasan, N., Eckels, K. H., Putnak, J. R., King, A. D., et al. (2003). Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. *Am J Trop Med Hyg, 69*(6 Suppl), 24-31.

Thomas, S., Nisalak, A., Anderson, K., Libraty, D., Kalayanarooj, S., Vaughn, D., et al. (2009). 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., 81*(5), 825-833.

Zhou, H., & Deem, M. (2006). Sculpting the immunological response to dengue fever by polytopic vaccination. *Vaccine, 24*(14), 2451-2459.

Table 1. Dengue vaccine formulations

Vaccine strains			
	Monovalent recipients * (N)	Doses	Passage History
DENV-1	8	Low (L) = $10^{4.5}$ pfu High (H) = 10^{6} pfu	PDK20
DENV-2	8	Low (L) = $10^{4.5}$ pfu High (H) = 10^{6} pfu	PDK50
DENV-3	8	Low (L) = $10^{3.5}$ pfu High (H) = 10^{5} pfu	PDK20
DENV-4	7	Low (L) = $10^{3.5}$ pfu High (H) = 10^{5} pfu	PDK20
Tetravalent formulations			
Formulation	Recipients (N)		
LLLL	3		
HLLL	4		
LHLL	3		
LLLH	3		

LLHL	4
LHLH	3
HLHL	4
LHHL	4
LLHH	4
HLLH	4
HHLL	3
LHHH	4
HLHH	4
HHLH	4
HHHL	3
HHHH	10

* Note : monovalent recipients received 'high-dose' formulations of DENV1-4

Table 2. Multivariate logistic regression models for seroconversion to DENV-1 – DENV-4 (four individual models were constructed). Seroconversion is defined as a PRNT50 titer of >=10 after one dose of a tetravalent formulation.

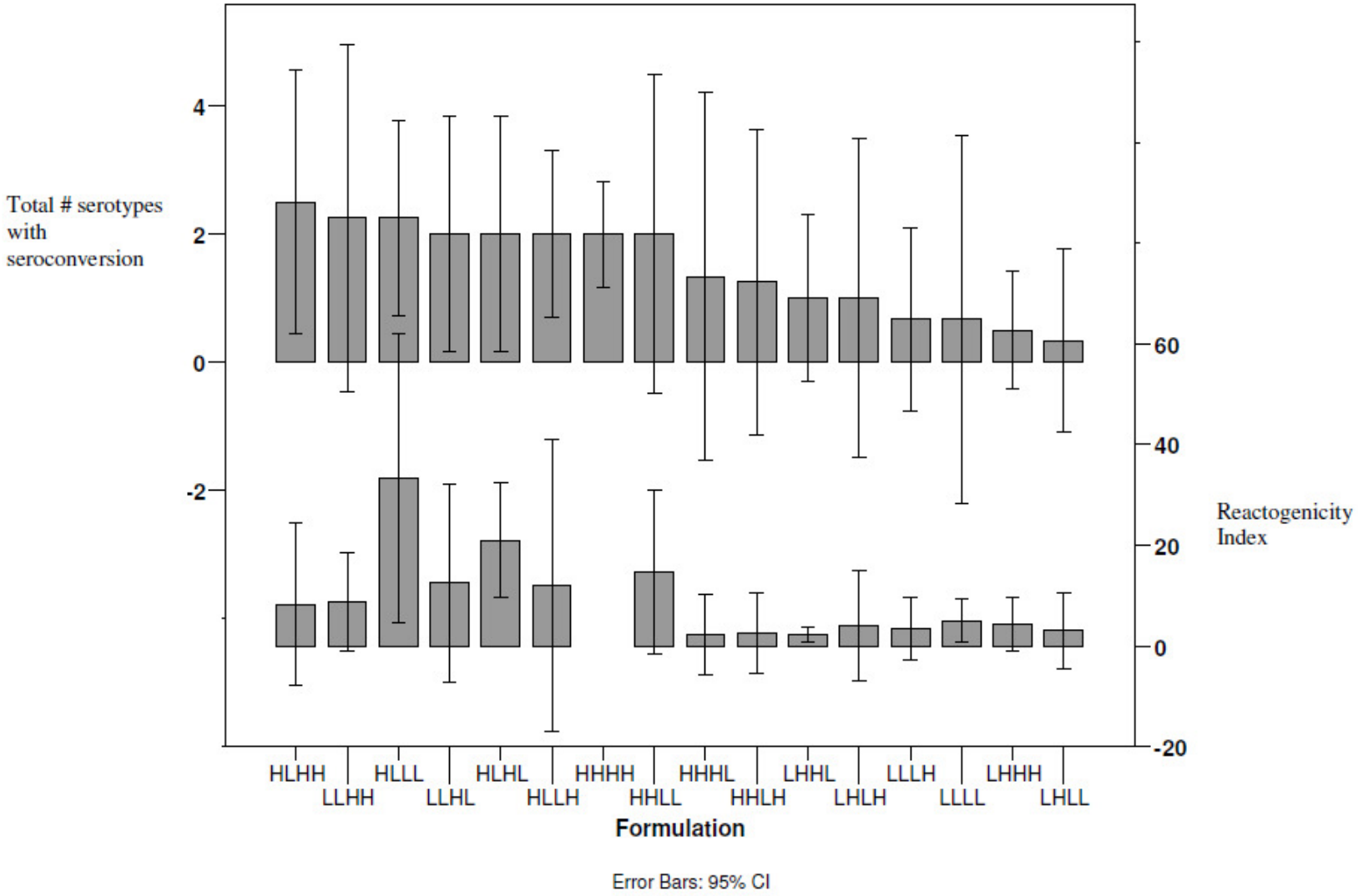
	Model 1: ***Seroconversion to DENV-1***		***Model 2:*** ***Seroconversion to DENV-2***		***Model 3:*** ***Seroconversion to DENV-3***		***Model 4:*** ***Seroconversion to DENV-4***	
Predictors:	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Den1: High versus low dose	**64.8 (6.73 - >999)**	**<0.001**	0.65 (0.21 – 2.02)	0.563	**3.58 (1.00 – 15.20)**	**0.050**	**11.35 (1.16 – 616.90)**	**0.031**
Den2: High versus low dose	**0.04 (<0.01 – 0.34)**	**<0.001**	1.34 (0.43 – 4.26)	0.756	0.43 (0.11 – 1.50)	0.223	0.21 (0.02 – 1.51)	0.154
Den3: High versus low dose	3.31 (0.61 – 24.03)	0.211	1.71 (0.55 – 5.65)	0.439	1.33 (0.38 – 4.92)	0.825	**11.27 (1.14 – 616.85)**	**0.033**
Den4: High versus low dose	**0.16 (0.02 – 0.96)**	**0.043**	1.11 (0.35 – 3.57)	1.00	1.89 (0.53 – 7.31)	0.406	4.48 (0.57 – 66.49)	0.214

Table 3. Logistic regression model comparing seroconversion to three or more serotypes to seroconversion to zero-two dengue serotypes following a single dose of tetravalent vaccine

Seroconversion to 3 or 4 serotypes compared to 0 to 2 serotypes		
	OR (95% CI)	**p-value**
Den1: High versus low dose	**6.369 (1.131 – 25.860)**	**0.036**
Den2: High versus low dose	0.218 (0.047 – 1.006)	0.051
Den3: High versus low dose	3.431 (0.713 – 16.505)	0.124
Den4: High versus low dose	1.884 (0.424 – 8.379)	0.405

Table 4. Multivariate linear regression model of reactogenicity index following a single dose of tetravalent vaccine

	Regression parameter	p-value
Den1: High versus low dose	**23.604**	**<.001**
Den2: High versus low dose	-4.500	.182
Den3: High versus low dose	3.104	.360
Den4: High versus low dose	-.643	.848
Den1*Den2	**-10.427**	**.035**
Den1*Den3	**-12.041**	**.016**
Den1*Den4	**-13.187**	**.008**
Intercept	6.405	0.071


Total # serotypes with seroconversion
4
2
0
-2
60
40
20
0
-20
Reactogenicity Index
HLHH
LLHH
HLLL
LLHL
HLHL
HLLH
HHHH
HHLL
HHHL
HHLH
LHHL
LHLH
LLLH
LLLL
LHHH
LHLL
Formulation
Error Bars: 95% CI

* No reactogenicity data available for formulation HHHH

Figure 1. Mean reactogenicity index (lower right y-axis) and total # of serotypes with seroconversion at day 28 (upper left y-axis), sorted in descending order of immunogenicity

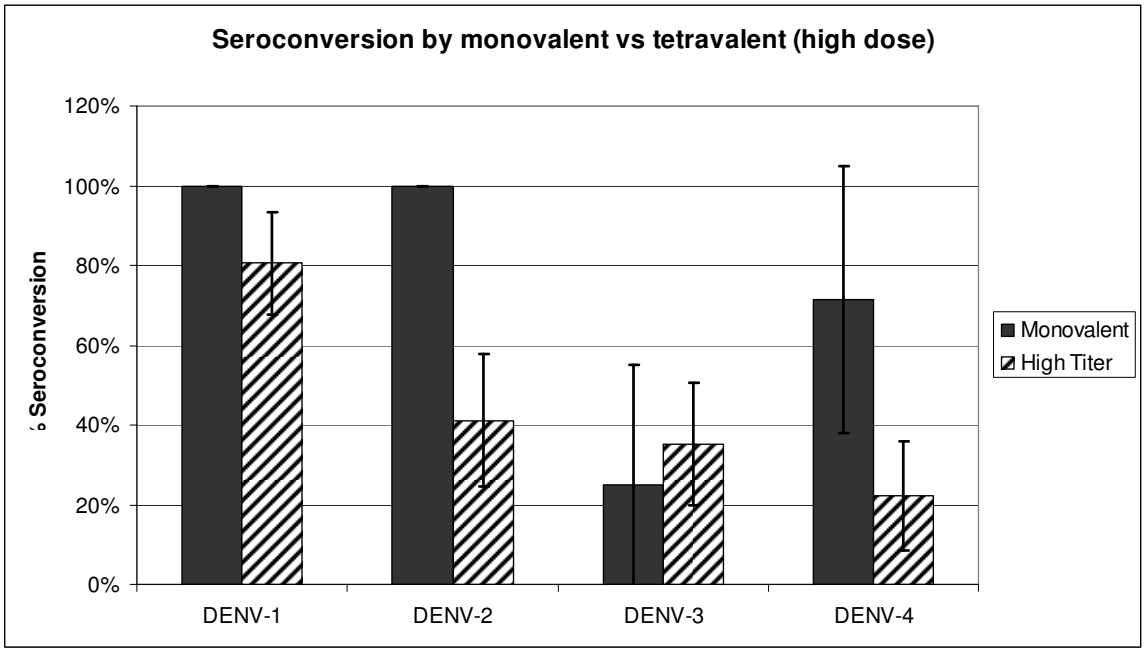


Figure 2a.

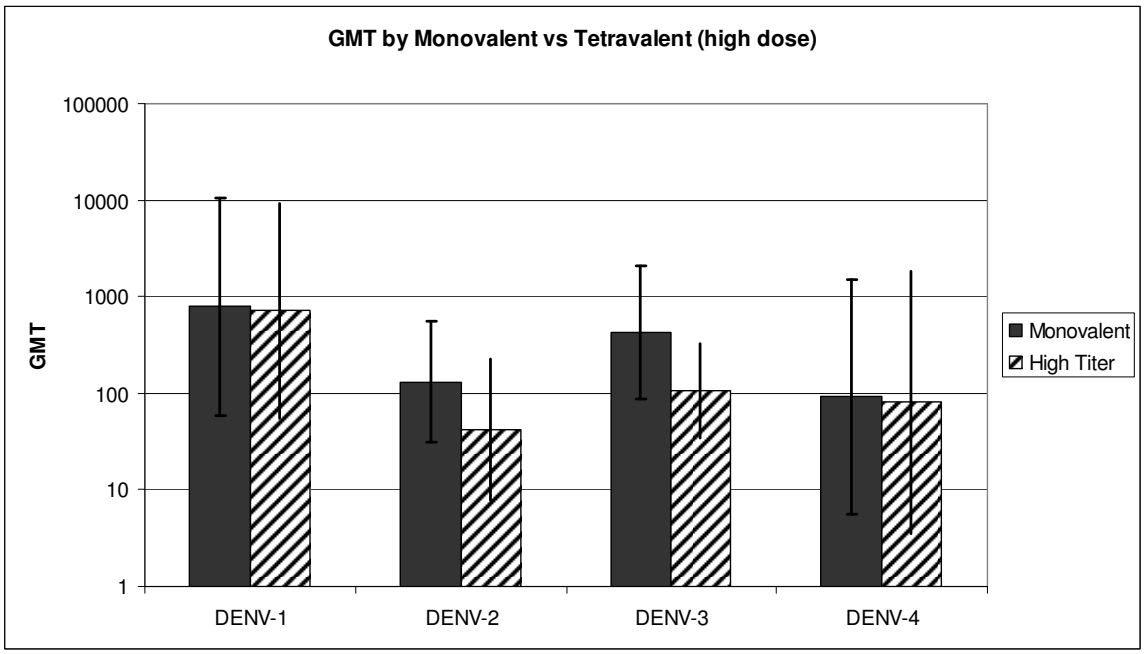
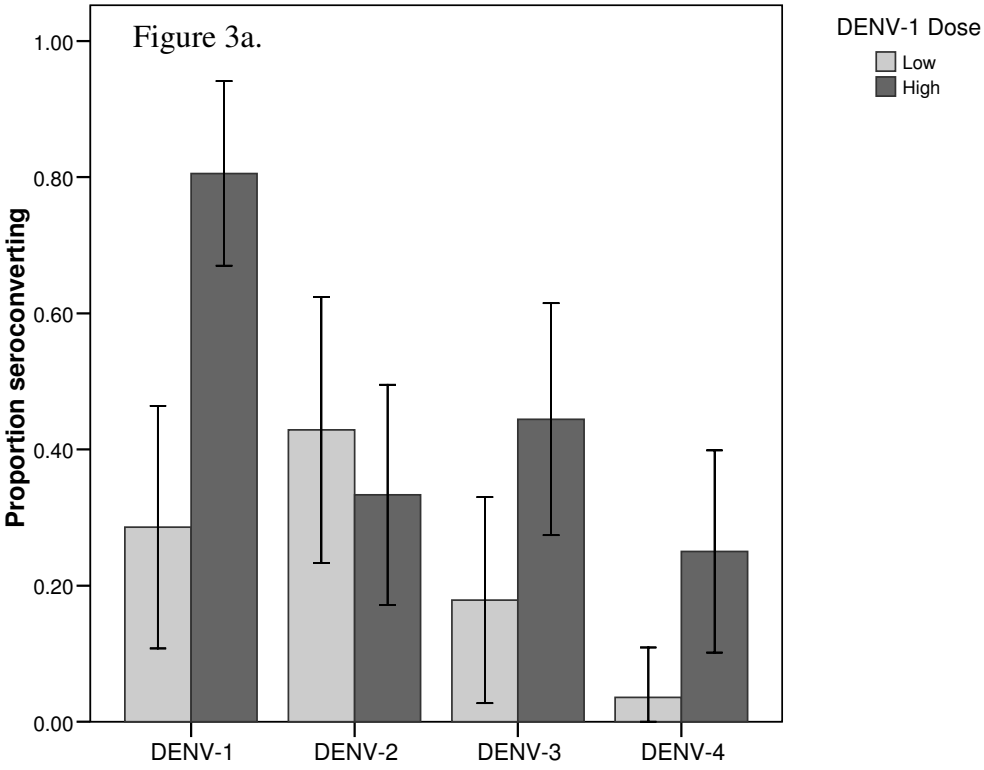


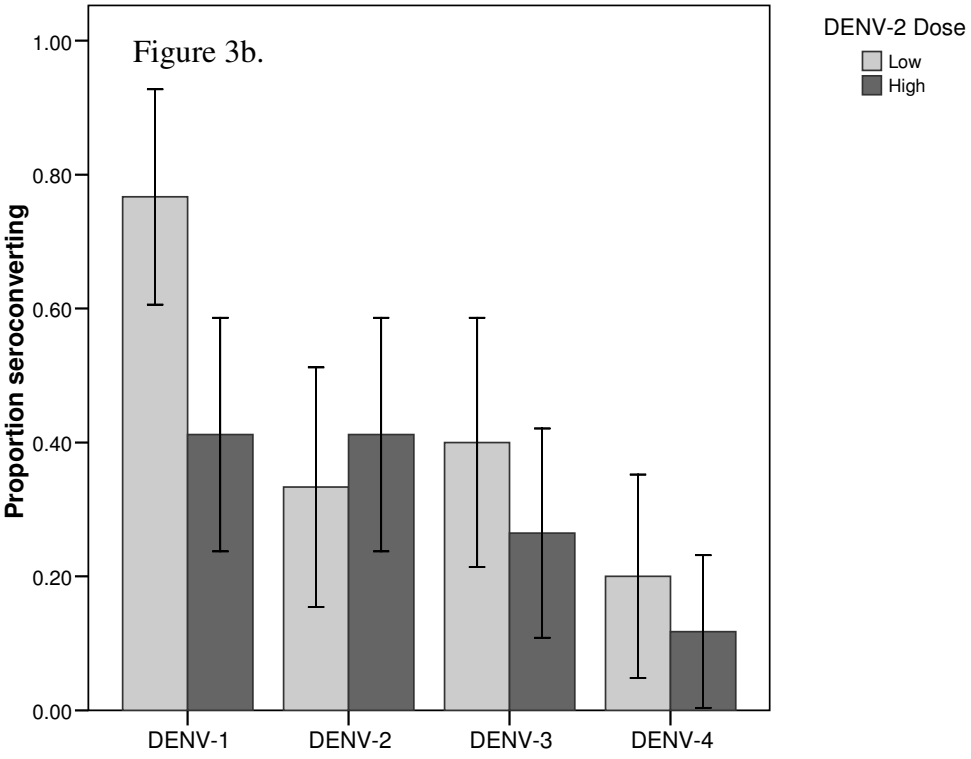
Figure 2b.

Figure 2. Proportion of individuals seroconverting to DENV-1 – DENV-4 (Figure 2a) and the geometric mean titer (GMT) of the PRNT50 for DENV-1 – DENV-4 (among individuals that seroconverted to that serotype) (Figure 2b), by whether they received each

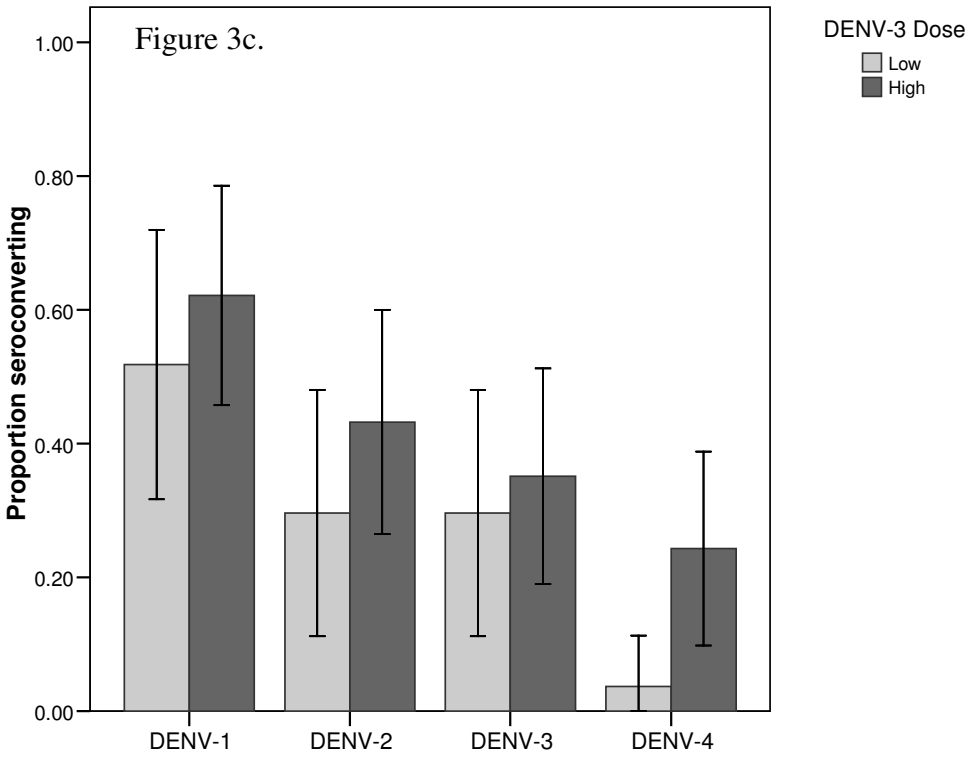
serotype as a high-dose monovalent vaccine or as a high-dose component of a tetravalent vaccine. Error bars show 95% confidence intervals for the percentage seroconverting against each serotype.



Seroconversion to DENV-1, -2, -3, and -4 at day 28 post-vaccination



Proportion seroconverting to DENV-1, -2, -3, and -4 at day 28 post-vaccination



Seroconversion to DENV-1, -2, -3, and -4 at day 28 post-vaccination

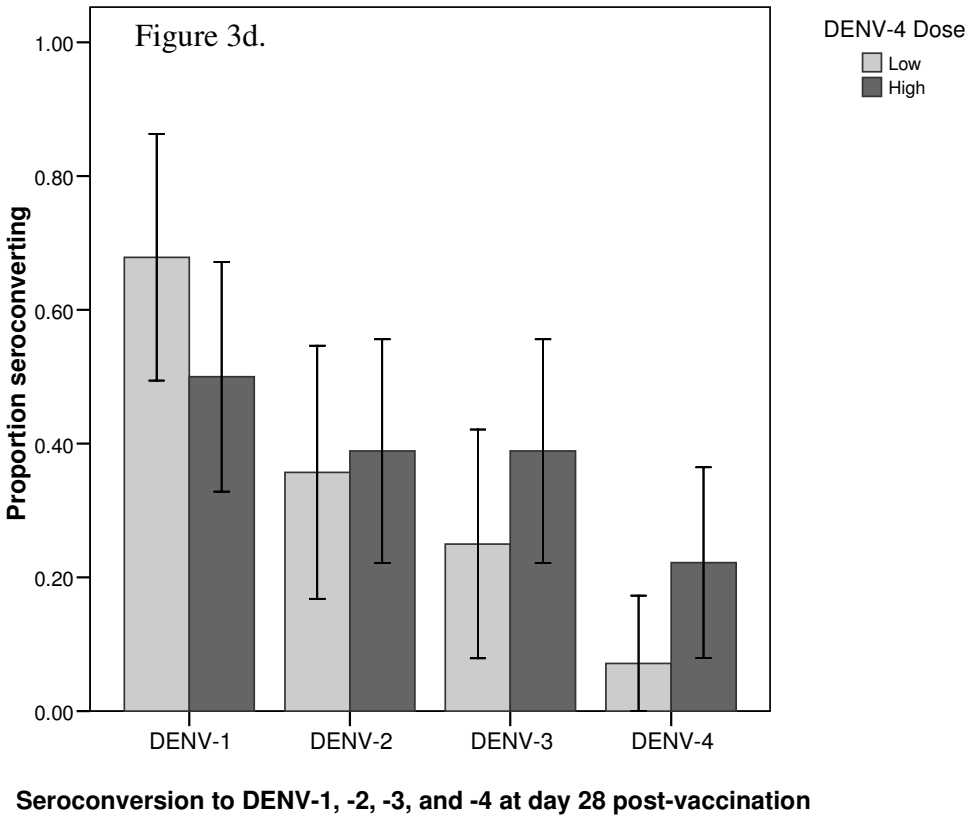


Figure 3. Proportion of individuals, across all tetravalent formulations, seroconverting to DENV-1 – DENV-4 (x-axis) depending upon whether **DENV-1** (Figure 3a), **DENV-2** (Figure 3b), **DENV-3** (Figure 3c), or **DENV-4** (Figure 3d) were high or low dose in the formulation. Error bars show 95% confidence intervals for the proportion seroconverting against each serotype.

CHAPTER 6 – CONCLUSIONS

Infection with dengue viruses (DENVs) has been identified as an important cause of human morbidity and mortality for decades, with reported epidemics of dengue-like illnesses dating back centuries (Gubler, 1997). In recent decades, there has been a dramatic increase in the co-circulation of dengue serotypes, linked to both an increased incidence of infection and the emergence of the clinically-severe dengue hemorrhagic fever (DHF) (Mackenzie, et al., 2004). DHF is hypothesized to result when preexisting, cross-reactive antibodies from a previous DENV exposure can facilitate subsequent infection with a different DENV serotype, resulting in an increased viral load, increased immune activation, and ultimately plasma leakage and shock (Halstead & Simasathien, 1970). A cross-protective role of preexisting DENV immunity has also been demonstrated (Sabin, 1952). The complex factors involved in whether a post-primary infection with DENV will be subclinical, mild, or develop into DHF are poorly understood but have been suggested to be both time- and titer-dependent (R. V. E. Gibbons, T P; Nisalak , A.;Chunsutthiwat, S.; Vaughn, D W; Green, S; Ennis, F. A.; Rothman, A.; Libraty, D H, 2004; Halstead, et al., 2002; Sabin, 1952). The ability to discriminate between pathologic DENV immune profiles, predisposing to illness with subsequent infection, and protective DENV immune profiles remains a central issue in DENV immunology, and one that looms large as DENV vaccines approach licensure and implementation.

Several unique aspects of DENV epidemiology and immunology have complicated the study of prior immunity and the clinical outcome of DENV infections. First, serological assays cannot discern between specific antibodies, developed in response to infection with a given serotype, and cross-reactive antibodies, which react with serotypes other than the infecting

serotype (Anantapreecha, et al., 2007; Halstead & Simasathien, 1970). For example, a child having antibodies to all four serotypes may have experienced four DENV infections and be solidly protected from illness with subsequent exposure, or the child may have experienced a single DENV infection which resulted in a broadly cross-reactive response, placing the child at a heightened risk of DHF with subsequent exposure. That is, a "snapshot image" of an individual's DENV antibody status may be poorly informative with respect to their disease risk.

Second, DENVs co-circulate with other flaviviruses such as JEV and yellow fever virus (YFV) throughout much of their geographic range and their antibodies are highly cross-reactive in serological assays (Makino, et al., 1994; Martin, et al., 2002). This cross-reactivity makes it difficult to isolate the burdens of each in endemic locales and to assess the possible clinical implications of these serological interactions.

Third, many DENV infections are clinically asymptomatic, with an estimated symptomatic : asymptomatic ratio of 1:2 (T. Endy, et al., 2010). Most previous studies of preexisting DENV immunity and clinical outcome have focused on hospitalized cases, with asymptomatic and mild infections passing undetected, thereby missing the vast majority of DENV infections (Chairulfatah, Setiabudi, Agoes, van Sprundel, & Colebunders, 2001; Dechant & Rigau-Perez, 1999; R. V. Gibbons, et al., 2007; Hammond, et al., 2005). Four studies have sought to characterize the full spectrum of clinical severities with DENV infection: (1) A two-year school-based study conducted in 1800 children from 1980-1981 in Bangkok, Thailand (Burke, et al., 1988), (2) a four-year school-based study conducted in 3800 children from 2005-2008 in Managua, Nicaragua (Balmaseda, Standish, Mercado, & al.,

2010), (3) a three-year study conducted in 1500 adults in West Java, Indonesia (Porter, et al., 2005), and (4) five-year (1998-2002) and four-year (2004-2007) school-based studies following ~2000 children per year for DENV infection in Kamphaeng Phet, Thailand (T. P. Endy, Chunsuttiwat, et al., 2002; Mammen, et al., 2008).

The availability of longitudinal antibody data and the capture of dengue infections across a range of clinical severities in the school-based cohort studies conducted in Kamphaeng Phet, Thailand, provided a unique opportunity to assess immunological factors associated with disease severity for dissertation studies one and two. Studies one and two focused on antibody – virus interactions, considering how (1) preexisting JEV antibodies and (2) time between DENV infections, as a presumed proxy for the decay of cross-reactive antibodies over time, may influence the clinical severity of DENV infection. Study three extended to virus - antibody interactions, considering the more applied question of how DENV serotypes interact and compete with regards to the antibody response to tetravalent vaccination.

DISSERTATION STUDIES

Study 1:

The first study of this dissertation sought to characterize the association between preexisting JEV antibodies and the clinical severity of a subsequent DENV infection. Heterologous flaviviruses are highly cross-reactive in serological assays, posing challenges for diagnosis in co-endemic locales such as Thailand, where DENV and JEV co-circulate and JEV vaccination is common. The possible clinical and epidemiological effects of these serological interactions were a topic of great interest in the 1960s, 1970s, and 1980s (Bond & Hammon,

1970; Hammon, 1969; Tarr & Hammon, 1974); these questions have since been largely abandoned though they remain far from resolved. The dominant paradigm has been that prior exposure to heterologous flaviviruses is either clinically inconsequential or perhaps mildly protective (R. V. Gibbons, et al., 2007; Sabin, 1952). The most influential observation regarding JEV immunity and DENV infection was made following a large Phase III trial of JE-VAX in Thailand, where it was observed that children who received JEV vaccine exhibited a non-significant decrease in DHF in the years following vaccination (Hoke, et al., 1988).

This study used data from a school-based cohort study of dengue infections that was conducted in Kamphaeng Phet, Thailand, from 1998-2002 (T. P. Endy, Chunsuttiwat, et al., 2002). The cohort study was unique in that it captured not only hospitalized illnesses, but also non-hospitalized illnesses (through fever surveillance) and asymptomatic seroconversions. Further, it was possible to characterize the pre-infection neutralizing antibody (NAb) profiles to DENV and JEV. Based upon these pre-infection data, children were classified as JEV NAb positive or negative, and DENV-naïve (seronegative for all four DENV serotypes), DENV-monotypic (positive for a single DENV serotype), or DENV-multitypic.

The presence of JEV NAbs prior to infection was associated with the increased occurrence of symptomatic versus asymptomatic DENV infection. This association was most notable in DENV-naives, in whom the presence of JEV NAbs was also associated with an illness of longer duration. Importantly, in subgroup analysis an increase in DHF occurrence with JEV NAbs was observed only in children younger than ten years with DENV-multitypic profiles.

These children would have had fewer years to accumulate DENV infections and specific immunity and their DENV-multitypic profiles, may, on average, have reflected more cross-reactivity than DENV-multitypic profiles in older children. It is possible that among these younger children with DENV-multitypic profiles, the presence of JEV NAbs simply served as a marker of a highly cross-reactive profile that was itself associated with the increased occurrence of DHF, instead of reflecting a true association between prior JEV exposure and DHF. Among those that were JEV NAb positive prior to infection, the titer of JEV NAbs did not differ between children with asymptomatic and symptomatic infections. This absence of a 'dose-response' may also suggest that detectable JEV NAbs were a marker of some other immune function, such as cell-mediated immunity, that may play a more direct biological role in the association between prior JEV exposure and DENV illness. A notable limitation of this study is that JEV NAbs could have arisen as a result of JEV vaccination (with live or inactivated vaccine), JEV exposure, or as a cross-reactive response to DENV infection; serological assays cannot discriminate these and it may be that the association with DENV illness may vary by source of JEV immunity.

The findings from this study suggest that the issue of heterologous flavivirus immunity and DENV infection merits renewed attention and interest. Specifically, DENV vaccine developers might incorporate detailed assessments of preexisting immunity to non-DENV flaviviruses and histories of vaccination against non-DENV flaviviruses in evaluating DENV vaccine safety and efficacy.

Study 2:

The second study of this dissertation investigated whether time interval between infections was an important predictor of infection severity. There is evidence of a titer-dependent association between cross-reactive antibodies and the clinical outcome of a subsequent DENV infection, with higher titers of cross-reactive antibodies able to attenuate infection and protect against illness, and lower titers possibly facilitating infection and increasing the risk of DHF (T. P. Endy, et al., 2004; Halstead, et al., 2002). The unavailability of a serological assay that can discriminate between cross-reactive and specific antibodies in a specimen makes it difficult to evaluate these associations using antibody titer data. It was postulated that these titer-dependent shifts in disease risk with waning cross-reactive antibodies may be evident as temporal shifts; following a DENV infection, an individual may pass through a temporary 'window of protection' (mediated by high-titer cross-reactive antibodies) and then through a 'window of risk' of disease (mediated by low to intermediate titer antibodies).

The major research question for this study was whether the interval between DENV infections was associated with infection severity and whether there were immunological differences between individuals with the same interval between infections that were associated with disease severity. The 1998-2002 prospective cohort study data were examined (as in study one), as was the second phase of the same cohort study that ran from 2004-2007 (Mammen, et al., 2008). 1696 children had a DENV infection detected during the studies; 268 of these children had a second infection detected during their enrollment. Hemagglutination inhibition (HI) antibody data were used to characterize subjects' baseline

DENV immunity prior to their first infection and to characterize immunological changes over time.

Among children that were HI-negative prior to their first observed infection, a shorter interval between the first and second observed infections was associated with asymptomatic infection (1.41 years, on average) and longer intervals were associated with DF (1.92 years) and DHF (2.12 years). This temporal trend in disease risk did not hold for children with evidence of at least partial DENV immunity by HI testing prior to their first infection in the study period, which could be due to long-term cross-protection that is presumed to occur with the accumulation of DENV infections. Asymptomatic second infection was further associated with a high total antibody response to the first observed infection. This is consistent with a model of disease risk where cross-protective immunity declines over time, with a more robust response to infection potentially increasing the duration of cross-protection Interval between infections remained a significant predictor of infection severity after controlling for immunological factors. This suggests that interval between infections may itself be associated with shifts in disease risk, perhaps through time-dependent qualitative shifts in antibody specificity.

These findings are consistent with a temporal/immunological model of disease risk where cross-reactive antibodies wane from higher-titer and protective to lower-titer and detrimental. This study suggests that there may be a temporary period of cross-protection following DENV infection, in accordance with Sabin's human challenge studies in the 1940s (Sabin, 1952).

Study 3:

The third study of this dissertation extended into the applied realm, asking whether there is evidence of interactions between DENV serotypes in tetravalent DENV vaccine formulations. The strong evidence of an enhanced risk of severe disease with sequential DENV exposures has warranted the development of tetravalent DENV vaccines to ensure safety. However, the generation of balanced immune responses with tetravalent DENV vaccination promises to be a significant challenge because of immunological and virological interference between serotypes (F. Guirakhoo, et al., 2002; Sabchareon, et al., 2002).

This study analyzed data from a unique factorial design clinical trial of tetravalent dengue (DENV) vaccine candidates conducted at the Walter Reed Army Institute of Research (WRAIR), that considered formulations of all combinations of high and low dose for each serotype (16 formulations in total) (Edelman, et al., 2003). The results were also compared against earlier clinical trials of the monovalent candidates (Kanesa-Thasan, et al., 2003). Possible immunological interactions between DENV serotypes were identified by comparing the antibody response following tetravalent and monovalent vaccination and by evaluating how changes in the dose of each serotype in tetravalent formulations affected the antibody response to all four serotypes as well as the reactogenicity (an aggregate measure of the severity and duration of adverse events) of each formulation.

The magnitude of the antibody response to DENV-1 dominated over the response for all other serotypes following monovalent vaccination. The magnitude of the response to both high and low dose DENV-1 in tetravalent formulations dominated over the antibody responses to other serotypes. DENV-2 was a relatively weak immunogen in both

monovalent and tetravalent formulations. However, when administered in high doses in tetravalent formulations, DENV-2 appeared to compete and interfere with seroconversion against DENV-1, demonstrating that the ability of a serotype to generate an antibody response may differ from its ability to exert virological interference.

There were two notable findings from this study that may inform future tetravalent DENV vaccine development efforts. First, increasing the dose of the dominant serotype (DENV-1) appeared to facilitate seroconversion to subdominant serotypes. It is unclear if this is a reflection of increased cross-reactivity or if a specific response to the subdominant components was elicited. Increased cross-reactivity would seem more probable, since increasing the dose of DENV-1 would likely not facilitate the replication of other serotypes and the generation of specific immunity to these serotypes. The findings from study 2 of this dissertation would suggest that cross-reactive antibodies can be protective for a window of time following infection (or perhaps, vaccination). Therefore, seroconversion from facilitation, whether as a result of a specific response or as a cross-reactive response, may be beneficial. Second, this study had the paradoxical finding that while the highest reactogenicity was observed with a high dose of DENV-1 in tetravalent formulations, increasing the dose of non-dominant serotypes dampened the overall reactogenicity of the formulations. It may be that when DENV-1 was high and all others low, this dominant strain was able to exert a replicative advantage over other serotypes and reproduce to high titers, resulting in increased reactogenicity. As the dose of non-dominant serotypes was increased, they were able to compete more effectively with DENV-1 and decrease its replicative advantage.

LIMITATIONS.

There were several limitations to the retrospective cohort data used for studies one and two. The first is the generalizability of these studies. To begin, the studies were conducted on a relatively limited timescale – from 1998-2002 and from 2004-2007. There is a great deal of unpredictability in the incidence of DENV infection; large epidemics have historically occurred in Thailand approximately every three to five years (Nisalak, et al., 2003). The clinical severity of DENV epidemics, as defined by the proportion of infections that are symptomatic, has also been suggested to vary unpredictably from year to year (T. Endy, et al., 2010) and there is evidence of longer-term shifts in the dominance of different serotypes (Zhang, et al., 2005). Second, these studies were limited to a rather narrow age range (five to sixteen years of age). These studies cannot be generalized to younger children or adults, who may contribute significantly to the burden of DENV illness in Kamphaeng Phet and who may play an important role in virus transmission. The number of DHF cases was very low in both phases of the cohort study and no deaths were observed. This limited the power to assess associations between JEV antibodies and the occurrence of severe DENV illness. Last, the epidemiology of DENVs differs importantly between Thailand and other regions, for example Latin America and Taiwan. Thailand has had all four DENV serotypes in circulation for many years, while Latin American countries are presently experiencing a re-introduction of serotypes and gradual increases in co-circulation, incidence, and DHF (Halstead, 2006). Thailand has a very high incidence of infection, while Taiwan is not endemic for DENV and experiences sporadic epidemics of a single DENV serotype (S. F. Chang, et al., 2008). The clinical findings and immunological trends observed in these studies may not be generalizable to other regions.

The cohort studies are retrospectively analyzed and therefore these dissertation studies were largely limited to the data that were collected at the time of the study. An example of this limitation is the unavailability of detailed information on history of JEV vaccination in study one. The JEV vaccination histories collected were based upon self-report only. Attempts to locate historical vaccination records for verification at public health offices were not successful.

Given the uncertainties inherent in interpreting DENV serological data, there is the possibility that some children were misclassified with respect to their immunological status. For example, in study one, JEV and DENV are quite cross-reactive in serological assays (Makino, et al., 1994; Martin, et al., 2002) and there is the possibility that some JEV NAbs arose as a cross-reactive response to DENV infection, with no prior JEV exposure or JEV vaccination. Conversely, given the reported high levels of JEV vaccine coverage in the study cohort and the low JEV seropositivity, it is probable that some JEV NAb-negative children had been vaccinated but that their JEV antibodies had since waned to undetectable levels. An anamnestic response to a subsequent DENV infection may still have occurred in JEV-negative children that had received JEV vaccine (Konishi, et al., 1999); this is supported by the observation that most JEV-negative / DENV-negative children exhibited a secondary-type response by IgM/IgG ELISA upon DENV infection. Approximately 50% of children who had no HI antibodies prior to infection were positive by PRNT; suggesting that the sensitivity of the HI assay used for many of these analyses is suboptimal. While the PRNT assay is considered the "gold standard" for characterizing protective immunity, PRNT is labor-intensive, has low specificity for identifying the infecting serotype and is highly sensitive to changes in assay conditions (Thomas, et al., 2009). The challenges in

interpreting DENV serological assays and serological data are, at present, unavoidable based on current technologies.

It is a unique feature of the cohort studies that asymptomatic infections were detected; however, there are important gaps in the information available for these cases. As they were identified based upon seroconversion between two routine blood specimens, it was not possible to determine precisely when cases were infected; only that they seroconverted within a window of time. Since the cases did not present with an acute illness, acute blood specimens were not collected and it is not possible to identify the infecting serotype with certainty. Attempts were made in studies one and two to impute the infecting serotype for asymptomatic infections, based upon what serotypes were detected among RT-PCR positive cases at their school during the year of their infection. It is possible that some serotypes can cause milder disease than others and the serotype distribution among acute, PCR-positive cases may not be representative of the distribution of serotypes among the asymptomatically-infected individuals.

The clinical trial data analysis (study three) was also subject to limitations. The numbers of subjects receiving each vaccine formulation was quite small (three to four per formulation) and it was not possible to assess higher-order statistical interactions between serotypes. Given that DENV vaccination regimens will likely involve the administration of multiple doses, a limitation of this analysis is that the nature of interactions following a second or third dose could not be evaluated. The protocol for this clinical trial provided a second dose of tetravalent vaccine 30 days after the first; it has since been concluded that cross-reactivity and persistent immune activation following an initial dose of vaccine interferes with

subsequent doses for up to 60 days (Guy, et al., 2009). Subsequently, no significant increases in seroconversion were observed following the second dose in the clinical trial; these data were not informative and therefore not analyzed. The clinical trials of monovalent and tetravalent vaccine candidates were conducted at different centers and at different points in time, and it is possible that results are not strictly comparable. One importance difference noted in the research paper is that the monovalent clinical trials used complement in the PRNT assay which possibly resulted in higher titers (Thomas, et al., 2009); for this reason, titers could not be compared between the monovalent and tetravalent clinical trials. Perhaps the greatest limitation of this study was that the nature of interactions between DENV serotypes will depend upon passage histories, strains used, and the nature of the vaccine (e.g., live-attenuated or chimeric) and the serotype interactions observed in this study may not be translatable to other vaccine candidates (Price & Thind, 1972).

STRENGTHS

A clear strength of the retrospective cohort studies used for studies one and two is that they captured the entire range of clinical severities with DENV infection. Most epidemiological studies of DENV infection are limited to hospitalized illnesses, which represent only a small proportion of all DENV infections (T. P. Endy, Chunsuttiwat, et al., 2002). The vast majority of symptomatic infections are non-hospitalized DF cases, which contribute significantly to the total burden of disease and virus transmission in the community (Anderson, et al., 2007). The sensitivity of the fever surveillance system in studies one and two was such that cases with transient fever and mild malaise were identified, as well as more severe hospitalized cases. The identification of asymptomatic infections in these studies was important because these cases can provide insight into what constitutes protective immunity

with DENV infection. The capture of all, or nearly all, DENV infections in the study cohort provided a unique opportunity to assess the factors associated with the clinical severity of DENV infection.

Analyses of the associations between serological factors and DENV disease severity in studies one and two are strengthened by the longitudinal nature of the cohort studies. It is not possible with hospital-based studies of acute illnesses to gain insight into which serological "exposures" preceded the event, since the antibody response at the time of infection is a highly cross-reactive mix of serotype specificities. It is a unique strength of these studies to have pre-infection antibody data available, for all four DENV serotypes as well as JEV. Further, the availability of sequential routine specimens and HI antibody data for every specimen provided the opportunity to evaluate the association between immunological factors and the severity of DENV infections at different time points following a previous exposure. Given the cross-reactivity and uncertainty associated with DENV antibody data (Anantapreecha, et al., 2007; Halstead & Simasathien, 1970), an additional strength of these analyses was the use of interval between infections as a presumed proxy for shifts in antibody titers and specificities over time, made possible by the sensitive nature of the surveillance system to capture the full range of DENV infections, over a period of multiple years.

The clinical trial data presented in study three are quite unique because of the fully factorial dosing design; other tetravalent candidates have been evaluated only in a small number of formulations with select dosing changes (F. Guirakhoo, et al., 2002; Sabchareon, et al., 2002). This allowed an unprecedented opportunity to look at how changes in the dose of one

serotype influenced the serological response to others. Additionally, it was valuable to have the clinical trial data from earlier evaluations of the same monovalent candidates, allowing assessment of how the serological response to vaccination with a given serotype strain was altered upon incorporation into tetravalent formulations.

PUBLIC HEALTH CONTRIBUTIONS.

Interactions between heterologous flaviviruses.

The finding in study one of an association between preexisting JEV immunity and symptomatic DENV infection is a novel discovery, and one that contradicts the currently-accepted paradigm that immunity to heterologous flaviviruses has no effect or possibly a protective effect with subsequent DENV infection. Specific to JEV immunity, previous studies have reported either a possible attenuating effect of prior JEV vaccination on the occurrence of DHF (Hoke, et al., 1988) or no effect of JEV vaccination on the risk of hospitalization with DENV infection (R. V. Gibbons, et al., 2007). These studies were limited to hospitalized cases or DHF cases, however, and would not have been able to detect less dramatic clinical effects. This is notable because in study one, the association between JEV antibodies and DENV illness was limited to an increase in non-hospitalized illnesses; no association was observed between JEV antibodies and hospitalized illness or DHF. Further, they were based upon JEV vaccination histories and not on the presence or absence of JEV antibodies.

In study one, it was not possible to discern JEV antibodies that arose as a result of vaccination, natural exposure, or as a cross-reactive response to DENV infection. However, it is notable that the most significant increases in symptomatic illness with JEV NAbs were

observed in DENV-naïve children, for whom JEV NAbs would have been the least likely to have arisen as cross-reactivity. Importantly, JEV NAbs were associated with increases in the occurrence of non-hospitalized DENV illness and associated with illnesses of longer duration. Non-hospitalized illnesses represent the vast majority of symptomatic infections and are responsible for a much greater portion of the total burden of DENV disease than DHF, which is in fact a relatively rare outcome of DENV infection (Anderson, et al., 2007). Therefore, if this association is a true finding, JEV NAbs could contribute importantly to the total burden of DENV illness.

JEV NAbs were only associated with the occurrence of DHF in the cohort of young children (less than ten years of age) with DENV-multitypic profiles. The multitypic profiles in these younger children are perhaps more likely to be a reflection of cross-reactivity than multitypic profiles in older children, as younger children would have had less time to accumulate DENV infections and serotype-specific immunity. It is possible that, in these children, JEV NAbs were simply markers of a highly cross-reactive profile that was itself associated with DHF rather than a reflection of a true association between prior JEV exposure and DHF.

These findings should be interpreted cautiously and additional studies must be performed to confirm this association in other cohorts. The JEV vaccine has been shown to be effective in decreasing the occurrence of JEV encephalitis (Hoke, et al., 1988) and there is insufficient evidence from this single study to suggest that any changes should be made in policies to vaccinate against JEV. The results of study one do, however, suggest that the issue of heterologous flavivirus immunity and DENV merits renewed interest and research. In

particular, these findings suggest that DENV vaccine developers should collect detailed information on heterologous flavivirus vaccination histories and assess how prior flavivirus exposure and immunity may influence the safety and efficacy of DENV vaccines. The findings from these studies may be important in shaping DENV vaccine policies and programs in regions where JEV co-circulates or JEV vaccination is common, for example by informing the optimal timing of DENV vaccine administration relative to the JEV vaccine.

Future studies should evaluate the possibility that JEV NAb immunity elicited by the inactivated JEV vaccine and the live-attenuated JEV vaccine may interact with DENV differently, perhaps by comparing the association between JEV antibodies and DENV illnesses between regions that use different JEV vaccines. Or similarly, it may be that a history of natural exposure to JEV may have a different association with the clinical severity of a subsequent DENV infection than does a history of JEV vaccination. These associations could possibly be discerned through the use of assays to detect antibodies to the DENV protein NS1 (absent in JE-VAX recipients, present with live JEV exposure) (Shu, et al., 2001). DENV co-circulates with other flaviviruses throughout much of its range (JEV in South and Southeast Asia, yellow fever virus in Africa and Latin America, West Nile virus throughout large parts of both hemispheres) and potential modulatory effects of immunity to other flaviviruses on the clinical presentation of DENV infections should be similarly evaluated. Lastly, this study considered only antibody-mediated immunity and future studies should evaluate possible associations between JEV vaccine-derived and JEV exposure-derived cell-mediated immunity and innate immunity.

Evidence of cross-protection and a temporal aspect to disease risk.

The identification in study two of an increased probability of asymptomatic DENV infection for an interval of time following a previous DENV infection, suggesting a "window of protection", is an important finding. Previous studies have suggested that the complex role of cross-reactive antibodies in modulating the clinical outcome of DENV infection is titer-dependent (T. P. Endy, et al., 2004; Halstead, et al., 2002). Two major roadblocks have limited the further characterization of cross-reactive antibodies as protective or pathologic. First, presently-available serological assays cannot distinguish specific from cross-reactive immunity, and second, few studies have collected longitudinal antibody and clinical outcome data to include asymptomatic infections.

Two prior studies have analyzed the interval between DENV infections as a risk factor for the severity of the second infection. Guzman et al analyzed data from three isolated DENV outbreaks in Cuba and concluded that individuals infected twenty years after their first exposure were significantly more likely to develop DHF than those infected four years after their first exposure (M. G. Guzman, et al., 2000). They hypothesized that this increase in DHF was due to a waning of cross-reactive antibodies from protective to enhancing levels. The most influential study of temporal trends in disease risk was conducted by Sabin during World War II, who administered serial inoculations of DENV to human volunteers and varied the amount of time elapsed between the first and second exposure (Sabin, 1952). On the basis of the clinical responses to the second inoculation with a heterologous DENV serotype, he concluded that following the initial exposure there was a six month window of protection from illness, mediated by cross-reactive DENV antibodies. This estimate of a six month window of protection has been a highly influential finding, being quoted widely and

used commonly in mathematical models of DENV transmission dynamics and often being the most important parameter in recreating the oscillations in DENV incidence observed in the field (Nagao & Koelle, 2008).

This is the first study since Sabin's 1952 paper to report a window of cross-protection following infection, using data from a unique longitudinal study that spanned years and captured both symptomatic and asymptomatic infections. The presumed protective capability of high-titer cross-reactive antibodies is an important finding. As DENV vaccine development efforts intensify, there has been concern that some antibody responses to vaccination may be a reflection of cross-reactivity and that these antibodies may have unintended disease-enhancing effects. The finding that cross-reactive antibodies may, at sufficiently high titers, provide some protection against DENV illness is encouraging. However, the analysis in study two also found an increased risk of illness with greater time elapsed. With antibody decay; it may be that boosting is critical to maintaining the immune responses necessary to effect a protective immune profile.

The description of a time-dependent component to the risk of disease with sequential DENV infections may lend insight into the complicated dynamics of DENV epidemics, which are characterized by unpredictable fluctuations in incidence and severity. It may be that these fluctuations are driven by shifts in population-level cross-protection. The analysis in study two provided evidence of both short-term (temporary cross-protection) and long-term effects of cross-reactive immunity. That is, the effects of temporary cross-protection were only evident for children who were HI-negative prior to their first infection. In children with some HI immunity prior to their first infection, no temporal trends in disease

risk were observed. This is consistent with suggestions that the cross-protection afforded by the accumulation of serotype-specific responses to multiple DENV infections can attenuate the clinical severity of a third or fourth infection. The findings in study two indicate that an appropriate mathematical model should account for both temporary cross-protection and account for the accumulation of longer-term cross-protection with sequential infections. Further, these findings suggest that the window of cross-protection used in these models should be longer than the six month estimate typically applied and should reflect a function that decreases over time rather than a set amount of time. While the time scale of this study was likely too brief to capture the full duration of residual cross-protection, these findings suggests cross-protection lasting at last one to two years following infection.

Study two assumed that serotype-specific sterile immunity resulted from infection and that natural boosting from re-exposure could not have influenced the observed patterns. Future studies should seek to identify whether natural boosting occurs and investigate its importance in contributing to individual cross-protection and population-level herd immunity. Boosting could be evaluated, for example, using long-term prospective observation of individuals; if there is evidence that they have been 'infected,' as indicated by four-fold rises in antibody titers, more than four times during follow-up this would suggest that antibody boosting can occur with re-exposure to DENV serotypes. This is an important question in that DENV vaccines, if effective, will serve to decrease DENV transmission and any effects of boosting would be diminished.

Identifying interactions in multivalent vaccines.

The generation of strong antibody responses to all four DENV serotypes in tetravalent formulations has proven a challenging task, due to immunological and/or viral interference between strains. Study three found evidence of interference as well as facilitation in the overall immune response to vaccination; specifically, that higher doses of the dominant serotype in a formulation could increase seroconversion to non-dominant serotypes. With the caveat that current assays cannot distinguish if these are serotype-specific seroconversions or cross-reactive responses to the dominant serotype, studies such as study two, above, suggest that high-titer cross-reactive antibodies may be protective. Therefore, whether cross-reactive or specific, one could define the optimal immune response to tetravalent vaccination to be one that is broad (against all four serotypes) and high-titer. While this cross-protection was demonstrated to decrease over time, administration of booster doses could possibly restore protective immunity to high levels. Study three suggests that facilitation between DENV serotypes in vaccine formulations, once identified, could possibly be exploited by increasing the doses of serotypes that demonstrate facilitation to increase overall seroconversion rates.

Secondly, the findings from study three suggest that the total dose of virus administered in a multivalent vaccine and reactogenicity (an aggregate measure of the severity and duration of adverse events) are not necessarily correlated. High doses of the dominant serotype DENV-1, when administered with low doses of the other serotypes, were associated with the highest reactogenicity observed with any of the formulations. However, the reactogenic effect of high dose DENV-1 was diminished with the incorporation of other high dose serotypes. This suggests that increasing the dose of sub-dominant strains may paradoxically serve to

dampen the overall reactogenicity associated with tetravalent formulations, perhaps due to an improved competitive advantage of the sub-dominant strains. These findings may inform future efforts to optimize vaccine doses for multivalent vaccines. They suggest that increasing the total amount of virus delivered may not necessarily increase the reactogenicity of the formula, if subdominant (and less reactogenic) strains are increased to compete with the dominant strain. This may provide a means to increase overall seroconversion rates without associated increases in the occurrence of vaccine-associated adverse events.

Study three illustrates the utility of factorial dose-ranging trials in assessing the nature and degree of interactions between component viruses in multivalent formulations. This method should be considered for the development and dose-optimization of other multitypic viral vaccines. These findings in study three are of public health importance in that they contribute to an improved understanding of how DENV serotypes may interact in multivalent vaccines and further suggest that these interactions, once identified, could possibly be exploited to increase overall seroconversion rates while minimizing reactogenicity.

REFERENCES

A, A. N., Panthuyosri, N., Anantapreecha, S., Chanama, S., Sa-Ngasang, A., Sawanpanyalert, P., et al. (2008). Cross-reactive IgM responses in patients with dengue or Japanese encephalitis. *J Clin Virol, 42*(1), 75-77.

Almond, J., Clemens, J., Engers, H., Halstead, S., Khiem, H., Pablos-Mendez, A., et al. (2002). Accelerating the development and introduction of a dengue vaccine for poor children, 5-8 December 2001, Ho Chi Minh City, VietNam. *Vaccine, 20*(25-26), 3043.

Anantapreecha, S., A, A. N., Prakrong, S., Chanama, S., Sa-Ngasang, A., Sawanpanyalert, P., et al. (2007). Dengue virus cross-reactive hemagglutination inhibition antibody responses in patients with primary dengue virus infection. *Jpn J Infect Dis, 60*(5), 267-270.

Anderson, K. B., Chunsuttiwat, S., Nisalak, A., Mammen, M. P., Libraty, D. H., Rothman, A. L., et al. (2007). Burden of symptomatic dengue infection in children at primary school in Thailand: a prospective study. *Lancet, 369*(9571), 1452-1459.

Anonymous. (1997). *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control, 2nd edition*. Geneva: World Health Organization.

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., et al. (2006). Vascular Leakage in Severe Dengue Virus Infections: a Potential Role for the Nonstructural Viral Protein NS1 and Complement. *J Infect Dis, 193*(8), 1078-1088.

Balmaseda, A., Standish, K., Mercado, J., & al., E. (2010). Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua. . *J Infect Dis, 201*, 5-14.

Berger, R., & Just, M. (1988 Oct). Interference between strains in live virus vaccines. II: Combined vaccination with varicella and measles-mumps-rubella vaccine. *J Biol Stand, 16*(4), 275-279.

Berger, R., Just, M., & Glück, R. (1998 Oct). Interference between strains in live virus vaccines, I: Combined vaccination with measles, mumps and rubella vaccine. *J Biol Stand, 16*(4), 269-273.

Bhamarapravati, N., & Yoksan, S. (2000). Live attenuated tetravalent dengue vaccine. *Vaccine, 18 Suppl 2*, 44-47.

Blaney, J. E., Jr., Matro, J. M., Murphy, B. R., & Whitehead, S. S. (2005). 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, 79*(9), 5516-5528.

Blaney, J. E., Jr., Sathe, N. S., Hanson, C. T., Firestone, C. Y., Murphy, B. R., & Whitehead, S. S. (2007). Vaccine candidates for dengue virus type 1 (DEN1) generated by replacement of the structural genes of rDEN4 and rDEN4Delta30 with those of DEN1. *Virol J, 4*, 23.

Blok, J., McWilliam, S. M., Butler, H. C., Gibbs, A. J., Weiller, G., Herring, B. L., et al. (1992). Comparison of a dengue-2 virus and its candidate vaccine derivative: sequence relationships with the flaviviruses and other viruses. *Virology, 187*, 573-590.

Bond, J. O., & Hammon, W. M. (1970). Epidemiologic studies of possible cross protection between dengue and St. Louis encephalitis arboviruses in Florida. *Am J Epidemiol, 92*(5), 321-329.

Burke, D. S., & Monath, T. P. (2001). Flaviviruses. In D. M. Knipe & P. M. Mowley (Eds.), *Fields Virology* (4th edition ed., pp. 1043-1125). Philadelphia, PA: Lippincott, Williams, Wilkins.

Burke, D. S., Nisalak, A., Johnson, D. E., & Scott, R. M. (1988). A prospective study of dengue infections in Bangkok. *Am J Trop Med Hyg, 38*(1), 172-180.

Cardosa, J., Ooi, M. H., Tio, P. H., Perera, D., Holmes, E. C., Bibi, K., et al. (2009). Dengue virus serotype 2 from a sylvatic lineage isolated from a patient with dengue hemorrhagic fever. *PLoS Negl Trop Dis. , 3*(4), e423.

Case definitions. Dengue fever. (2000). *Epidemiol Bull, 21*(2), 14-15.

Chairulfatah, A., Setiabudi, D., Agoes, R., van Sprundel, M., & Colebunders, R. (2001). Hospital based clinical surveillance for dengue haemorrhagic fever in Bandung, Indonesia 1994-1995. *Acta Trop, 80*(2), 111-115.

Chang, K. J. (1997). Studies on the serological cross-reaction between dengue and Japanese encephalitis. *J Microbiol Immunol Infect, 30*(4), 207-218.

Chang, S. F., Huang, J. H., Chen, L. K., Su, C. L., Liao, T. L., Chien, L. J., et al. (2008). Retrospective serological study on sequential dengue virus serotypes 1 to 4 epidemics in Tainan City, Taiwan, 1994 to 2000. *J Microbiol Immunol Infect, 41*(5), 377-385.

Chareonsook, O., Foy, H. M., Teeraratkul, A., & Silarug, N. (1999). Changing epidemiology of dengue hemorrhagic fever in Thailand. *Epidemiol Infect, 122*(1), 161-166.

Clarke, D. H., & Casals, J. (1958). Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am J Trop Med Hyg, 7*, 561-573.

Cologna, R., & Rico-Hesse, R. (2003). American genotype structures decrease dengue virus output from human monocytes and dendritic cells. *J Virol, 77*(7), 3929-3938.

Cummings, D. A., Iamsirithaworn, S., Lessler, J. T., McDermott, A., Prasanthong, R., Nisalak, A., et al. (2009). The impact of the demographic transition on dengue in Thailand: insights from a statistical analysis and mathematical modeling. *PLoS Med, 6*(9), e1000139.

de Thoisy, B., Lacoste, V., Germain, A., Munoz-Jordan, J., Colon, C., Mauffrey, J. F., et al. (2009). Dengue infection in neotropical forest mammals. *Vector Borne Zoonotic Dis, 9*(2), 157-170.

Dechant, E. J., & Rigau-Perez, J. G. (1999). Hospitalizations for suspected dengue in Puerto Rico, 1991-1995: estimation by capture-recapture methods. The Puerto Rico Association of Epidemiologists. *Am J Trop Med Hyg, 61*(4), 574-578.

DeRoeck, D., Deen, J., & Clemens, J. D. (2003). Policymakers' views on dengue fever/dengue haemorrhagic fever and the need for dengue vaccines in four southeast Asian countries. *Vaccine, 22*(1), 121-129.

Dick, G., & Horgan, E. (1952). Vaccination by scarification with combined 17D yellow fever and vaccinia vaccine. *J Hyg (Camb), 50*, 376-383.

Division of Vector-Borne Infectious Diseases, N. C. f. I. D., Centers for Disease Control and Prevention. (1997, 1 June). Information on dengue fever and dengue hemorrhagic fever. Retrieved 3 September, 2000

dos Santos, L. U., & de Andrade, C. F. (1997). Survey of cyclopids (Crustacea, Copepoda) in Brazil and preliminary screening of their potential as dengue vector predators. *Rev Saude Publica, 31*(3), 221-226.

Edelman, R., Wasserman, S. S., Bodison, S. A., Putnak, R. J., Eckels, K. H., Tang, D., et al. (2003). Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. *Am J Trop Med Hyg, 69*(6 Suppl), 48-60.

Ellis, R. D., Fukuda, M. M., McDaniel, P., Welch, K., Nisalak, A., Murray, C. K., et al. (2006). Causes of Fever in adults on the thai-myanmar border. *Am J Trop Med Hyg, 74*(1), 108-113.

Endy, T., Anderson, K., Nisalak, A., Yoon, I., Green, S., Rothman, A., et al. (2010). Determinants of Inapparent and Symptomatic Dengue Infection in a Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *Manuscript submitted for publication*.

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

Endy, T. P., & Nisalak, A. (2002). Japanese encephalitis virus: ecology and epidemiology. *Curr Top Microbiol Immunol, 267*, 11-48.

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). 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, 189*(6), 990-1000.

Endy, T. P., Nisalak, A., Chunsuttiwat, S., Libraty, D. H., Green, S., Rothman, A. L., et al. (2002). Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. *Am J Epidemiol, 156*(1), 52-59.

Fried, J., Gibbons, R., Kalayanarooj, S., Thomas, S., Srikiatkhachorn, A., Yoon, I., et al. (2010). 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., 4*(3), e617.

From the Centers for Disease Control and Prevention. Dengue fever at the US-Mexico border, 1995-1996. (1996). *JAMA, 276*(18), 1464-1465.

Gambel, J. M., Drabick, J. J., Swalko, M. A., Henchal, E. A., Rossi, C. A., & Martinez-Lopez, L. (1999). Dengue among United Nations mission in Haiti personnel, 1995: implications for preventive medicine. *Mil Med, 164*(4), 300-302.

Gibbons, R. V., Kalanarooj, S., Jarman, R. G., Nisalak, A., Vaughn, D. W., Endy, T. P., et al. (2007). Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences. *Am J Trop Med Hyg, 77*(5), 910-913.

Gibbons, R. V. E., T P; Nisalak , A.;Chunsutthiwat, S.; Vaughn, D W; Green, S; Ennis, F. A.; Rothman, A.; Libraty, D H. (2004, 4-6 October 2004). *The relationship of preexisting dengue virus neutralizing antibody levels to viremia and disease severity in secondary dengue infections.* Paper presented at the WHO/TDR and PDVI Joint Workshop on Dengue Diagnostics and Dengue Classification/Case Management, Geneva, Switzerland.

Gratz, N. G. (2004). Critical review of the vector status of Aedes albopictus. *Med Vet Entomol, 18*(3), 215-227.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever: Its history and resurgence as a global public health problem. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 1-22). New York: CAB International.

Gubler, D. J. (2002a). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol, 10*(2), 100-103.

Gubler, D. J. (2002b). The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res, 33*(4), 330-342.

Gubler, D. J., & Clark, G. G. (1995). Dengue/Dengue Hemorrhagic Fever: The Emergence of a Global Health Problem. *Emerg Infect Dis, 1*(2), 55-57.

Guirakhoo, F., Kitchner, S., Morrison, D., Forrat, R., McCarthy, K., Nichols, R., et al. (2006). Live attenuated chimeric yellow fever dengue type 2 (Chimerivax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity. *Human Vaccines, 2*(2), 60-67.

Guirakhoo, F., Pugachev, K., Arroyo, J., Miller, C., Zhang, Z. X., Weltzin, R., et al. (2002). Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. *Virology, 298*(1), 146-159.

Gulati, S., & Maheshwari, A. (2007). Atypical manifestations of dengue. *Trop Med Int Health, 12*(9), 1087-1095.

Guy, B., Barban, V., Mantel, N., Aguirre, M., Gulia, S., Pontvianne, J., et al. (2009). Evaluation of interferences between dengue vaccine serotypes in a monkey model. *Am J Trop Med Hyg, 80*(2), 302-311.

Guzman, M. G., Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Montes, T., Vazquez, S., et al. (2007). Neutralizing antibodies after infection with dengue 1 virus. *Emerg Infect Dis, 13*(2), 282-286.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazquez, S., et al. (2000). Epidemiologic studies on dengue in Santiago de Cuba, 1997. *Am J Epidemiol, 152*(9), 793-799.

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Vazquez, S., & Halstead, S. B. (2002). Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica, 11*(4), 223-227.

Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S., & Morier, L. (1990). Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg, 42*, 179-184.

Halstead, S. B. (1980). Dengue haemorrhagic fever-a public health problem and a field for research. *Bull World Health Organ, 58*(1), 1-21.

Halstead, S. B. (1980). Immunological Parameters Of Togavirus Disease Syndromes. *The Togaviruses*, 107-173.

Halstead, S. B. (1989). Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev Infect Dis, 11*(Suppl 4), S830-S839.

Halstead, S. B. (1997). Epidemiology of dengue and dengue hemorrhagic fever. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 23-44). New York: CAB International.

Halstead, S. B. (2006). Dengue in the Americas and Southeast Asia: do they differ? *Rev Panam Salud Publica, 20*(6), 407-415.

Halstead, S. B., Chow, J., & Marchette, N. J. (1973). Immunologic enhancement of dengue virus replication. *Nature New Biology, 243*(122), 24-26.

Halstead, S. B., Lan, N. T., Myint, T. T., Shwe, T. N., Nisalak, A., Kalyanarooj, S., et al. (2002). Dengue hemorrhagic Fever in infants: research opportunities ignored. *Emerg Infect Dis, 8*(12), 1474-1479.

Halstead, S. B., Nimmannitya, S., & Cohen, S. N. (1970). Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J Biol Med, 42*, 311-328.

Halstead, S. B., Rojanasuphot, S., & Sangkawibha, N. (1983). Original antigenic sin in dengue. *Am J Trop Med Hyg, 32*(1), 154-156.

Halstead, S. B., & Simasathien, P. (1970). Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. *Yale J Biol Med, 42*, 276-292.

Hammon, W. M. (1969). Observations on dengue fever, benign protector and killer: a Dr Jekyll and Mr Hyde. *Am J Trop Med Hyg, 18*(2), 159-165.

Hammon, W. M., Rudnick, A., & Sather, G. E. (1960). Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. *Science, 131*, 1102-1103.

Hammond, S. N., Balmaseda, A., Perez, L., Tellez, Y., Saborio, S. I., Mercado, J. C., et al. (2005). Differences In Dengue Severity In Infants, Children, And Adults In A 3-Year Hospital-Based Study In Nicaragua. *Am J Trop Med Hyg, 73*(6), 1063-1070.

Hawley, W. A., Reiter, P., Copeland, R. S., Pumpuni, C. B., & Craig, G. B., Jr. (1987). Aedes albopictus in North America: probable introduction in used tires from northern Asia. *Science, 236*(4805), 1114-1116.

Hohdatsu, T., Yamada, H., Ishizuka, Y., & Koyama, H. (1993). Enhancement and Neutralization of Feline Infectious Peritonitis Virus Infection in Feline Macrophages by Neutralizing Monoclonal Antibodies Recognizing Different Epitopes. *Microbiol Immunol, 37*, 499-504.

Hoke, C. H., Jr., Nisalak, A., Sangawhipa, N., Jatanasen, S., Laorakpongse, T., Innis, B. L., et al. (1988). Protection against Japanese encephalitis by inactivated vaccines. *N Engl J Med, 319*, 608-614.

Innis, B. L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Suntayakorn, S., et al. (1989). An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg, 40*(4), 418-427.

Irvin, N., Hoddle, M. S., O'Brochta, D. A., Carey, B., & Atkinson, P. W. (2004). Assessing fitness costs for transgenic Aedes aegypti expressing the GFP marker and transposase genes. *Proc Natl Acad Sci U S A*.

Jarman, R. G., Holmes, E. C., Rodpradit, P., Klungthong, C., Gibbons, R. V., Nisalak, A., et al. (2008). Microevolution of Dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. *J Virol, 82*(11), 5494-5500.

Kalayanarooj, S., Gibbons, R. V., Vaughn, D., Green, S., Nisalak, A., Jarman, R. G., et al. (2007). Blood group AB is associated with increased risk for severe dengue disease in secondary infections. *J Infect Dis, 195*(7), 1014-1017.

Kalayanarooj, S., Rimal, H. S., Andjaparidze, A., Vatcharasaevee, V., Nisalak, A., Jarman, R. G., et al. (2007). Clinical Intervention and Molecular Characteristics of a Dengue Hemorrhagic Fever Outbreak in Timor Leste, 2005. *Am J Trop Med Hyg, 77*(3), 534-537.

Kanesa-Thasan, N., Edelman, R., Tacket, C. O., Wasserman, S. S., Vaughn, D. W., Coster, T. S., et al. (2003). Phase 1 studies of Walter Reed Army Institute of Research candidate attenuated dengue vaccines: selection of safe and immunogenic monovalent vaccines. *Am J Trop Med Hyg, 69*(6 Suppl), 17-23.

Kanesa-thasan, N., Hernandez , L., Lyons, A., Putnak, R., Sun, W., McKinney, D., et al. (2002, 6-8 May 2002). *A phase I study of the WRAIR tetravalent live attenuated dengue vaccine in flavivirus-immune adult volunteers.* Paper presented at the 5th Annual Conference on Vaccine Research, Baltimore MD.

Kanesa-thasan, N., Sun, W., Kim-Ahn, G., Van Albert, S., Putnak, J. R., King, A., et al. (2001). Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. *Vaccine, 19*(23-24), 3179-3188.

Kim, H. W., Arrobio, J. O., Brandt, C. D., Jeffries, B. C., Pyles, G., Reid, J. L., et al. (1973). Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. *Am J Epidemiol, 98*(3), 216-225.

Kimura, R., & Hotta, S. (1944). Studies on dengue: anti-dengue active immunization experiments in mice. *Japanese Journal of Bacteriology, 1*, 96-99.

Kitchener, S., Nissen, M., Nasveld, P., Forrat, R., Yoksan, S., Lang, J., et al. (2006). Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. *Vaccine, 24*(9), 1238-1241.

Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L., & Burke, D. S. (1989). Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. *Am J Trop Med Hyg, 40*(4), 444-451.

Konishi, E., Yamaoka, M., Khin Sane, W., Kurane, I., Takada, K., & Mason, P. W. (1999). The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes. *J Virol, 73*(7), 5527-5534.

Kou, Z., Quinn, M., Chen, H., Rodrigo, W., Rose, R., Schlesinger, J., et al. (2008). Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. *Journal of Medical Virology, 80*(1), 134-136.

Kouri, G., Guzman, M. G., Valdes, L., Carbonel, I., del Rosario, D., Vazquez, S., et al. (1998). Reemergence of dengue in Cuba: a 1997 epidemic in Santiago de Cuba. *Emerg Infect Dis, 4*(1), 89-92.

Kouri, G., Mas, P., Guzman, M. G., Soler, M., Goyenechea, A., & Morier, L. (1983). Dengue hemorrhagic fever in Cuba, 1981: rapid diagnosis of the etiologic agent. *Bull Pan Am Health Organ, 17*, 126-132.

Kouri, G. P., Guzman, M. G., Bravo, J. R., & Triana, C. (1989). Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. *Bull World Health Organ, 67*, 375-380.

Kuno, G., Chang, G. J., Tsuchiya, K. R., Karabatsos, N., & Cropp, C. B. (1998). Phylogeny of the genus Flavivirus. *J Virol, 72*(1), 73-83.

Kuno, G., Gubler, D. J., & Oliver, A. (1993). Use of Original Antigenic Sin Theory to Determine the Serotypes of Previous Dengue Infections. *Trans R Soc Trop Med Hyg, 87*, 103-105.

Lewis, J. A., Chang, G. J., Lanciotti, R. S., Kinney, R. M., Mayer, L. W., & Trent, D. W. (1993). Phylogenetic Relationships of Dengue-2 Viruses. *Virology, 197*, 216-224.

Lobigs, M., Larena, M., Alsharifi, M., Lee, E., & Pavy, M. (2009). Live chimeric and inactivated Japanese encephalitis virus vaccines differ in their cross-protective values against Murray Valley encephalitis virus. *Journal of Virology, 83*(6), 2436-2445.

Locally Acquired Dengue --- Key West, Florida, 2009--2010. (2010). *MMWR Morb Mortal Wkly Rep, 59*(19), 577-581.

Mackenzie, J. S., Gubler, D. J., & Petersen, L. R. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med, 10*(12 Suppl), S98-S109.

Makino, Y., Tadano, M., Saito, M., Maneekarn, N., Sittisombut, N., Sirisanthana, V., et al. (1994). Studies on serological cross-reaction in sequential flavivirus infections. *Microbiol Immunol, 38*, 951-955.

Mammen, M. P., Pimgate, C., Koenraadt, C. J., Rothman, A. L., Aldstadt, J., Nisalak, A., et al. (2008). Spatial and temporal clustering of dengue virus transmission in Thai villages. *PLoS Med, 5*(11), e205.

Martin, D. A., Biggerstaff, B. J., Allen, B., Johnson, A. J., Lanciotti, R. S., & Roehrig, J. T. (2002). Use of immunoglobulin m cross-reactions in differential diagnosis of human flaviviral encephalitis infections in the United States. *Clin Diagn Lab Immunol, 9*(3), 544-549.

Mascola, J. R., Mathieson, B. J., Zack, P. M., Walker, M. C., Halstead, S. B., & Burke, D. S. (1993). Summary Report - Workshop on the Potential Risks of Antibody- Dependent Enhancement in Human HIV Vaccine Trials. *AIDS Res Hum Retroviruses, 9*, 1175-1184.

Meyer, H., Hopps, H., Bernheim, B., & Douglas, R. (1967). Combined measles-smallpox and other vaccines. First International Conference on Vaccines Against Viral and Rickettsial Diseases in Man. Pan American Health Organization. *Scientific Publ. No. 147, Washington, DC, Pan American Health Organization*, 336-342.

Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., et al. (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med, 9*(7), 921-927.

Morens, D. M. (1994). Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin Infect Dis, 19*, 500-512.

Morrison, D., Legg, T., Billings, C., Forrat, R., Yoksan, S., & Lang, J. (2010). A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naive adults. *J Infect Dis, 201*(3), 370-377.

Mukhopadhyay, S., Kuhn, R. J., & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol, 3*(1), 13-22.

Nagao, Y., & Koelle, K. (2008). Decreases in dengue transmission may act to increase the incidence of dengue hemorrhagic fever. *Proc Natl Acad Sci U S A, 105*(6), 2238-2243.

Nagao, Y., Thavara, U., Chitnumsup, P., Tawatsin, A., Chansang, C., & Campbell-Lendrum, D. (2003). Climatic and social risk factors for Aedes infestation in rural Thailand. *Trop Med Int Health, 8*(7), 650-659.

NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report. *U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Allergy and Infectious Disease.*

Nimmannitya, S. (1997). Dengue Hemorrhagic Fever: Diagnosis and Management. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 133-145). Cambridge: CAB International.

Nimmannitya, S., Halstead, S. B., Cohen, S., & Margiotta, M. R. (1969). Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *Am J Trop Med Hyg, 18*(6), 954-971.

Nisalak, A., Endy, T. P., Nimmannitya, S., Kalayanarooj, S., Thisayakorn, U., Scott, R. M., et al. (2003). Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *Am J Trop Med Hyg, 68*(2), 191-202.

Nishiura, H., & Halstead, S. B. (2007). Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. *J Infect Dis, 195*(7), 1007-1013.

Oliphant, T., Nybakken, G. E., Engle, M., Xu, Q., Nelson, C. A., Sukupolvi-Petty, S., et al. (2006). Antibody Recognition and Neutralization Determinants on Domains I and II of West Nile Virus Envelope Protein *J Virol. , 80*(24), 12149-12159.

Ong, A., Sandar, M., Chen, M. I., & Sin, L. Y. (2007). Fatal dengue hemorrhagic fever in adults during a dengue epidemic in Singapore. *Int J Infect Dis, 11*(3), 263-267.

Patriarca, P., Wright, P., & John, T. (1991). Factors affecting immunogenicity of oral poliovirus vaccine in developing countries: review. *Rev Infect Dis, 13*(926-939).

Payne, A. (1960). Oral immunization against poliomyelitis. *Bull WHO, 23*, 695-703.

Porter, K. R., Beckett, C. G., Kosasih, H., Tan, R. I., Alisjahbana, B., Rudiman, P. I., et al. (2005). Epidemiology Of Dengue And Dengue Hemorrhagic Fever In A Cohort Of Adults Living In Bandung, West Java, Indonesia. *Am J Trop Med Hyg, 72*(1), 60-66.

Price, W. H., & Thind, I. S. (1972). The mechanism of cross-protection afforded by dengue virus against West Nile virus in hamsters. *J Hyg Camb, 70*, 611-617.

Ramos, M. M., Mohammed, H., Zielinski-Gutierrez, E., Hayden, M. H., Lopez, J. L., Fournier, M., et al. (2008). Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico border: results of a household-based seroepidemiologic survey, December 2005. *Am J Trop Med Hyg, 78*(3), 364-369.

Rauh, L., & Schmidt, R. (1965). Measles immunization with killed virus vaccine. Serum antibody titers and experience with exposure to measles epidemic. *American Journal of Diseases of Children, 109*, 232-237.

Rico-Hesse, R. (1990). Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology, 174*, 479-493.

Rico-Hesse, R., Harrison, L. M., Salas, R. A., Tovar, D., Nisalak, A., Ramos, C., et al. (1997). Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology, 230*, 244-251.

Rodhain, F., & Rosen, L. (1997). Mosquito vectors and dengue virus-vector relationships. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 45-60). New York: CAB International.

Rush, B. (1789). An account of the bilious remitting fever, as it appeared in Philadelphia, in the summer and autumn of the year 1780 *Medical Inquires and Observations* (1 ed., pp. 89-100). Philadelphia: Prichard and Hall.

Russell, P. (1978, May 1978). *Dengue in the Caribbean, 1977*. Paper presented at the Dengue in the Caribean, 1977. Proceedings of a workshop held in Montego Bay, Jamaica., Montego Bay, Jamaica.

Russell, P. K., Nisalak, A., Sukhavachana, P., & Vivona, S. (1967). A plaque reduction test for dengue virus neutralization antibodies. *J Immunol, 99*(2), 285-290.

Sabchareon, A., Lang, J., Chanthavanich, P., Yoksan, S., Forrat, R., Attanath, P., et al. (2002). Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. *Am J Trop Med Hyg, 66*(3), 264-272.

Sabin, A. B. (1952). Research on dengue during World War II. *Am J Trop Med Hyg, 1*, 30-50.

Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., et al. (1984). Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol, 120*, 653-669.

Shope, R. E. (1997). Concepts of control of Japanese encephalitis and dengue. *Southeast Asian J Trop Med Public Health, 28*(Suppl 2), 131-134.

Shu, P. Y., Chen, L. K., Chang, S. F., Yueh, Y. Y., Chow, L., Chien, L. J., et al. (2001). Antibody to the nonstructural protein NS1 of Japanese encephalitis virus: potential application of mAb-based indirect ELISA to differentiate infection from vaccination. *Vaccine, 19*(13-14), 1753-1763.

Siler, J. F., Hall, M. W., & Hitchens, A. P. (1926). Dengue: Its history, epidemilogy, mechanism of transmission, etiology, clinical manifestations, immunity, and prevention. *The Philippine Journal of Science, 29*, 1-304.

Simasathien, S., Thomas, S. J., Watanaveeradej, V., Nisalak, A., Barberousse, C., Innis, B. L., et al. (2008). Safety and Immunogenicity of a Tetravalent Live-attenuated Dengue Vaccine in Flavivirus Naive Children. *Am J Trop Med Hyg, 78*(3), 426-433.

Simmons, J. S., St John, J. H., & Reynolds, F. H. K. (1931). Experimental studies of dengue. *The Philippine Journal of Science, 44*, 1-252.

Skowronski, D. M., De Serres, G., Crowcroft, N. S., Janjua, N. Z., Boulianne, N., Hottes, T. S., et al. (2010). Association between the 2008-09 seasonal influenza vaccine and pandemic H1N1 illness during Spring-Summer 2009: four observational studies from Canada. *PLoS Med, 7*(4), e1000258.

Smith, C. E. (1956). The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. *J Trop Med Hyg, 59*(10), 243-251.

Stephens, H. A., Klaythong, R., Sirikong, M., Vaughn, D. W., Green, S., Kalayanarooj, S., et al. (2002). HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. *Tissue Antigens, 60*(4), 309-318.

Stephenson, J. R. (2005). Understanding dengue pathogenesis: implications for vaccine design. *Bull World Health Organ, 83*(4), 308-314.

Strategic direction for dengue research: Disease burden and epidemiological trends. (Feb 2002.). *World Health Organization.* .

Sun, W., Edelman, R., Kanesa-Thasan, N., Eckels, K. H., Putnak, J. R., King, A. D., et al. (2003). Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. *Am J Trop Med Hyg, 69*(6 Suppl), 24-31.

Tarr, G. C., & Hammon, W. M. (1974). Cross-protection between group B arboviruses: Resistance in mice ot Japanese B encephalitis and St. Louis encephalitis viruses induce by dengue virus immunization. *Infect Immun, 9*(5), 909-915.

Thein, S., Aung, M. M., Shwe, T. N., Aye, M., Zaw, A., Aye, K., et al. (1997). Risk factors in dengue shock syndrome. *Am J Trop Med Hyg, 56*(5), 566-572.

Thisyakorn, U., & Nimmannitya, S. (1993). Nutritional Status of Children with Dengue Hemorrhagic Fever. *Clin Infect Dis, 16*, 295-297.

Thomas, S., Nisalak, A., Anderson, K., Libraty, D., Kalayanarooj, S., Vaughn, D., et al. (2009). 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., 81*(5), 825-833.

U.S. Department of Health and Human Services, N. I. o. H., National Institute of Allergy and Infectious Disease. NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report.

Vorndam, V., & Kuno, G. (1997). Laboratory diagnosis of dengue virus infections. In D. J. Gubler & G. Kuno (Eds.), *Dengue and dengue hemorrhagic fever* (pp. 313-333). Cambridge: CAB International.

Wahala, M. P. B., Wahala, E. F., Donaldson, R. A., Accavitti-Loper, M. A., Baric, R. S., & deSilva, A. M. (2010). Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses. *PLoS Pathog. , 6*(3), e1000821.

Watts, D. M., Harrison, B. A., Pantuwatana, S., Klein, T. A., & Burke, D. S. (1985). Failure to detect natural transovarial transmission of dengue viruses by Aedes aegypti and Aedes albopictus (Diptera: Culicidae). *J Med Entomol, 22*(3), 261-265.

Weaver, S. C., & Barrett, A. D. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nat Rev Microbiol, 2*(10), 789-801.

Weaver, S. C., & Vasilakis, N. (2009). Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infection, Genetics and Evolution 9*, 523-540.

Whitehead, S. S., Blaney, J. E., Durbin, A. P., & Murphy, B. R. (2007). Prospects for a dengue virus vaccine. *Nat Rev Microbiol, 5*(7), 518-528.

Yasuno, M., & Tonn, R. J. (1970). A study of biting habits of Aedes aegypti in Bangkok, Thailand. *Bull World Health Organ, 43*(2), 319-325.

Zhang, C., Mammen, M. P., Jr., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Monkongdee, P., et al. (2005). Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. *J Virol, 79*(24), 15123-15130.

Zhou, H., & Deem, M. (2006). Sculpting the immunological response to dengue fever by polytopic vaccination. *Vaccine, 24*(14), 2451-2459.