Dengue: a trilogy of people, mosquitoes and the virus. Current epidemiology and pathogenesis in (non-)endemic settings
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Dengue consists of a spectrum of disease manifestations caused by four serotypes of Dengue virus, the most prevalent arthropod-borne virus affecting humans in the tropics and subtropics. The incidence of dengue and its geographical distribution have increased dramatically in the past 6 decades. While the majority of patients recover following a self-limiting non-severe clinical course, a small proportion progresses to severe, potentially fatal disease. The disease burden is high; the economic impact of dengue is considerable in terms of medical care, mosquito control measures and the loss of working hours. Due to the increase of population sizes, uncontrolled urbanization, migration and mobility of the human host, proliferation of vector breeding sites, unsuccessful vector control and the current lack of an effective vaccine, it is likely that dengue will continue to represent an important public health problem for many years to come. This thesis consists of a series of investigations into the aspects of the human, mosquito and viral factors that contribute to the epidemiology, persistence and pathogenesis of endemic dengue in Vietnam.

Khoa T.D. Thai was born on April 25th, 1980 in Can Tho City, Vietnam. Due to the political and economic instability after the Vietnam war, his family fled Vietnam (1983) and arrived in the Netherlands in 1986. Khoa obtained his atheneum diploma at the Willem Blaeu College in Alkmaar in 1999, after which he studied medicine at the Academic Medical Center - University of Amsterdam. During his medical study, he participated in a research project from 2005 to 2007, which was funded by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO). He worked as a medical intern in Tanzania and received his medical degree in May 2007. Khoa was awarded a Mozaïek fellowship by the Netherlands Organization for Scientific Research (NWO) in 2007 and conducted parts of this thesis in the Oxford Clinical Research Unit (OUCRU), Ho Chi Minh City, Vietnam. Khoa Thai is married to Thi Ngoc Nu, Huynh (2007) and they have a son, Hoang Minh, Thai.
PHÒNG CHỐNG BỆNH SỐT XUART HUYẾT

MUỐI VĂN TRUYỀN BỆNH
HAY DIỆT CHUNG

VỌNG BỘI CỦA MUỖI VĂN

THÁ CA
BE DIỆT LẠNH QUẢNG
LA BIỆN PHÁP TỐT NHẤT

THU GOM VA HUY BỘ CÁC
VẬT PỆ THẢI

VIỆN PASTEUR NHA TRANG
TRUNG TÂM Y TẾ ĐỊ PHÒNG BỆNH TRƯỜNG

Khoa

Patient

Incident start
measurement cohort

Epistemology

20000 /ka/yr

xa2

xa3
Dengue: a trilogy of people, mosquitoes and the virus.
Current epidemiology and pathogenesis in (non-)endemic settings.

Thesis, University of Amsterdam, the Netherlands


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Dengue: a trilogy of people, mosquitoes and the virus
Current epidemiology and pathogenesis in (non-)endemic settings.

ACADEMISCH PROEFSCHRIFT
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aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 8 mei 2012, te 10:00 uur

door

Thai Dang Khoa Thai

geboren te Can Tho, Vietnam
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|                  | Prof. dr. M.D. de Jong |
| Copromotors:     | dr. P.J. de Vries  
|                  | dr. H.R. van Doorn |
| Overige leden:   | Prof. dr. M.P. Grobusch  
|                  | Prof. dr. H. Zaaijer  
|                  | Prof. dr. N. Nagelkerke  
|                  | Prof. dr. M. Borgdorff  
|                  | Prof. dr. Tran Tinh Hien  
|                  | Prof. dr. A. Osterhaus |

Faculteit der Geneeskunde

“It is difficult to say what is impossible, for the dream of yesterday is the hope of today and the reality of tomorrow.”  
(Robert H. Goddard 1882 - 1945)
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Dengue consists of a spectrum of disease manifestations caused by four serotypes of Dengue virus, the most prevalent arthropod-borne virus affecting humans in the tropics and subtropics. The incidence of dengue and its geographical distribution have increased dramatically in the past 6 decades. While the majority of patients recover following a self-limiting non-severe clinical course, a small proportion progresses to severe, potentially fatal disease. The disease burden is high; the economic impact of dengue is considerable in terms of medical care, mosquito control measures and the loss of working hours. Due to the increase of population sizes, uncontrolled urbanization, migration and mobility of the human host, proliferation of vector breeding sites, unsuccessful vector control and the current lack of an effective vaccine, it is likely that dengue will continue to represent an important public health problem for many years to come. This thesis consists of a series of investigations into the aspects of the human, mosquito and viral factors that contribute to the epidemiology, persistence and pathogenesis of endemic dengue in Vietnam.
1. Dengue Epidemiology, Burden of Disease and Transmission Dynamics

The World Health Organization (WHO) ranks dengue among the most important infectious diseases with major impact on international public health. The geographical distribution is expanding and the transmission rates are increasing. Recent estimates indicate that approximately 3.5 billion people, ~55% of the world’s population live in countries at risk for locally acquired dengue virus (DENV) infection. DENV transmission and disease are determined by a complicated combination of factors involving the (i) virus, (ii) mosquito vector, (iii) human host and (iv) environment. Many inter-related factors such as biological and demographics issues influence dengue epidemiology and transmission.

1.1 The Virus

Dengue viruses belong to the genus *Flavivirus*, family *Flaviviridae*, and exist as four closely related but antigenically distinct viruses denoted DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virions are spherical particles 40-50 nm in diameter, with a lipid envelope enclosing an isometric nucleocapsid 30 nm in diameter. The virion envelope has a fringe of fine surface projections that consist of the envelope and membrane structural proteins. Similar to other flaviviruses, DENVs are single stranded, positive-sense, RNA viruses with a genome of approximately 11 Kb. The RNA contains a single long open reading frame which encodes three structural proteins (C, prM, E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Short untranslated regions (UTRs) at each end of the genome are required for replication. Mature virions contain three structural proteins, the capsid protein C, membrane protein M, and the envelope protein E. The E protein has three distinct structural domains. Domain I is structurally positioned between domain II, the homodimerization domain, and the immunoglobulin-like domain III.

1.2 The Mosquito Vector

All dengue serotypes are transmitted to humans through the bites of infected female *Aedes* mosquitoes of the subgenus *Stegomyia*. *Aedes aegypti* is the principle vector for human disease and is closely associated with human dwelling; larvae are mostly found in artificial water-filled containers or natural sites. Most biting activity occurs in the early morning or late afternoon, and the mosquito becomes infective after an extrinsic incubation period of 10-12 days. In recent decades *Ae. albopictus* has spread from Asia to Africa, the Americas and Europe, notably aided by the international trade in used tyres or ornamental plants (e.g. lucky bamboo (*Dracaena sanderiana*) in which eggs are deposited when they contain rainwater.

1.3 The Host

After an incubation period of 4-7 days, clinical manifestations vary broadly, ranging from asymptomatic, mild undifferentiated febrile illness through to severe dengue (i.e. dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) of which DSS is the most common life threatening syndrome. Dengue has been described as the tip of the iceberg, as less than 10% of symptomatic dengue cases are reported and ~85% of all DENV infections are asymptomatic or subclinical. Individual risk factors determine the severity of disease and include secondary infection with a different serotype, age, ethnicity and possibly chronic underlying diseases. Young children in particular may be less able than adults to compensate for capillary leakage and are consequently at greater risk of DSS.

1.4 The Environment

Although dengue transmission dynamics are multifactorial, environmental factors such as temperature and rainfall play a prominent role. More specifically, temperature affects the length of the gonotrophic cycle. Because the majority of breeding sites are outdoors, warm temperature and high moisture contribute to increased adult survival. Daily, seasonal and interannual variability in temperature, humidity and rainfall can influence mosquito populations and vectorial competence. Interannual variability in climate has also been associated to the state or the intensity of the El Niño Southern Oscillation (ENSO). The ENSO is an atmosphere-ocean coupled system that produces quasi-periodic short-term climate and sea surface temperature changes in the Pacific region that impact on weather worldwide. One indicator statistic of the ENSO state is the Southern Oscillation Index (SOI), which is the normalized difference in atmospheric pressure between Darwin and Tahiti. Vector abundance and *Ae. aegypti* infestations are not uniformly distributed throughout residential areas, resulting in spatial heterogeneity of dengue incidence. Sequential probing and feeding of the vector also contribute to spatial heterogeneity. Once an infective mosquito enters a house or a member of a household becomes infected, the probability of multiple infections in the household increases and may result in clusters of DENV infections.
2. Dengue Epidemiology in Vietnam

Dengue is a growing public health problem in Vietnam. Dengue-like illness was first recorded in Vietnam in 1913 and epidemics occurred in the northern and central provinces, whereas southern Vietnam experienced its first dengue epidemic in 1929. Thereafter, the number of reports and the number of DENV-infected patients reported by the Vietnamese Ministry of Health have increased. Over time, the morbidity and mortality of dengue have increased and DHF epidemics occur throughout all provinces. The outbreak trend of DHF in the country has become irregular, with a high inter-epidemic background since 1963. All dengue serotypes are circulating. The case-fatality rate is dropping over time, probably reflecting increased awareness and improved supportive care protocols. More recently, an increasing proportion of adolescents and adults develop DHF, compared to the days when DHF was considered primarily a paediatric illness.

2.1 Dengue in Binh Thuan province, Southern Vietnam

Dengue is hyperendemic in Binh Thuan province, southern Vietnam. DF accounts for one-third of cases of acute undifferentiated fever. The estimated annual incidence of DENV infection in this province is 11.7%. The majority of uncomplicated infections are not recognised as dengue cases, which leads to substantial under-reporting of dengue in the health information systems. Cases of complicated dengue are routinely notified to the Provincial Center for Preventive Medicine, mostly without laboratory confirmation. The annual incidence of notified cases of complicated dengue fever (i.e. DHF/DSS) varied in communities from 0.2/1,000 to 7.9/1,000 population between 1999 and 2003.

2.2 Basic Reproduction Number (R₀)

A key parameter for understanding the epidemiology of dengue in a community is its basic reproduction number, denoted as R₀. R₀ is the average number of secondary infections produced when one infected individual is introduced into a naïve population. If R₀ is greater than one, the number of people infected increases, and if R₀ is less than one, that number declines. Thus, if sustained disease control reduces transmission intensity by a factor that exceeds R₀, dengue will eventually be eliminated. The R₀ is related to 1) the average age at infection or 2) herd immunity. Basic reproduction numbers estimated for dengue range between 1.33 and 11.6. Using the age-stratified prevalence of dengue neutralizing antibody obtained in an area of high dengue endemicity, dengue type-specific R₀ values ranged from 4.3–5.8, whereas using epidemic data from Brazil, R₀ ranged from 3.8 to 5.1.

3. Dengue Manifestations, Diagnosis, Treatment and Prevention

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. Dengue is a self-limiting disease in most (~80%) DENV infected patients; some (~5%) infections may require hospital care. Main principles of clinical management are early detection of severe disease, supportive management, and adequate nursing care of patients. Appropriate triage reduces hospital admissions, and also saves the lives (reduction of the burden of disease). Diagnosis of dengue cases is based on clinical symptoms, hematology and laboratory findings. The definitive diagnosis is made using laboratory techniques. Typically, symptoms develop after an incubation period of 4–7 days with an abrupt onset of fever often accompanied by exanthema, erythema, arthralgia and headache with severe retro-orbital pain. Flushed skin, with petechiae may appear in the ‘critical phase’ and a macular rash in early convalescence phase. Leucopenia is invariable present with an excess of plasma-cytoid cells or a relative monocytosis. Minor bleeding from skin and mucosal surfaces may be seen in uncomplicated infections. Biochemical hepatitis is frequently seen in DENV infection. Disease manifestations in adults and children show differences.
3.1 CASE CLASSIFICATION

The WHO classification scheme of 1997 divided symptomatic DENV infections into three groups, i.e. (i) undifferentiated fever, (ii) dengue fever (DF) and (iii) dengue haemorrhagic fever (DHF), in which DHF was sub-classified into four severity grades; among these, grades III and IV were defined as dengue shock syndrome (DSS). This classification scheme is now widely used. However, the use of this WHO dengue case classification in clinical practice has been shown to be difficult and impractical. In the 2009 WHO guidelines, the classification guidelines has been revised which seems to overcome most of these misclassifications. DENV infections are separated into two main groups, i.e. dengue and severe dengue. The dengue group is further divided into two subgroups – patients with warning signs and those without warning signs. Severe dengue shows one or more of the following manifestations: a) plasma leakage that may lead to shock and/or fluid accumulation, with or without respiratory distress, b) severe bleeding, and/or c) severe organ impairment (e.g. hepatic damage, renal impairment, cardiomyopathy, encephalopathy or encephalitis).

3.2 DIFFERENTIAL DIAGNOSIS

Dengue fever can easily be confused with other infectious diseases, especially in the early phase of disease. Depending on the geographical origin of the patient and other etiologies that may be considered include malaria, leptospirosis and typhoid. Other diseases that mimic DENV infection are flu-like syndromes (influenza, measles, Chikungunya, etc.), illnesses with a rash (rubella, measles, Chikungunya, drug reactions), diarrhoeal diseases (enteroviruses) or illnesses with neurological manifestations (Meningo/encephalitis).

3.3 TREATMENT, PREVENTION AND CONTROL

No specific treatment available and treatment is supportive and symptoms-specific. With appropriate supportive therapy (oral rehydration solution intake or adequate intravenous fluid replacement), mortality may be reduced to less than 1%. Attempts to find an anti-viral therapy for dengue have been made. Compounds or anti-viral drugs which block the viral entry pathway or virion replication have also been considered in an attempt to reduce viraemia and limit disease complications.

In general, three main strategies are considered integral to the prevention and control of dengue: 1) control of mosquito vectors, 2) development of vaccines and 3) discovery of effective antiviral drugs. Problems with homotypic immunity, immune enhancement and lack of a suitable animal model for dengue disease have hampered vaccine development. At least five dengue vaccines, including monovalent and tetravalent vaccines and using live-attenuated or chimeric viruses, were being investigated in phase I or II clinical trials.

3.4 DENGUE LABORATORY DIAGNOSIS

A definitive diagnosis of DENV infection can solely be made in the laboratory and therefore relies on appropriate laboratory capacity. Dengue virus infection can be diagnosed, based on cell culture, serological and antigen detection tests and molecular techniques in serum or plasma. Serological tests such as enzyme-linked immunosorbent assay (ELISA), rapid diagnostic tests (RDTs) and immunofluorescence assay (IFA) are commonly used for confirmation. A new laboratory test based on the detection of dengue virus NS1 antigen is available for confirmation of dengue in early stages of disease. NS1 antigen (NS1 Ag) is a conserved glycoprotein and is both group-specific and DENV serotype specific.

3.5 DISTINGUISHING BETWEEN PRIMARY AND SECONDARY DENV INFECTION

Epidemiological studies indicate that severe dengue occurred more often in secondary DENV infection. Secondary DENV infection and age are the most important risk factors for developing severe manifestations of dengue. Anti-dengue IgM and IgG antibody concentrations differ in primary and secondary dengue virus infection. IgM antibody concentrations are significantly higher is primary than in secondary infections. A small proportion of patients hardly produces IgM antibodies after a secondary infection. It is possible to distinguish primary from secondary infection with the use of antibody concentrations and the IgM/IgG ratio. This ratio is higher in primary infection. Unfortunately, this approach is not useful in clinical settings as detection of antibody concentrations are low in the early phase of infection may hamper diagnosis. Dengue specific IgG avidity test is another method which measures the affinity of antibody-antigen bond by testing at different concentrations of urea. It is thought that the avidity is higher in secondary dengue infections.

4. DENV INFECTION PATHOGENESIS

The severity of dengue infections is multifactorial. Disease severity is influenced by the age and genetic background of the host, the strain and serotype of the infecting virus and the prior history of DENV infections of the host. The mechanisms for the variable clinical course are not completely elucidated, but interactions between virus and host immunity and hyperendemicity of multiple serotypes are believed to play an important role in determining the outcome of disease. In the last decades, several theories have been postulated, including immune complex disease, antibodies cross-reacting with vascular endothelium, immune response enhancing antibodies, selection of virulent strains and virulence.

4.1 HOST SUSCEPTIBILITY

Host genetic factors may also be relevant and predispose some individuals to DHF. Some individuals may have a genetically determined predisposition to DHF/DSS, possibly mediated by differences in
human leucocyte antigen (HLA). To activate the cellular host immune responses, human leucocyte antigens (HLAs), encoded by the major histocompatibility complex (MHC), display antigen-proteins to antigen receptors of host T-lymphocyte. HLA genes have shown great variability and several studies have found associations with disease severity. For example, Vietnamese patients with HLA-A33 (class I) were less likely to develop DHF and Mexican individuals homozygote for DRB1*04 (HLA class II) are associated with an altered risk of DHF. Other possible host factors include age, sex, and pre-existing chronic diseases, but the role of host genetics in determining disease outcome is unclear.

4.2 THE ANTIBODY-DEPENDENT ENHANCEMENT THEORY (ADE)

Infection with one DENV serotype presumably results in lifelong immunity to that serotype, but fails to confer immunity to the other serotypes. One of the most important clinical aspects of DHF/DSS is that these syndromes often occur in patients experiencing a secondary infection with a heterologous serotype. The antibody-dependent enhancement theory proposes that due circulating antibodies from a previous infection induce a complex reaction during a second infection with a different serotype, which may lead to more severe disease.

This theory is supported by several epidemiological studies. In vitro studies have demonstrated that non-neutralizing concentrations of serotype cross-reactive, DENV-specific antibodies enhance viral replication, suggesting that antibodies produced during previous infection or passively acquired, contribute to DHF/DSS via ADE. Moreover, cross-reactive T cells may also contribute to the immune-pathogenesis. Low-affinity T cells against the original infecting serotype dominate during a secondary heterologous infection in a phenomenon termed ‘original antigenic sin’. Activation of cross-reactive memory T cells likely contributes to severe disease via the activation of innate immune cells and enhanced cytokine production notably interferon-γ (IFN-γ).

Other factors such as complement activation, platelet activation, and the production of potentially cytotoxic cytokines, including tumour necrosis factor-α4, interleukin (IL)-1 and -6, by macrophages, lymphocytes and endothelial/epithelial cells will contribute to and exacerbate this cascade of inflammatory events.

4.3 DENGUE VIRUS GENETIC DIVERSITY

There are different levels of DENV genetic diversity. (i) DENV exists as four closely related antigenically distinct serotypes, DENV-1 through DENV-4. (ii) Genetic variation within each of the four serotypes is defined by “genotypes” or “subtypes”. (iii) RNA viruses exhibit a high degree of variation in the genomic sequences as a consequence of error-prone RNA replication.

4.4 DENGUE SEROTYPES AND CLINICAL OUTCOME

DENV-2 viruses have most commonly been associated with DHF/DSS, followed by DENV-1 and DENV-3 viruses. DENV-4 appears to be the most clinically mildest, although it too can cause severe disease. DENV-2 and DENV-4 have been associated with increased disease severity as a secondary infection, whereas DENV-1 and DENV-3 seem to cause more severe disease in primary infection.

The association of some DENV genotypes with increased disease severity has been documented, in particular involving certain genotypes of DENV-2 and DENV-3. In general, Southeast Asia appears to serve as a source for viral diversity, generating a multitude of strains, some of which are inherently more virulent and perhaps more successful than others. Evidence from phylogenetic studies suggests that only DENV-2 strains that originated in Southeast Asia are associated with DHF/DSS in the Americas, and not the native American strains that originated from the South Pacific.

4.5 DENV SEQUENCE VARIABILITY

Comparisons of nucleotide sequence from infected individuals have revealed the existence of at least four major genetic groups in DENV-1. DENV-2 is divided into six genotypes (Sylvatic, American, Cosmopolitan, Asian 1, Asian 2, and Asian-American). DENV-3 has been divided into four genotypes (I–IV) and DENV-4 is divided into two endemic genotypes (I–II) and one sylvatic genotype and shows the least genetic diversity among the serotypes, at least among available strains. The lowest sequence variability between genotypes is found in the 5’ UTR, where specific sequences and RNA secondary structures are required for replication and translation functions.

4.6 DENV INTRA-HOST DIVERSITY

Viral RNA-dependent RNA polymerases are of notoriously low fidelity; incorporation of mutations into the progeny RNA strand, coupled with the lack of a second strand for proofreading, results in the generation of a cloud of closely related variant viral sequences. Although most often associated with chronic infections by RNA viruses, such as hepatitis C virus, it has become clear that also acute RNA virus infections also result in significant intra-host sequence diversity. Lately, studies focusing on the C, E, and NS2B genes have indicated that DENV also exhibits substantial sequence diversity in humans and to a lesser extent in mosquitoes. An intriguing report recently demonstrated that a defective DENV1 lineage was disseminated and maintained in human populations in Myanmar over at least 2 years, not only providing further evidence of intra-host diversity of viral species but also implying complementation of the defective genome by co-infection of cells with functional viruses. Now that it has been established that DENV does exist as a closely related viral population, the question naturally arises as to whether the degree of intra-host sequence diversity or particular sequence signatures that are not represented in isolated viruses are associated with viral pathogenesis.
RESEARCH QUESTIONS, AIM AND OUTLINE OF THIS THESIS

Dengue is of major public health importance in Vietnam. It affects mainly children and young adolescents. The aim of the studies presented in this thesis is to provide better understanding into dengue epidemiology, disease transmission, clinical and viral pathogenesis.

Specific research questions are:

1. To quantify the dengue epidemiology.
   a. What are the incidence, prevalence, burden of disease and disease transmission patterns at village and provincial level in Vietnam?

2. To improve the understanding of dengue pathogenesis.
   a. What are the clinical and virological characteristics with respect to serotype, antibody response and viremia?
   b. What is the frequency of plasmacytosis in DENV infections?
   c. What is the extent of intra-host diversity of DENV?

The first part of this thesis contributes to the understanding of different aspects of the burden of disease, its epidemiology and disease transmission in southern Vietnam. Chapter 2 addresses the burden of disease in Binh Thuan province, by compiling different data sources. In this study we aim to quantify the dengue-attributable disease burden in Binh Thuan and present this by a pyramid-shaped presentation. The aim of chapter 3 was to measure serum dengue specific IgG antibodies in serum of healthy children and to determine the association of dengue IgG with environmental risk factors, by conducting a household survey. Because dengue IgG is a marker for previous exposure and based on the proportion of dengue IgG seroprevalence by age, we estimated the annual incidence with a complementary log-log link method. In chapter 4, we report on the incidence by sero-conversion while controlling for cross-reactivity with other flaviviruses. We followed children of two rural communities and thereby validated our previous findings (in chapter 3). In chapter 5, available data from a cross-sectional, a two year follow-up study and a household survey in southern Vietnam were used to explore the hypothesis that dengue virus transmission is spatially focal.

Age at primary and secondary dengue infections is considered as one of the most important modulators for clinical dengue attack and disease severity. Chapter 6 quantifies the relationship between age at infection with dengue and the risk of developing clinical attacks by estimating the conditional probability of clinical dengue with primary and secondary infections. Investigations of dengue transmission dynamics are reported in chapter 7. Wavelet analyses were performed on time series of monthly notified dengue cases to investigate dengue periodicity, patterns of synchrony in both time and space, dengue travelling waves and to associate the relationship between dengue incidence with global and local climate variables. Chapter 8 reviews the current state of knowledge on the associations between climate variability, climate change and dengue transmission, and the tools that are used to quantify these associations.

In the second part of the thesis, we describe clinical observational studies for a better understanding of dengue pathogenesis. In chapter 9, we report PCR results for patients presenting at primary health care settings with serologically confirmed dengue and analyze the epidemiology and clinical and virological characteristics with respect to serotype, antibody response and viraemia. Despite the general bone marrow suppression (leucopenia and thrombocytopenia), blood plasmacytosis has been reported in a few patients with DENV infection. A prospective observational study among ill-returned Dutch travellers is presented in chapter 10, to quantify and describe the kinetics and phenotype of peripheral blood plasma cells (Pcs) in these patients. In chapter 11, we investigated the frequency of viral variants, the genetic distance between the different variants, and the evolution of DENV diversity, in adult Vietnamese patients with DENV-1 infections by clonal sequencing of domain III (DIII) of the envelop (E) gene. The findings in chapter 2-16 are evaluated and summarized in the general discussion in chapter 12.
PART I
DENGUE EPIDEMIOLOGY,
BURDEN OF DISEASE
AND TRANSMISSION DYNAMICS
DENGUE VIRUS INFECTIONS IN VIET NAM: TIP OF THE ICEBERG

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Dengue is highly endemic in Binh Thuan province, southern Viet Nam. To quantify the dengue-attributable disease burden in Binh Thuan, data from different sources was compiled. In 2003, 688 220 patients consulted 112 public primary health facilities. A total of 86 449 patients had fever, of whom 7399 (8.6%, 95% CI 8.4-8.8) were booked without classifying diagnosis; this corresponds to 7.7 per 100 person years. Serological diagnosis confirmed that dengue contributed to approximately one quarter of all undifferentiated fevers presented to the public primary health facilities. The annual incidence of acute primary and secondary dengue among the total population was substantially higher and estimated to range from 5.5 to 11.1 per 100 person years. The number of notified cases of dengue in 2003 was only 527 cases, less than 1% of the total incidence of dengue.

1. INTRODUCTION
Dengue is the most common arthropod-transmitted viral infection in the world. The geographical distribution of dengue is steadily expanding, and in many areas the epidemiology is changing stratum from epidemic to endemic. Estimations of the incidence and thus the geographical distribution of dengue is steadily expanding, and in many areas the epidemiology disease burden attributable to dengue are variable. The main reason is the variability of the clinical presentation of dengue virus infections, which ranges from a mild unspecific febrile illness to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). These complications are mainly associated with secondary dengue virus infections. Immunity against dengue virus is determined by production of neutralizing antibodies. There are four antigenically distinct dengue virus serotypes. The immune response is monotypic; it does not protect against an infection by another serotype. The immune response to secondary infections, which does not neutralize the virus, may even increase the risk of complications.

2. METHODOLOGY
2.1. STUDY SITE AND POPULATION
The study was carried out in 2003, in Binh Thuan province in southern Viet Nam. Binh Thuan had a population of approximately 1.12 million, divided over 122 administrative units including 97 communities in semi-rural areas, 14 wards (in Phan Thiet city – the capital of Binh Thuan province), and 11 small towns (nine of which are recognized as district centres). Phu Quy – an island off the coast and governed as a separate district (22 594 inhabitants), was not included in this study.

The climate in Binh Thuan is a tropical monsoon climate, with the rainy season lasting from May until approximately October. In 2003, the total rainfall was 1135 mm; the mean temperature was 26.9 °C and the relative humidity 80%. (Table 1) (Source: Statistical Yearbook 2003 – Binh Thuan Statistics Office, Phan Thiet). Public health care in Binh Thuan is provided by a provincial hospital in Phan Thiet and nine district hospitals. Primary health care is provided by 103 commune and 13 regional health facilities (further called health posts). (Source: Statistical Yearbook 2003 – Binh Thuan Statistics Office, Phan Thiet).

2.2. DATA SOURCES
2.2.1. Total burden of disease and fever
The total disease burden was extracted from the routine health information system (HIS). The HIS of the public health services in Viet Nam reports at three levels: community, district and province. At community level, health data are recorded in a Health Examination Notebook (HEN) in which all patient consultations are being recorded, including patient identifiers, occupation and ethnic group. Diarrhoea and acute respiratory tract infections are recorded in a separate column; all other diagnoses are grouped under “other”. Treatment is specified by the given medication and by whether the patient was ambulatory, had to be admitted to the health posts, or was referred to a district or provincial hospital. Malaria is recorded in a separate file. For this study an extra column was added to the HEN to identify patients who presented with fever (an axillary temperature ≥ 38.0 °C). The presumptive diagnosis of febrile patients was recorded. When no classifying diagnosis was made, this was recorded as “acute undifferentiated fever” (AUF). AUF was defined as any febrile illness of duration less than 14 days, confirmed by an axillary temperature ≥ 38.0 °C, without indication of either severe systemic or organ-specific disease. Malaria was excluded by microscopic examination of a thick blood smear. The data in the HEN were aggregated in monthly reports and then sent to the district health services where they were collected by the research team.

2.2.2. Dengue as a cause of undifferentiated fever
The contribution of dengue as a cause of undifferentiated fever was extracted from a separate study. The details of that study have also been explained previously.

In brief, in twelve non-adjacent commune health posts and the clinic of the provincial malaria station, we determined the diagnosis of patients who presented with AUF by performing serological tests on “acute” and “convalescence” serum samples. An “acute” serum sample was collected at first a second,
presentation; “convalescence”, serum sample was collected three weeks later. Serum samples were stored at −20 °C at the study sites until monthly transfer to Cho Ray hospital, where they were stored at −70 °C. Complete pairs of acute and convalescence serum samples were tested for dengue with IgG and IgM-capture ELISA (Focus Technologies Inc., Cypress, CA, USA), as described previously. ELISA was performed at the Department of Microbiology, Cho Ray Hospital, Ho Chi Minh City, Viet Nam. The results of ELISA were classified as “acute primary dengue”, “acute secondary dengue”, “past (not acute) dengue” and “no dengue”.

2.2.3. Incidence of first dengue virus infections
The annual incidence of primary dengue in the general population was assessed by measuring the sero-prevalence of IgG dengue antibodies among primary-school children, as described previously. The age-dependent increase of the IgG sero-prevalence was used to calculate the annual incidence of primary dengue virus infections. In a second survey two years later among the same population, we calculated the incidence of primary dengue as the proportion of children who experienced sero-conversion between January 2003 and April 2005, while excluding cross-reactions with Japanese encephalitis virus infections.

2.2.4. Notification of dengue
The 2003 routine dengue notification data were used to compare with the other data. Routine surveillance of dengue is based on an algorithm supplied by the National Dengue Control Program that basically follows the guidelines of WHO, but does not require haematology support (haematocrit and/or platelets count). By using this algorithm, in principle only dengue haemorrhagic fever and dengue shock syndrome are notified and uncomplicated dengue fever is not recognized. The Department of Preventive Medicine of Binh Thuan province collects monthly cumulative reports of dengue cases from all health posts, follows trends in notification and warns for outbreaks in the province; in addition, the department also applies preventive measures. Serological confirmation is only done in some complicated cases that need referral to the provincial hospital. Sometimes serum samples are transferred to Institute Pasteur, Ho Chi Minh City, for isolation of dengue virus, but not on a routine basis.

2.3. ETHICAL CONSIDERATIONS
The study was approved by the Review Board of the Cho Ray Hospital, Ho Chi Minh City. The study was explained and discussed in meetings with provincial authorities and staff of the health posts. All patients, or, for children, the parents or guardians, gave their written informed consent.

2.4. STATISTICAL ANALYSIS
Statistical analysis was performed using statistical software (SPSS 11.5, SPSS Inc., Chicago, IL, USA). Binary regression was applied to calculate the annual incidence of DENV infection as described previously. Descriptive statistics were used to describe the distribution of the demographic and incidence data. A univariate generalized linear model was used to find the association between climate factors and monthly incidence.

3. RESULTS
3.1. TOTAL BURDEN OF DISEASE AND FEVER
In 2003, 688 220 patients consulted the 112 public primary health facilities which is, on average, 17 consultations per health post per day. A total of 86 449 patients had fever, of whom 7399 (8.6%, 95% CI 8.4–8.8) were booked without classifying diagnosis and were thus classified as AUf. The mean of the number of fever and AUf cases, divided by the total population of the respective communities, is presented in Figure 1. Overall, the number of consultations for fever, divided by the population, was 7.7%. The data did not specify the number of patients, only the number of consultations. Thus, if patients would present their fevers not more than one time per year to the health posts, the average incidence of AUf would be 7.7 per 100 person years.

The mean number of consultations for fever per month, for children and adults, is shown in the table 1, together with monthly rainfall and temperature. The mean monthly number of malaria
cases (due to *P. falciparum* and *P. vivax*) is also shown for comparison. Malaria contributed to 2.8% of all fevers (including adults and children). Over the year, fever was the reason for 11.1% (range 9.1–15.0%) of consultations by adults and 15.0% (range: 6.7–24.3%) of children's consultations. The diagnosis was classified as AUF in 9.2% (range: 7.5–14.8%) of the consultations by adults and 7.8% (range: 6.3–9.6%) by children. There was no correlation between the total number of consultations and rainfall or temperature.

### 3.2. Dengue as a Cause of Undifferentiated Fever

In 2003, paired serum samples were collected from 1636 patients with AUF who attended the 13 study sites. Of these, two cases per health post and per month were randomly selected totalling 275 (16.8%) paired serum samples. These samples were tested for dengue virus IgM- and IgG-specific antibodies with ELISA. Acute dengue was found in 70 (25.5%) cases, including 23 (8.4%) cases of acute primary dengue [21 (7.7%) children < 15 years; 2 (0.7%) ≥ 15 years] and 47 (17.1%) cases of acute secondary dengue [19 (18.4%) and 28 (16.3%) respectively]. A past dengue virus infection was detected in 161 (58.5%) cases [36 (35.0%) < 15 years and 125 (72.7%) ≥ 15 years]. In 44 (16.0%) patients [27 (26.2%) < 15 years and 17 (9.9%) ≥ 15 years] the tests were negative (Chi-square on two age groups and four diagnoses: 55.043 (df = 3); *P* value <0.001). Figure 2 shows the serological diagnoses per age group.

### Table 1. Mean monthly number of consultations for fever and acute undifferentiated fever at all public primary health facilities of Binh Thuan.

<table>
<thead>
<tr>
<th>Month</th>
<th>Temp. (°C)</th>
<th>Rain-fall (mm)</th>
<th>Number of consultations (&lt;1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adult</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total For fever¹ (%)</td>
</tr>
<tr>
<td>1</td>
<td>24.9</td>
<td>-</td>
<td>33.91 3.79 (11.2)</td>
</tr>
<tr>
<td>2</td>
<td>25.8</td>
<td>-</td>
<td>34.02 4.06 (11.9)</td>
</tr>
<tr>
<td>3</td>
<td>27.1</td>
<td>-</td>
<td>39.06 4.29 (11.0)</td>
</tr>
<tr>
<td>4</td>
<td>28.6</td>
<td>15</td>
<td>41.11 4.33 (10.5)</td>
</tr>
<tr>
<td>5</td>
<td>28.1</td>
<td>249</td>
<td>38.84 3.98 (9.2)</td>
</tr>
<tr>
<td>6</td>
<td>28.0</td>
<td>102</td>
<td>46.58 4.26 (8.1)</td>
</tr>
<tr>
<td>7</td>
<td>27.2</td>
<td>281</td>
<td>41.03 4.24 (10.3)</td>
</tr>
<tr>
<td>8</td>
<td>27.4</td>
<td>97</td>
<td>39.01 4.00 (10.3)</td>
</tr>
<tr>
<td>9</td>
<td>27.2</td>
<td>168</td>
<td>31.41 3.51 (11.2)</td>
</tr>
<tr>
<td>10</td>
<td>27.1</td>
<td>106</td>
<td>31.85 4.16 (13.1)</td>
</tr>
<tr>
<td>11</td>
<td>26.7</td>
<td>108</td>
<td>27.39 3.65 (13.3)</td>
</tr>
<tr>
<td>12</td>
<td>25.1</td>
<td>9</td>
<td>25.71 3.86 (15.0)</td>
</tr>
<tr>
<td></td>
<td>26.9</td>
<td>1135</td>
<td>429.92 47.72 (11.1)</td>
</tr>
</tbody>
</table>

¹ (%) percentage of fever among all consultations
² percentage of AUF among all consultations for fever
³ percentage of malaria (*P. falciparum* and *P. vivax*) among total of fever consultations (adults and children)
Figure 2 shows the monthly distribution of the proportion of acute dengue cases among the total of cases with AUF. The number of cases with dengue was higher in the rainy season than in the dry season [49 (31.0%) vs. 21 (17.9%); chi-square 6.046, df 1, P value = 0.014].

3.3. Incidence of First Dengue Virus Infections
The annual incidence of acute primary dengue was calculated by binary regression with a log-log link function, thus applying a model of loglinear decrease of the proportion of dengue IgG-naive children among primary-school children.272 The overall annual incidence of primary infections (sero-conversion) was 11.7%. In the serum bank of patients with AUF, we observed similar patterns. The dengue IgG prevalence in convalescent samples increased by age from 60% among children younger than 10 years to 94% in adults older than 41 years.

Figure 2. The serological diagnoses of dengue per age group. The serological diagnosis, confirmed by ELISA on paired serum samples, in 275 patients with acute undifferentiated fever, who presented at primary health facilities in Binh Thuan

3.4. Dengue Notification
In 2003, a total of 527 dengue cases were notified. This was not further specified or broken down into age groups.

4. Discussion
This study showed that dengue was a very common disease in the area, and that routine notification data grossly underestimated its true incidence.

In this study, we applied ELISA for the serological confirmation of dengue. ELISA, though not recognized as a gold standard, has sufficient sensitivity and specificity for both serodiagnosis in patients as well as for epidemiological studies, in comparison to the plaque reduction neutralization test (PRNT) and haemagglutination inhibition assay (HI).42,92,215 Previous studies indicated that dengue is highly endemic in southern Vietnam and can therefore be considered a disease of childhood.96,98 In Binh Thuan province, dengue is the most frequent cause of all fevers presented to the public primary health services.219

Based on the findings in this study, we constructed a model that quantifies and illustrates several echelons at which dengue can present to the public health services, analogous to the Piot model that was developed for tuberculosis control and other diseases (Figure 4).222 The base of this pyramidal model depicts the total population. Superposed on that are four levels that refer to disease and health consumption: the total number of patients in Binh Thuan with AUF, the total number of cases with acute dengue, the number of patients with dengue that present to the public primary health services, and the number that actually becomes notified as dengue.

In order to calculate these totals, we needed to make some assumptions and approximations. The total population of Binh Thuan was 1.12 million in 2003, but the age distribution was not known. At the next layer of the pyramid, we observed that 86 449 consultations were booked for fever. This corresponds to 7.7 per 100 person years among the general population if we assume that no patient consults the health posts for fever more than once a year and that no patient goes
to other health providers. The total number of dengue cases was derived from the annual incidence. The school surveys showed that the true annual incidence of first infections was approximately 12% among subjects who were never infected before. The total number of primary dengue infections can be calculated from the total population of sero-naïve subjects if we make a few assumptions. Here, we assumed that (i) half of the population of Bình Thuận was 25 years of age or younger and (ii) that in this age group the loglinear decrease of sero-naïve subjects was constant over the years, similar to what we found in primary-school children. By doing so, we easily calculated that approximately 21,600 (3.7%) persons younger than 25 years suffered from primary dengue every year. This number had to be increased by the number of cases of acute primary dengue among persons older than 25 years, but since the incidence of dengue was lower in older age groups, the proportions decreased, but not lower than its half, 1.8%. Thus, every year, approximately 1.8% to 3.7% of the total population suffered from acute primary dengue.

In the study on the causes of fever, we observed that acute secondary dengue was approximately twice as common as acute primary dengue, so that the total number ofcases with acute primary or secondary dengue should range from 5.5% to 11.0% of the total population of Bình Thuận. In absolute terms, this is approximately 60,000 to 120,000 cases. This number would increase if two dengue virus types circulate simultaneously. In 2003, the blood of 15 cases of dengue was sent to Institute Pasteur in Ho Chi Minh City for virus isolation. In three cases DENV-2 was isolated. In 2001, six cases of DENV-2 and three cases of DENV-3 infections were identified by virus isolation in 61 blood samples (Institute Pasteur Ho Chi Minh City, unpublished data). For drawing the pyramidal figure, we assumed the circulation of only one serotype.

The third level was the total number of dengue-infected patients who sought help at public primary health services. From the serological studies on the causes of acute undifferentiated fever, we know that one quarter of the patients with acute undifferentiated fever actually had dengue, which corresponds to 1.9 per 100 person years among the total population. This is a 2.9 to 5.8-fold difference with the total number of cases of acute dengue (5.5% to 11.1% of the total population). These subjects also suffered from dengue but apparently did not seek help or did so from other health providers. Furthermore, if this also applies to all other causes of fever, then the total number of cases of AUF could also be 2.9 to 5.8-fold higher. Lastly, only 527 cases of complicated dengue were notified, which corresponds to 0.4% to 0.9% of the total number of cases with dengue.

Figure 4 shows that the burden of disease attributable to dengue was much greater than what was being notified as such, even if our assumptions contain large deviations from the reality. The routine notification system, however, grossly underestimates the true incidence. Our findings, however, suggest that the unspecific clinical presentation is the main reason why the notified data represent a very small fraction of the total number of dengue infections in the world.

Our findings reflect the recent estimations of the global incidence of dengue. It is estimated that, annually, between 50 and 100 million cases of DF occur among the more than 2.5 billion people at risk. The annual total number of DHF cases is estimated at 250,000, approximately 2% of the total of dengue virus infection. The consequences for surveillance are two-fold. First, notifications based on the case definitions of complicated dengue grossly underestimate the total burden of the disease. Secondly, the complication rate of dengue is very low in highly endemic regions as long as the number of secondary infections is low. The latter could lead to the conclusion that the main focus of surveillance should be the detection of new serotypes entering an endemic area, for example, by using molecular tools at some sentinel sites, so that a sudden increase in the incidence of acute secondary dengue can be anticipated.

In conclusion, dengue is highly endemic in southern Vietnam and leads to much health consumption. The routine notification system, however, grossly underestimates the true incidence. This study underscores the need for effective dengue control measures that would limit the transmission of the virus till a vaccine becomes available, and makes a case for other methods of surveillance that would anticipate outbreaks of secondary infections.

5. ACKNOWLEDGEMENT
This study was carried out with the support of the Netherlands Foundation for the Advancement of Tropical Research (WOTRO). We thank our colleagues in the Department of Virology, Cho Ray Hospital, Ho Chi Minh City, for their contribution with laboratory analysis. We are very grateful to the doctors and other personnel of the Bình Thuận Provincial Malaria Centre, Phan Thiet, and health posts for their cooperation.
Chapter 3

The age specific prevalence of dengue antibodies, annual incidence and risk factors among children in southern Vietnam

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Dengue is highly endemic in southern Vietnam and all four serotypes of dengue virus have already been identified. To determine age specific prevalence of dengue and associated risk factors we conducted a serological study at two primary schools and assessed risk factors by analyzing children’s questionnaires and household surveys. Sera were collected from 961 primary school children in the Binh Thuan province and were tested for the presence of dengue virus serum antibodies using an indirect IgG enzyme-linked immunosorbent assay (ELISA). Antibody prevalence of the total population was 65.7% (n = 631) which increased from 53.0% to 88.2% with increasing age. The annual incidence of a first dengue infection, estimated by binary regression of the sero-prevalence by age, was 11.7%. Interestingly, prevalence of dengue IgG antibodies was significantly higher in children who confirmed using a pit latrine (RR 1.467, 95% CI = 1.245-1.730) and whose domestic environment was littered with discarded cans (RR 1.238, 95% CI = 1.042-1.470) and pigs (RR 1.228, 95% CI = 1.002-1.504). The epidemiology of dengue in southern Vietnam is stable with a constantly high annual incidence of first infections. Transmission occurs mainly peri-domestic which has important public health implications.

1. INTRODUCTION

Dengue has become one of the most important vector borne diseases over the last six decades with a steady rise of global incidence, increasing geographic distribution and a transition from epidemic transmission with long inter-epidemic intervals to endemic with seasonal fluctuation. This is caused, not in the least, by the enlarging habitat of the main dengue vectors *Aedes aegypti* and *Aedes albopictus* to almost all tropical and sub-tropical zones of the world. *Ae. aegypti*, in particular, is highly adapted to human settlement and is the main reason why dengue also thrives in urbanized areas. To date it is estimated that over two fifths (2.5 billion) of the world population live in dengue endemic areas, of whom fifty million (2%) are infected annually. The four antigenically distinct serotypes of dengue virus, DENV-1, DENV-2, DENV-3 and DENV-4, can be distinguished neither on the basis of clinical disease nor on epidemiologic characteristics. Infection with a dengue serotype provides life-long immunity without cross-protective immunity to the other serotypes. IgG antibodies against dengue are detectable after 10 to 14 days and remain for life. It is assumed that in Vietnam the incidence of dengue increased over the last years, presumably by the rapid socioeconomic changes and increased urbanization of the last decade. However, the validity of the existing incidence data is unclear because notification and surveillance were only recently put into place. In addition, the high population density and ecological conditions in the rural areas of Vietnam have been favourable for dengue transmission since many decades, suggesting a rather stable, endemic transmission pattern. Binh Thuan, a rural province in southern Vietnam, is highly endemic for dengue and all four serotypes have already been identified. However, the real infection pressure and household and environmental risk factors were not known. In order to estimate the annual incidence of primary infections and fluctuations in the previous years and to determine risk factors for dengue infection, surveys were held in the primary schools of two rural communes.

2. MATERIAL AND METHODS

2.1 STUDY SITE AND POPULATION

The study was conducted at two primary schools in Binh Thuan province, Vietnam. Binh Thuan, is one of the most arid provinces of Vietnam, ranging from the Truong Son forests mountains in the west to the South Chinese sea, 150 km northeast of Ho Chi Minh city. Binh Thuan has a tropical climate with a mean temperature of 27 °C, a rainfall of 1.152 mm per year and the rainy season is from May to October. The primary schools are situated in the villages Ham Kiem and Ham Hiep, 5 km west and 15 km south of the provincial capital Phan Thiet respectively. From 2002 census data, the total populations in Ham Kiem and Ham Hiep were 6.467 and 11.131 respectively. In co-operation with the people’s committee of the village, the local health post staff and school teachers, all pupils of the primary school and their parents were informed about the study. The study protocol was approved by the provincial Ministry of Health and the Health Department of Ham Kiem and Ham Hiep. All parents provided verbal informed consent to draw blood from their children. All children from 7 until 14 years of age of the two primary schools belonged to the study population. This is 60.8% (n = 508) of the entire children population in Ham Kiem and 32.5% (n = 469) in Ham Hiep.

Using a standardized questionnaire, children were questioned and a selection of children was visited at their home. Dengue virus specific IgG serum antibodies were determined in all samples, whereas children with fever were also tested for dengue virus specific IgM serum antibodies. Children who mentioned not feeling well, were examined and their temperature was measured.

2.2 DENGUE VIRUS SERODIAGNOSIS

One ml of blood was collected by finger puncture in plain vials (Greiner, Minicollect), left to clot at ambient temperature, centrifuged at 1000 rpm for 15 minutes and transferred to a sterile vial for storage at –20 °C until testing. All samples were tested for the presence of dengue specific serum antibodies against dengue virus using a commercial available indirect IgG ELISA. Dengue specific IgM serum antibodies were screened in febrile children with an IgM-capture enzyme linked immunosorbent assay (MAC-ELISA). Both the MAC-ELISA and IgG ELISA were performed according to the manufactures instructions (Focus Technologies Inc., Cypress, CA, USA). Optical density (OD) values were measured at 450 nm with 620 nm as a reference with a Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were expressed as the ratio between the sample OD value and the OD value of the kit calibration benchmark microplate reader (Bio-rad laboratories, inc., hercules, Ca, usa). Results were expressed as the ratio between the sample OD value and the OD value of the kit calibration benchmark microplate reader (Bio-rad laboratories, inc., hercules, Ca, usa).
serum (ODR), both after subtraction of the OD of an enclosed blank specimen. ODR values > 1 were considered positive. Acute primary dengue in febrile children was diagnosed when IgM ODR was greater than 1 and exceeded ODR of the IgG; if the IgM ODR was positive but lower than ODR of the IgG, this was interpreted as a possible acute secondary infection.

2.3 QUESTIONNAIRE
All children were interviewed using a questionnaire concerning age and gender, recent episodes of fever and certain risk factors. The following risk factors were addressed: the presence of domestic animals (chicken, oxen or pig) on the compound of the children’s houses, the source of water used for consumption and personal hygiene, exposure to surface water and soil, location of the house near a market (assumed to be a predilection spot for different mosquitoes), peri-domestic mosquito breeding sites, such as littering coconut husks, plastics and cans and the presence of plants with stagnant water spaces such as bromeliads and the saucers on which potted plants are placed, and vector control measures taken. The presence of a pit latrine which is being flushed by pouring water from a barrel was also questioned. All questions were filled in by the interviewing medical doctor or by the child with the help of the teacher.

2.4 HOUSEHOLD SURVEY
Both villages were divided in four quarters. In every quarter, 50 houses were randomly selected from the households of the children in the study population, before the serological results were available. These 400 houses, home to 533 primary school children, were visited for obtaining information on peri-domestic risk. In Ham Kiem we included 199 houses, inhabited by 269 children. In Ham Hiep 201 houses were enrolled inhabited by 264 children. The household survey was performed two months after drawing blood. Detailed data were collected, based on observation, on house construction, condition and type of toilets facilities around the house.

2.5 STATISTICAL ANALYSIS
The parameter of interest, the annual incidence of dengue infection, was calculated by binary regression of the prevalence of IgG anti-dengue antibodies to age, using a complementary log-log link function, using S-plus 2000 for Windows (Mathsoft Inc, Seattle, WA, USA). The probability \( p_i \) of testing positive for antibodies of an individual \( i \), experiencing a constant incident rate (force of infection) \( \lambda_i \), at age \( t_i \) is an exponential function of the incident rate:

\[
1 - p_i = e^{-\lambda t_i}
\]  
Hence,

\[
\log(-\log(1 - p_i)) = \log(t_i) + \log(\lambda_i)
\]  (2)

Co-variables affecting the force-of-infection of individual \( i \) are taken into account by letting \( \log(\lambda_i) \) depend linearly on observed co-variables (risk factors) \( X_i \)

\[
\log(-\log(1 - p_i)) = \log(t_i) + \alpha + \sum \beta_j X_{ij}
\]  (3)

in which constant \( \alpha \) and the coefficients \( \beta_j \) are estimated by maximum likelihood.

If in (2) age is chosen for \( t_i \), then binary regression of the sero-prevalence over the consecutive ages yields the overall annual incidence. Independently, the effect of risk factors, \( \beta_j \), can also be analysed in the same model, univariate or multivariate, and expressed as relative risks (RR). All tests were carried out at a significance level of 0.05. For analysis of risk factors, the questions with unclear or unknown answers were omitted.

3. RESULTS
A total of 977 children were registered as pupils of both schools, 508 in Ham Kiem and 469 in Ham Hiep. They were all included in this study except 16 children (7 girls, 9 boys) who were absent at the time of sampling blood, thus leaving 961 (98.4%) subjects for analysis. Table 1 shows their characteristics. The male: female ratio was approximately equal in all age groups. Twenty one (2.2%) children had fever at the time of the examination. Dengue specific IgM antibodies were detected in 11 (1.1%) children, of whom 6 (0.6%) were classified as having acute primary dengue and 5 (0.5%) as probably a secondary dengue infection.
Table 1. Characteristics of 977 primary school children in two villages in Binh Thuan province, Vietnam, participating in a dengue sero-survey.

<table>
<thead>
<tr>
<th></th>
<th>Ham Kiem</th>
<th>Ham Hiep</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total village population (males/females)</td>
<td>6467 (3215 / 3252)</td>
<td>11131 (5343 / 5788)</td>
<td>17598 (8558 / 9040)</td>
</tr>
<tr>
<td>Total number of children (age 0 to 15 years)</td>
<td>2084</td>
<td>2891</td>
<td>4975</td>
</tr>
<tr>
<td>Total primary school children</td>
<td>835</td>
<td>1442</td>
<td>2277</td>
</tr>
<tr>
<td>Number of children included</td>
<td>508</td>
<td>469</td>
<td>977</td>
</tr>
<tr>
<td>Number of children tested for dengue IgG (males/females) by age group (years)</td>
<td>496 (256 / 240)</td>
<td>465 (260 / 205)</td>
<td>961 (516 / 445)</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>62</td>
<td>132</td>
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<td>8</td>
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<tr>
<td>Total households in</td>
<td>1246</td>
<td>2168</td>
<td>3414</td>
</tr>
<tr>
<td>Households surveyed (representing % of tested school children)</td>
<td>199 (54.2)</td>
<td>201 (56.8)</td>
<td>400 (55.5)</td>
</tr>
</tbody>
</table>

3.1 SERO-PREVALENCE STRATIFIED BY AGE

Of all school children 631 (65.7%, 95% CI = 62.6-68.6) tested positive for IgG antibodies against dengue, with comparable proportions in Ham Kiem 307 (62%, 95% CI 58-66) and Ham Hiep 324 (70%, 95% CI 65-74). Figure 1 shows the sero-prevalence per age group. The total sero-prevalence of dengue IgG of the total population increased from 53% in children of 7 years of age to 88% in children of 13 years of age. In Ham Kiem, the sero-prevalence increased from 53% in children of 7 years of age to 86% in children of 13 years of age. Similarly, the sero-prevalence in Ham Hiep increased from 53% at the age of 7 years to 90% at 13 years. The dengue IgG prevalence was similar among boys and girls.

3.2 INCIDENCE RATE

The overall annual incidence of primary infections (sero-conversion), applying binary regression on the sero-prevalence data, was 11.7% (Table 2) with small differences between Ham Kiem (10.5%) and Ham Hiep (13.1%).

3.3 EPIDEMIOLOGICAL AND HOUSEHOLD RISK FACTORS

Several variables in the questionnaires were significantly associated with the presence of dengue IgG antibodies in univariate binary regression analysis (Table 2). These were peri-domestic littering of discarded cans and pigs. The type of sanitary facilities was also significantly associated with the prevalence dengue IgG antibodies. In general there are three types in use in the two communes. The most common type is a pit latrine on the premise outside the house, flushed by pouring water from an open water barrel. Other less frequently used types are a wooden facility for squatting above an open ditch or stream and, rarely, an in-house water closet with a closed flushing system. Children who use the pit latrine type had a higher prevalence of dengue antibodies. Children who confirmed of have the questionnaire variables which were significant in the individual univariate analysis (P < 0.05) were entered altogether in a multivariate model. Of all the variables, recorded by household visits, on house construction and environment, none was associated with the presence of dengue IgG antibodies (Table 3).
Table 2 Relative risk of age (annual incidence), household and environmental factors, assessed by interview, for positive dengue IgG antibodies. The results of multivariate models are presented only for factors which were significant in the univariate models.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency</th>
<th>Univariate binary regression</th>
<th>Multivariate binary regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>IgG pos (%)</td>
<td>RR</td>
</tr>
<tr>
<td>Age (annual incidence)</td>
<td>0.117</td>
<td>0.108-0.127</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hm Nen</td>
<td>0.105</td>
<td>0.094-0.118</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hm Hgp</td>
<td>0.131</td>
<td>0.116-0.147</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Gender</td>
<td>0.981</td>
<td>0.906-1.064</td>
<td>0.647</td>
</tr>
<tr>
<td>Peri-domestic animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>0.63</td>
<td>0.62</td>
<td>1.022</td>
</tr>
<tr>
<td>Oven</td>
<td>0.36</td>
<td>0.35</td>
<td>0.880</td>
</tr>
<tr>
<td>Pigs</td>
<td>0.19</td>
<td>0.21</td>
<td>1.238</td>
</tr>
<tr>
<td>Water source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>River Water</td>
<td>0.5</td>
<td>0.4</td>
<td>0.841</td>
</tr>
<tr>
<td>Tap Water</td>
<td>0.22</td>
<td>0.21</td>
<td>0.955</td>
</tr>
<tr>
<td>Well Water</td>
<td>0.80</td>
<td>0.80</td>
<td>1.025</td>
</tr>
<tr>
<td>Water tanks</td>
<td>0.59</td>
<td>0.59</td>
<td>1.040</td>
</tr>
<tr>
<td>Water and soil contact</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living near rice field</td>
<td>0.15</td>
<td>0.131</td>
<td>1.311</td>
</tr>
<tr>
<td>Bare feet contact with mud</td>
<td>0.35</td>
<td>0.36</td>
<td>1.061</td>
</tr>
<tr>
<td>Washed through river</td>
<td>0.15</td>
<td>0.15</td>
<td>1.013</td>
</tr>
<tr>
<td>Swam/washed in river</td>
<td>0.12</td>
<td>0.12</td>
<td>1.021</td>
</tr>
<tr>
<td>Living near market</td>
<td>0.12</td>
<td>0.14</td>
<td>1.021</td>
</tr>
<tr>
<td>Peri-domestic hygiene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromeliads</td>
<td>0.45</td>
<td>0.47</td>
<td>1.114</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Frequency</th>
<th>Univariate binary regression</th>
<th>Multivariate binary regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>IgG pos (%)</td>
<td>RR</td>
</tr>
<tr>
<td>Coconut husk</td>
<td>0.59</td>
<td>0.59</td>
<td>1.011</td>
</tr>
<tr>
<td>Discarded cans</td>
<td>0.40</td>
<td>0.44</td>
<td>1.238</td>
</tr>
<tr>
<td>Plant saucers</td>
<td>0.45</td>
<td>0.47</td>
<td>1.116</td>
</tr>
<tr>
<td>Littering plastics</td>
<td>0.31</td>
<td>0.32</td>
<td>1.118</td>
</tr>
<tr>
<td>Sanitary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toilet</td>
<td>0.43</td>
<td>0.49</td>
<td>1.467</td>
</tr>
<tr>
<td>Vector borne transmission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of bed net</td>
<td>0.98</td>
<td>0.97</td>
<td>0.772</td>
</tr>
<tr>
<td>Bed net recently re-impregnated</td>
<td>0.4</td>
<td>0.4</td>
<td>1.116</td>
</tr>
<tr>
<td>Spraying of residual insecticides</td>
<td>0.29</td>
<td>0.25</td>
<td>0.901</td>
</tr>
<tr>
<td>Modern convenience</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>0.82</td>
<td>0.84</td>
<td>1.196</td>
</tr>
<tr>
<td>Television</td>
<td>0.70</td>
<td>0.77</td>
<td>1.082</td>
</tr>
</tbody>
</table>
Table 3. Relative risk of observed house construction and environmental factors for positive dengue IgG antibodies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative Risk</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>House situation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near highway</td>
<td>1.246</td>
<td>0.851-1.825</td>
<td>0.258</td>
</tr>
<tr>
<td>Near paved road</td>
<td>0.633</td>
<td>0.446-1.046</td>
<td>0.080</td>
</tr>
<tr>
<td>Near unpaved road</td>
<td>1.013</td>
<td>0.768-1.336</td>
<td>0.927</td>
</tr>
<tr>
<td>Near red dragon fruit or chard</td>
<td>0.961</td>
<td>0.770-1.199</td>
<td>0.723</td>
</tr>
<tr>
<td>Roof type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flat roof</td>
<td>0.974</td>
<td>0.606-1.566</td>
<td>0.914</td>
</tr>
<tr>
<td>Diagonal roof</td>
<td>1.027</td>
<td>0.639-1.650</td>
<td>0.914</td>
</tr>
<tr>
<td>Flat/Diagonal roof</td>
<td>1.373</td>
<td>0.626-3.011</td>
<td>0.429</td>
</tr>
<tr>
<td>Roof construction material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrugated iron</td>
<td>0.885</td>
<td>0.662-1.184</td>
<td>0.412</td>
</tr>
<tr>
<td>Cement</td>
<td>0.979</td>
<td>0.587-1.631</td>
<td>0.934</td>
</tr>
<tr>
<td>Wall construction material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cement</td>
<td>1.110</td>
<td>0.893-1.393</td>
<td>0.337</td>
</tr>
<tr>
<td>Bricks</td>
<td>0.990</td>
<td>0.638-1.535</td>
<td>0.963</td>
</tr>
<tr>
<td>Wood</td>
<td>0.916</td>
<td>0.688-1.219</td>
<td>0.548</td>
</tr>
<tr>
<td>Clay</td>
<td>0.924</td>
<td>0.681-1.252</td>
<td>0.608</td>
</tr>
<tr>
<td>Other</td>
<td>0.927</td>
<td>0.495-1.737</td>
<td>0.813</td>
</tr>
<tr>
<td>Floor construction material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cement</td>
<td>0.977</td>
<td>0.780-1.225</td>
<td>0.943</td>
</tr>
<tr>
<td>Clay</td>
<td>0.941</td>
<td>0.729-1.217</td>
<td>0.640</td>
</tr>
<tr>
<td>Tiles</td>
<td>1.163</td>
<td>0.850-1.592</td>
<td>0.347</td>
</tr>
<tr>
<td>Door construction material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wood</td>
<td>0.954</td>
<td>0.743-1.225</td>
<td>0.712</td>
</tr>
<tr>
<td>Metal</td>
<td>1.152</td>
<td>0.884-1.509</td>
<td>0.291</td>
</tr>
<tr>
<td>Other</td>
<td>0.741</td>
<td>0.435-1.264</td>
<td>0.272</td>
</tr>
</tbody>
</table>

4. DISCUSSION

Our study shows that the prevalence of dengue antibodies among primary school children in Binh Thuan province, Vietnam, increases at a rather constant rate with age, indicating high, relatively stable, transmission rates over many years. Binary regression of the sero-prevalence data gave an estimate of the sero-conversion rate, corresponding to the incidence rate of first dengue infections, with a narrow confidence interval. Other studies in dengue endemic areas with similar sero-prevalence, also showed that antibody prevalence increases with age, but did not calculate the annual incidence from this.14,97,229,261,303 Although, the indirect IgG ELISA used is a suitable tool to detecting dengue IgG antibodies and has shown high sensitivity and specificity95, cross-reactivity may occur with other flavivirus antibodies.235,288 It cannot be ruled out that infection with other flaviviruses such a Japanese Encephalitis virus (JEV) may have caused some of the sero-conversions. This number is probably low because the reported incidence of clinical encephalitis is generally low.

The incidence rate, calculated on the basis of the point prevalence of febrile children with acute primary dengue, is a very crude estimate. Several assumptions would be needed to translate the point prevalence of acute primary dengue to incidence rates. For example, assuming that dengue fever lasts 1 week87,235, and that the dengue season in Binh Thuan lasts approximately 5 months (21 weeks) then the annual incidence of primary dengue would be approximately 21*0.6% = 13%. This calculation is unrealistic because it does not take into account absent children, who may also have had fever, and the delay between onset of disease and IgM becoming detectable. This also applies to the seasonality of dengue. All samples were collected in March, at the end of the dry season, when the incidence is at its lowest. Therefore the IgG sero-prevalence data are a better basis for calculating the annual incidence than using IgM.

A key parameter for understanding the epidemiology of dengue in a community is its basic reproductive number, \( R_b \). In the case of dengue this estimation is complicated by the cross-reactivity of dengue IgG antibodies among the four strains and that IgG seroconversion thus demonstrates primary infection by any of the four strains. IgG antibodies provide only strain specific, life long, immunity and thus the incidence of acute, primary and secondary, dengue is higher than the sero-conversion rate. Moreover, prior exposure to heterogenous strains initially provides some short lived protection, but, conversely, later enhances viral replication.163 In endemic areas with random mixing, \( R_b \) is inversely related to the proportion of susceptibles in the population. Hence, for a stationary population \( R_b \) equals \( 1 + L/A \) where \( L \) is the average life-span of the host and \( A \) is the average age of infection (= 1/annual risk of infection). Had there been only a single dengue strain, then our data would suggest that the \( R_b \) of this one dengue virus serotype in Vietnam lies between 5 to 7. However, as all four strains circulate this is unrealistic. Assuming all four serotypes are equally represented and cross-reactivity between the strains did not occur, estimates of
For each strain, the estimated $R_0$ would range from 1.25 to 1.75. These estimates are somewhat uncertain and depend critically on assumptions such as random mixing and equal $R_0$ for all 4 strains. For example, Ferguson et al. (1999) analyzed multitypic sero-prevalence data from Thailand, where overall age sero-prevalence patterns are similar to our findings. Using mathematical models that take into account the interaction between different strains and immunological response, their strain specific estimates of $R_0$ ranged from approximately 4 to 8.

The results show that dengue transmission is high and stable over the years in Binh Thuan. The experience of the health service indicates that there is a strong seasonal fluctuation, starting with the highest incidence in the rainy season. There are no firm data to support this clinical experience because dengue surveillance and notification are still in their infancy in Vietnam. However, seasonal fluctuation is a common finding in most high transmission areas with monsoon climates.

Risk factor analysis was based on questionnaires, filled out by all children guided by the interviewing medical doctor or teacher. This approach and the added value of the actual observation of households ensure that the results of the risk factor analysis are accurate. Moreover, the large sample size and the incorporation of age in the risk factor analysis were strategies to ensure that findings were also precise. Among the investigated risk factors, pit latrines and littering discarded cans on the domestic premises were associated with a higher prevalence of dengue antibodies. An explanation for having pit latrines may be that uncovered water storage containers were found in or next to every latrine used for flushing. Long term storage of water provides breeding sites for *Ae. aegypti*. In Vietnam, people are inclined to cover the containers with drinking water to keep it clean. Instructions to improve the lids so that mosquitoes cannot enter are easilyuptaken. The containers in pit latrines are usually not covered and the *Aedes* control programs in Vietnam will have to pay special attention this. Littering rubbish, such as discarded cans and plastics around the house, may also contain water, especially in the rainy season and also this requires special attention. The weak but significant association between the presence of pigs and seropositivity, is possibly an effect of cross reactivity between dengue and Japanese encephalitis B (JEB). The pig is an amplifying host for JEB. However an association between raising pigs and creating habitats for *Aedes larvae* is also possible. This needs further exploration. The malaria control program in Vietnam has a much longer history than dengue control. It makes use of insecticide treated bed nets and spraying of residual insecticides. It is interesting to see that in our study area so many people use bed nets and so few re-impregnate these. This probably contributed to the wide confidence intervals of these risk estimates. The relative risk of the use of residual insecticides showed a smaller confidence interval, but of all three vector control measures, aiming at *Anopheles* spp., the vector of malaria, we did not find a protective effect on dengue. This illustrates that vector control measures for dengue should focus on larval control of *Aedes*, and that it requires a different approach than malaria vector control.

The relatively high $R_0$ estimates and the gradual increase in sero-prevalence with age, suggest that dengue in our area is already hyper-endemic and that the likelihood of inter-current epidemics is slim. This obviates the need for disease surveillance and not so much of early warning of outbreaks. Despite the recognition of environmental risk factors for dengue antibody prevalence, disease control by means of sanitary measures may be extremely difficult, and it may even increase the risk of major epidemics by decreasing the replication number. For Binh Thuans province, elimination of DF, DHF and DSS, is probably best achieved via mass dengue immunization programmes when vaccines become available.

5. ACKNOWLEDGEMENTS

We thank Chantal Burghoorn (Institute of Virology, Erasmus Medical Center Rotterdam) for her technical advises with ELISA. We thank the staff at the malaria station, Phan Thiet, in particular Tran Thi Lieu, Nguyen Ngoc Tien and Bui Van Thang for their support with the household survey. We also thank doctors and personnel of the health posts in Ham Kiem and Ham Hiep for their assistance with drawing blood. Their support was of great value.
INCIDENCE OF PRIMARY DENGUE VIRUS INFECTIONS IN SOUTHERN VIETNAMESE CHILDREN AND REACTIVITY AGAINST OTHER FLAVIVIRUSES

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**1. INTRODUCTION**

Mosquito-borne flavivirus infections such as dengue have rapidly become important emerging diseases globally, in terms of morbidity and mortality.\(^8^8\)\(^3^0^7\) Dengue is transmitted by mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus*.\(^2^2^1\) It is a public health problem with growing global incidence and geographical distribution to almost all tropical and subtropical regions; its transmission is changing from epidemic to endemic.\(^7^7\) World Health Organization (1997) estimates that more than 2.5 billion (two-fifths) of the world population are at risk of dengue, of whom 50 million (2%) are infected annually. Dengue fever (DF) can be caused by any of the four distinct serotypes of dengue virus (DENV).\(^2^3^5\) Immunity against dengue is mainly based on production of neutralizing antibodies.\(^1^0^5\) IgG antibodies become detectable 10–14 days after infection and remain lifelong. Primary infection with any one of the four serotypes provides lifelong immunity against that serotype but confers only partial or transient immunity against the other serotypes.\(^2^3^5\) In endemic areas dengue is typically a disease of children and young adults with a mean age of 5–10 years.\(^9^7^,^3^0^7\) A correct diagnosis is essential for case management of individual patients and for control programs that rely on accurate incidence figures. The diagnosis of dengue by primary healthcare workers is usually a clinical diagnosis, but this is notoriously inaccurate.\(^5^6^,^2^1^9\) Therefore, serological confirmation of DF, by demonstrating dengue-specific antibodies, is a very important tool. However, most tests do not distinguish between the four dengue virus types and show more or less cross-reactivity with other flaviviruses, such as West Nile virus (WNV), Yellow Fever virus (YFV) and Japanese Encephalitis virus (JEV). The serological distinction between DENV and JEV especially is difficult where DENV and JEV are endemic, such as in Southeast Asia.\(^2^3^0\) Binh Thuan, a rural province in southern Vietnam, is highly endemic for DF.\(^7^7^2\) In 2003, a cross-sectional study among primary school children of two rural villages showed that the dengue IgG sero-prevalence increased by age, corresponding to an annual incidence primary dengue infections of 11.7%. The true incidence of primary dengue infection, measured by sero-conversion rates, has not been studied. Therefore, we repeated the sero-survey at the primary schools of two rural communes, to measure the true incidence of primary dengue infection by sero-conversion, while controlling for cross-reactivity with other flaviviruses, notably JEV.

**2. MATERIAL AND METHODS**

**2.1. STUDY SITE AND POPULATION**

Binh Thuan Province is located along the south-eastern coast of Vietnam, 150 km north-east of Ho Chi Minh City. It covers 7828 km\(^2\), spread over a rather flat coastal zone, where small-scale agricultural activities prevail, and the forested Truong Son Mountains, an extension of the Anamite mountains ridge. In 2004, the estimated population was 1.1 million inhabitants.\(^2^3^2\) This study was conducted at the primary schools of Ham Kiem and Ham Hiep, 5 km west and 15 km south of the provincial capital Phan Thiet. In 2002, the population of the two communities counted 6,467 and 11,131 members, respectively. The study cohort included all children >7 years of age attending the primary schools of Ham Kiem and Ham Hiep. This accounted for approximately 55% of the entire children’s population in Ham Kiem and 26% in Ham Hiep. Children who had no dengue virus-specific IgG serum antibodies in a sero-survey of 2003 were retested in 2005, provided they still attended school.\(^7^7^2\) Dengue virus-specific IgG serum antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Children who showed sero-conversion between 2003 and 2005 were also tested for other flaviviruses with an immunofluorescence assay (IFA). In cooperation with the People’s Committee of the village, the local health post-staff and school teachers, all pupils of the primary school and their parents were informed about the study and consent was obtained from all. The study followed a protocol approved by the Provincial Health Services, the community health centres of Ham Kiem and Ham Hiep and the Scientific Committee of Cho Ray Hospital, Ho Chi Minh City. Blood collection and dengue virus ELISA Approximately 1 ml of blood was collected by finger puncture into plain vials (Greiner; Minicollect\(^1^7^0\)). Vials were centrifuged on the spot; serum was transferred to a sterile vial and transported immediately to the laboratory of the Provincial Centre for Malaria and Goiter Control, where it was stored at -20 °C. After collecting all specimens, they were transported on ice packs in a cooling box to the Virology Laboratory of Cho Ray Hospital, Ho Chi Minh City, where they were stored at -70 °C. A commercial direct IgG ELISA (Focus Diagnostics Inc., Cypress, CA, USA) was used to demonstrate dengue virus-specific IgG serum antibodies. The tests were performed according to the manufacturer’s instructions.\(^2^3^2\) Optical density (OD) values were measured at 450 nm with 620 nm as a reference with a Benchmark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). OD results were corrected by subtracting the OD values of blank samples which are included in every test kit. The ratio between the sample OD value and...
the OD of the kit calibration serum is expressed as optical density ratio (ODR), by the manufacturer referred to as ‘index value’. ODR >1 was considered positive. There were no samples with ODR = 1.00.

2.2. FLAVIVIRUS IFA

Serum samples taken in 2005 from children who showed sero-conversion in the dengue IgG ELISA between 2003 and 2005 were tested with commercially available IFA slides (PanBio Diagnostics Inc., Baltimore, MD, USA), coated with antigens of WNV, Venezuelan Equine Encephalitis virus (VEE), JEV, YFV and as a control DENV. These slides were used for the detection of IgG serum antibodies to the respective virus antigens. In brief, the serum samples were first screened at dilutions 1:16 and 1:32 in veronal buffer supplemented with 5% guinea-pig serum. Serum was applied on the IFA slides, which were incubated in a moist chamber for 2h at 37 °C. Slides were then washed in phosphate-buffered saline (PBS) twice and rinsed twice with de-ionized water. After air-drying of the slides, anti-human IgG fluorescein-labelled conjugate (DAKO Denmark A/S, Glostrup, Denmark) was applied, and slides were incubated in a moist chamber for 30 min at 37 °C, followed by a second wash in PBS and rinsed with de-ionized water. The IFA slides were evaluated at x 400 using a fluorescence microscope (Axioskop 40; Carl Zeiss AG, Jena, Germany). Fluorescence was graded using the following scale: cytoplasmic fluorescence was graded from moderate to intense (apple green), 2–4. Low intensity or ‘dim’, but definite, cytoplasmic fluorescence was graded as 1. Samples with grade 2 or more were considered positive. Samples which were found positive to DENV and to other flaviviruses were further tested at dilutions 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024.

2.3. STATISTICAL ANALYSIS

The age-dependent sero-prevalence was modelled under the assumption that dengue is endemic in Southern Vietnam, that the force of infection was constant over the years and that antibodies remain detectable lifelong. Thus the proportion of sero-negative subjects that sero-converted per year is also constant:

\[
\frac{dy}{dt} = -\lambda y
\]

where \( y \) is the prevalence of sero-negative subjects and \( k \) is a measure of the force of infection. This can be rewritten as:

\[
y(t) = y(0) \exp(-\lambda t)
\]

The annual incidence rate is thus, when \( t = 1 \) year:

\[
1 - \exp(-\lambda)
\]

The true annual incidence of primary dengue was calculated using the prevalence of sero-negatives in 2003 and 2005, where \( t \) was 23 months after the first measurement on \( t = 0 \) (March 2003). Statistical analysis was performed using the software package S-plus 2000 Professional (Release 2; Mathsoft Inc., Seattle, WA, USA). Overall, age-specific and gender specific sero-prevalence was calculated with 95% confidence intervals; chi-square tests were used for proportions. All tests were carried out at a significance level of 0.05.

3. RESULTS

SAMPLE POPULATION

In 2005, 831 children were registered as pupils at both schools, 459 in Ham Kiem and 372 in Ham Hiep. From the cross-sectional study in 2003, a total of 330 (34.3%, 330/961) serum samples of children tested negative for dengue virus-specific IgG. These children were included in this study except for 99 children (57 from Ham Kiem and 43 from Ham Hiep) who had graduated, 1 who had dropped out of school and 14 who were absent at the time of sampling blood. This left 216 (93.9%, 95% CI: 90.0–96.3) subjects for analysis, 124 from Ham Kiem and 92 from Ham Hiep, with a male:female ratio of 1:1.2. Sero-conversion of dengue IgG and incidence rate Of 216 serum samples, 66 (30.6%, 95% CI: 24.8–37.0) tested positive for IgG antibodies against dengue in 2005: 37 in Ham Kiem and 29 in Ham Hiep. The true annual incidence of primary dengue, calculated from the sero-conversion rate was 17.3% (95% CI: 13.8–21.4). IFA reactivity pattern
of serum samples from children Reactivity against other flaviviruses was tested with IFA in sera of children who showed sero-conversion for dengue virus-specific IgG in the ELISA. Sixty-two of the 66 samples (93.9%, 95% CI: 85.4 – 97.6) obtained in 2005 from children who sero-converted by ELISA also tested positive by IFA for DENV. Of these 62 samples, eight (12.1%) reacted to YFV (12.1%), three (4.6%) reacted to JEV and one (1.5%) to WNV. The antibody titres to YFV, JEV and WNV did not exceed the DENV antibody titres in the IFA and were thus interpreted as cross-reactivity because of a primary dengue infection (Table 1).

Table 1. Immunofluorescence reactivity against flaviviruses in serum of nine out of sixty-six primary school children who demonstrated sero-conversion by dengue IgG- ELISA.

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Flavivirus specific serum IgG antibody serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex / Age (y)</td>
<td>ELISA (ODR)</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>M / 11</td>
<td>4.7</td>
</tr>
<tr>
<td>M / 11</td>
<td>3.6</td>
</tr>
<tr>
<td>M / 10</td>
<td>7.8</td>
</tr>
<tr>
<td>M / 11</td>
<td>3.6</td>
</tr>
<tr>
<td>M / 11</td>
<td>1.5</td>
</tr>
<tr>
<td>F / 11</td>
<td>4.3</td>
</tr>
<tr>
<td>M / 11</td>
<td>4.1</td>
</tr>
<tr>
<td>F / 9</td>
<td>7.4</td>
</tr>
<tr>
<td>F / 10</td>
<td>8.5</td>
</tr>
</tbody>
</table>

1 ODR: optical density ratio; ODR > 1 is considered positive; 2 IFA: immunofluorescence assay; titers are shown as reciprocal values; titers ≥ 1:64 were considered positive.

4. DISCUSSION

This study confirms that dengue is a very common disease in southern Vietnam, with an annual incidence of first infections as high as 17.3%. This is only slightly higher than the incidence of first DENV infections that we estimated in our first sero-survey: 11.7% in children aged 7–14 years. This result confirms that the analysis that we used in our first survey, i.e. binary regression of sero-prevalence to age, using a complementary log–log link function, yields a rather accurate estimate of the incidence of DENV infection. Much has been written about the sero-prevalence, but few studies in south-eastern Asia have investigated the incidence of DENV infection in healthy children. The incidence found in our study was similar to that reported in Thailand and Indonesia among healthy children in prospective cross-sectional studies. Other data in this region showed variable (overall) incidences of DENV infection in different endemic areas for dengue. These incidences are lower than results from our study, because they were based on individuals with minimally symptoms to severe cases of DENV infection seeking for medical aid. A more recent study that also investigated sero-conversion in children in Nicaragua reported similar incidence rates, but, surprisingly, found a much higher prevalence of DENV antibodies. The sample size in sero-epidemiological studies is a crucial aspect, because sample size can influence precision or power of the study. Using a sero-prevalence of 65.7%, as observed previously, and assuming a precision of 5% and a confidence level of 95%, the calculated sample size for our study would be 179 children. We included 330 children of whom 216 were retested for specific IgG antibodies against dengue. This sample size was sufficient and could be used for calculation of the annual incidence. Serological technique is the method of choice for routine diagnosis of flavivirus infections. Many commercially available immunoassays are available for the diagnosis of DENV and other flavivirus infections. Although the indirect IgG-ELISA for DENV, used in this study, is a suitable tool for detecting dengue IgG antibodies with high sensitivity and high specificity; cross-reactivity may occur with other flavivirus antibodies. In areas where multiple flaviviruses, e.g. DENV and JEV, are endemic, samples may show reactivity to different antigens. This may be not only due to cross-reactivity but also due to the presence of specific antibodies acquired by different flavivirus infections. In this study, we found some reactivity to differently flaviviruses with IFA but because the titres were lower than those to DENV, this was interpreted as cross-reactivity. Although southern Vietnam is endemic for JEV and Japanese encephalitis cases occur sporadically throughout the year, its public health importance is minor.287 Secondly, as YFV and WNV infections have never been reported in south-eastern Asia, the IFA reactivity to those viruses should be considered as cross-reactivity, whose degree also confirmed previous findings. Knowledge of the sero-prevalence and incidence of DENV infections is essential for planning public health interventions such as vector control or possible future use of a vaccine. But, it is also important information to clinicians who are involved in individual case management. In areas without facilities and laboratory confirmation of common infectious diseases doctors base their clinical diagnosis on signs and symptoms. Clinical diagnosis of dengue is highly inaccurate, and the WHO classification system for clinicians is not very accurate either. However, it is not yet clear which case definition could better identify cases of (potentially) severe dengue at the primary healthcare level. This study shows that the large burden of dengue disease presented to primary health facilities is underpinned by a much greater incidence of asymptomatic to mild dengue infection in the general population. Inappropriate, too sensitive case detection could lead to unaffordable intervention policies that may not always be necessary. Knowledge of the incidence of largely asymptomatic infections does not need to reflect the incidence of symptomatic infections presented to the health services, but this knowledge may help sensitizing primary healthcare workers to making the presumptive diagnosis of dengue. In conclusion, our data established a high incidence rate of DENV infections. Although DENV and JEV are both endemic in Binh Thuan province, JEV infection is of minor importance in children. Using a binary regression with a
log–log link function, applied on sero-prevalence in cross-sectional sero-survey data, yields a rather accurate estimate of the true annual incidence in endemic areas such as Vietnam.

5. ACKNOWLEDGEMENTS
This study was funded by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO). We thank Thea Philipse (Institute of Virology, Erasmus Medical Center, Rotterdam) for her technical advises with IFA, and our colleagues at the Department of Virology, Cho Ray Hospital, Vietnam, for their carefully performed diagnostic testing. Thanks are given to clinicians and co-workers of Ham Kiem and Ham Hiep health posts and Binh Thuan Provincial Malaria and Goiter Control Center.
CHAPTER 5

GEOGRAPHICAL HETEROGENEITY OF DENGUE TRANSMISSION IN TWO VILLAGES IN SOUTHERN VIETNAM

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This study was performed to test the hypothesis that there are “hotspots”, i.e. geographical heterogeneity, of dengue transmission. Data from two repeat sero-surveys in two villages in Vietnam were used to identify incident infections and to relate these to prevalence at baseline and thus assess geographical heterogeneity, i.e. clustering, in dengue transmission. A total of 400 households were surveyed; serological data from 521 children at baseline and from 119 children at follow-up were included in a spatial analysis. Geographical heterogeneity of dengue transmission was explored using a permutation null distribution test. This showed for the first time evidence of clustering of dengue virus transmission at the household level among asymptomatic children. Risk areas could be identified by sero-prevalence surveys combined with mapping. Control of dengue virus transmission could be supported by identification and control of hotspots.

1. INTRODUCTION

Recent estimates indicate that approximately 3.5 billion people, ~55% of the world’s population live in countries at risk for dengue.15 Dengue ranks among the most important infectious diseases with a major impact on public health in Vietnam and many other countries in the tropics and subtropics. Dengue virus transmission primarily takes place through bites by the mosquito vectors, Aedes aegypti and Aedes albopictus, which feed preferentially on human blood, and are often found in and around human dwellings.112,245 Infection with dengue virus results in either (almost) asymptomatic infection, undifferentiated febrile illness, dengue fever (DF) or even life-threatening manifestations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).235 To date, no vaccine or chemotherapy is yet available. Prevention and control of dengue transmission therefore depend on vector control (larvicide treatment, insecticide sprays and elimination of breeding sites) and avoidance of bites. The national dengue control program in Vietnam recommends vector control by larvae elimination. However, these measures are usually only implemented after notification of severe cases (DHF and DSS).307 This local policy is based on the assumptions that such cases reflect locally increased vector densities with higher infection rates. It remains unclear to what extent this approach controls further transmission, because the majority of dengue virus infections (~80%) are mild/atypical or even asymptomatic.57 It is therefore likely that such measures are not adequate to prevent sustained dengue virus transmission in the community.5,17,40 In fact, its geographical distribution is spreading and transmission rates have increased over the last decades.157

Previous studies showed that dengue disease tends to cluster either in the same household or in nearby neighbourhoods.198,212,281 However, while suggestive of clustering of transmission, clusters of disease may be due to diagnostic biases or heterogeneity in susceptibility to symptomatic disease following infection within families or households.

To explore the hypothesis that dengue virus transmission is spatially focal, we used available data from a cross-sectional sero-epidemiological study in 2003, a two year follow-up study and a household survey in two communes, Ham Kiem and Ham Hiep in southern Vietnam.272,274

2. METHODS

2.1. DATA SOURCES, STUDY SITES AND POPULATION

This study used available data from a cross-sectional, a follow-up study and a household survey in two communes, Ham Kiem and Ham Hiep, among primary school children.272,274 Briefly, we conducted a cross-sectional study in two communes in 2003, in which all primary school children at two primary schools in the two communes were included and their prevalence of antibodies to dengue measured. Additionally, a household survey was carried out in 400 houses. All children who had no dengue virus-specific IgG serum antibodies in a sero-survey of 2003 were retested in a follow-up study which was conducted in 2005. From 2002 census data, the total populations in Ham Kiem and Ham Hiep were 6,467 and 11,131. The population densities of the two communities were approximately 109 people/km² and 322 people/km² for Ham Kiem and Ham Hiep, respectively. It has a tropical climate with a mean temperature of 27 °C, an average monthly rainfall of approximately 100 mm and a rainy season that lasts from May until October.

2.2. GEOGRAPHIC MAPPING

During the household visits in 2003, geographic coordinates were recorded. The latitude and longitude of household visits were registered using a hand-held global positioning system (eTrex®, Garmin International Inc., USA). The coordinate system and datum used were degree decimal and WGS-84, respectively. MapInfo Professional (MapInfo Corp., 1998) was used to display the distribution of dengue serum specific IgG cases per household.

2.3. STATISTICAL ANALYSIS

We hypothesized that there is geographical heterogeneity in dengue transmission within communities with the occurrence of “hotspots”. If so, new infections, as indicated by observed seroconversion during follow-up, would occur near places where dengue IgG seroprevalence was highest at baseline (2003). If not (the null hypothesis) new infections would occur randomly. To test this hypothesis we looked at the geographical distance between old infections (i.e. children who were seropositive for dengue) at baseline and new infections observed during follow-up using a permutation analysis. Consider a child i at baseline living at coordinates Q_i = (x_i,y_i). Let P_i = 1 if the child was seropositive and -1 otherwise. Similarly, let consider a child j observed at follow-up, and let,
again, $I_i = 1$ if the child was seropositive and $=-1$ otherwise. The coordinates of this child are $Q_i = (x_i, y_i)$.

Now consider the statistic:

$$T = \sum_{i=1}^{N} \sum_{j=1}^{M} w_i P_i d(Q_i, Q_j)$$

where $d(Q_i, Q_j) = 1/(0.001 + \text{distance}(Q_i, Q_j))$ where the (Euclidean) distance is measured in degrees (i.e. approximately 110 km) so that $d(Q_i, Q_j)$ of sites a hundred meters (flying distance of vectors) apart is about half that of the $d()$ between a spot and itself.

Further, let $w_i$ (the “weight” of a baseline child) be taken $= \text{age}$ for $P_i = -1$ and $= 1/\text{age}$ for $P_i = 1$. This weighting was done because age is an important predictive factor for seropositivity since the seroprevalence increases strongly with increasing age.

The permutation null distribution (with separate permutations for the two communities in the study) was generated using a specially written computer program. Large values (relative to the permutation null distribution) reflect the existence of hotspots. A total of 100 draws from the permutation null distribution were generated using this program.

2.4. ETHICAL CONSIDERATION

The protocols for recruitment, testing and follow-up were approved by the Provincial Health Services, the community stations of Ham Kiem and Ham Hiep and the Scientific Committee of Cho Ray Hospital, Ho Chi Minh City. In cooperation with the People’s Committee of the villages, the health post-staff and school teachers, all children of the primary school and their parents were informed about the study and consent was obtained from all.

3. RESULTS

3.1. BASELINE SERO-PREVALENCE

The study design and data sources are shown in figure 1. Figure 2 shows the map of Binh Thuan province, Vietnam and location of the study areas. During the household survey in 2003, a total of 400 households, home to 533 children, were visited for obtaining geographical coordinates. Serological data were available for 521 children of which 339 (65%) were positive for dengue serum specific IgG. This was taken as background sero-prevalence in this study. The spatial distributions of the households of these 521 children in the villages are shown in figure 3 and 4.

3.2. GEOGRAPHICAL HETEROGENEITY

All children ($n = 216$) who had no dengue virus-specific IgG serum antibodies (dengue naïve) in a sero-survey of 2003 and who had been followed-up for 23 months were eligible for inclusion to exploring the heterogeneity of dengue transmission excluding 97 children whose geographical coordinates had not been recorded in 2003. Because only dengue naïve were included, any seroconversion of IgG was due to dengue infection during the 23 months of follow-up. In the permutation analysis, we considered 119 children, 65 and 54 from Ham Kiem and Ham Hiep, respectively. These children were living in 111 households. All children who have been followed-up lived in the same house as two years previously. Figure 5 shows the permutation null distribution of $T_{\text{observed}} / T_{\text{null}}$. The null distribution values exceeded 1 for every permutation, i.e. the observed clustering exceeded random draws from the null distribution 100/100 times. Clearly, this provides cogent evidence for the existence of geographical heterogeneity, i.e. that new infections occurred near places where prevalence was highest at baseline.

4. DISCUSSION

Results in this study showed that new dengue virus infections occurred near places where sero-prevalence was highest at baseline, suggesting important spatial heterogeneity in the transmission of dengue. This study overcomes methodological problems of earlier studies which looked at clustering of symptomatic cases.

There are several plausible explanations for the nearby simultaneous appearance of dengue cases at household level. First, entomological studies have shown that *Ae. aegypti* has a multi-feeding behaviour on multiple people during a single gonotrophic cycle. The implications of
Figure 1. Data sources overview

Figure 2. Map of Binh Thuan province, Vietnam and location of the study areas.
Figure 3. Distribution map of children per household in Ham My, Binh Thuan, Vietnam.

Figure 4. Distribution map of children per household in Ham Kiem, Binh Thuan, Vietnam.
this behaviour may include the occurrence of clusters of dengue cases in or nearby the same household and the rapid and sometimes explosive spread of dengue. However, this is unlikely to account for our observations in view of the probable (long) time lag between “baseline” infections and follow-up infections. Second, local occurrence of dengue clusters could also be due to locally elevated vector density. Cluster investigations in Thailand showed significant differences in the *Ae. aegypti* pupae/person ratio among dengue cases in comparison with non-dengue cases. However, no significant differences were shown for adult *Ae. Aegypti* population density. Abundance of pupae or adult female mosquitoes may be informative for routine surveillance or as an eradication measure, but these measures lack correlation between indices and dengue disease. Detection of DENV-infected adult *Ae. aegypti* female mosquitoes that can potentially infect multiple individuals may be more relevant for DENV transmission.

Third, focal spreading can also be explained by the movement of the infected mosquitoes with its restriction of the flying range of approximately 100 metres. The transmission through a neighbourhood is most likely caused by the activities, daily movements and social networks of infected people as cluster sizes often exceed the flying range of the mosquitoes. Apparently, undiagnosed asymptomatic dengue virus infections or unrecognized dengue cases with mild symptoms play a more predominant role for the spread of dengue virus and undetected persistence of transmission locally.

Though this study gives insight in the transmission dynamics of dengue virus within communes and at household level, there are some limitations: (1) it must be noted that it is impossible to ascertain whether these children were infected at home, at school, or somewhere else. Only household geographical coordinates were considered but children living close together often attend the same school, and make use of the same playgrounds etc. However, the likely role of households is suggested by observations from a prospective spatial cluster study in Thailand. Absenteeism of children due to fever tended to cluster in small geographical areas where dengue transmission was active, whereas those who were absent for other reasons were always from areas where dengue was not active. Other reports also showed that household members of dengue sero-converters had a higher relative risk for dengue virus infection. (2) While our study established geographical clustering, it was not designed to identify the key factors accounting for this clustering, such as environmental or entomological factors (water source, water storage, vector density), which have been known to contribute to dengue virus transmission.

Despite these limitations, results from spatial analysis provide insight in dengue virus transmission and control. Based on these data, we believe that sero-surveillance should play a role in identifying hotspots of transmission and that strategies that are centered only on severe clinical dengue cases will be ineffective in controlling transmission, as only a very small proportion (~5%) of dengue cases will develop severe disease. Such population based sero-prevalence surveillance among children combined with geographical information systems (GIS), is a rapid, easy-to-perform and affordable tool for identification of possible high exposure areas at community level. Where possible, identification of dengue risk areas should also be accompanied with vector surveillance and more importantly with the identification of DENV-infected mosquitoes in field settings. Presently, tools for detection of dengue virus in vectors are not yet available for field application. Thus in addition to infection hotspot identification, control measures should be guided by measurement and control of vector density, e.g. through breeding sites elimination with perifocal spraying in identified risk areas. However, the effectiveness of insecticidal treatments in open areas is limited by insufficient residual effect when applying spraying of ultralow volume of insecticides formulation per unit area, and insecticides application inside house where DENV-infected mosquitoes rest may be more cost-effective. Nevertheless, the success of dengue control cannot only rely on intermittent surveillance and insecticide spraying alone, and involvement of the community seems key. However, the best approach to this involvement is still unclear. Education campaigns have been used to increase awareness of dengue in Vietnam, but their effects on source reduction have never been studied.

**ACKNOWLEDGEMENTS**

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SUPPORTING INFORMATION

RANDOMIZE TIMER
rep = 100
iter = 100
loops = 0
OPEN "gis3.txt" FOR INPUT AS #1
OPEN "gis4.dat" FOR OUTPUT AS #2
DIM x(521, 7)
FOR i = 1 TO 521
INPUT #1, x(i, 1), x(i, 2), x(i, 3), x(i, 4), x(i, 5), x(i, 6)
PRINT x(i, 1), x(i, 2)
NEXT i
n1 = 1: n2 = 261: n3 = 262: n4 = 521
10 dist2 = 0
FOR k = 1 TO 2
IF k = 1 then m1 = n1: m2 = n2 else m1 = n3: m2 = n4
FOR i = m1 TO m2
IF (x(i, 5) <> 9) then gosub 1000
NEXT i
NEXT k
PRINT #2, dist2
loops = loops + 1: print loops
IF (loops <= iter) then gosub 2000: goto 10
Close #1
Close #2
STOP
1000 REM subroutine
FOR j = m1 TO m2
dist = (ABS(x(i, 5) = x(j, 5)) - ABS(x(i, 5) <> x(j, 5))) / (.001 + SQR((x(i, 1) - x(j, 1))^2 +
(x(i, 2) - x(j, 2))^2)))
dist2 = dist2 + dist
REM PRINT i; j; ABS(x(i, 5) = x(j, 5)); ABS(x(i, 5) <> x(j, 5)); dist; dist2
NEXT j
RETURN
2000 REM permute
FOR m = 1 TO rep
FOR j = n1 TO n2
FOR l = n1 TO n2
x = RND
IF (x(j, 5) <> 9 AND x(j, 5) <> 9) THEN IF x > .5 THEN c = x(l, 5): x(l, 5) = x(j, 5): x(j, 5) = c
NEXT l
NEXT j
FOR j = n3 TO n4
FOR l = n3 TO n4
AGE-SPECIFICITY OF CLINICAL DENGUE DURING PRIMARY AND SECONDARY INFECTION

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Background: Although age at dengue virus (DENV) infection is recognized as playing a key role in characterizing the risks of clinical attack and disease severity, the contributions of age to disease development have yet to be quantified in detail. The present study aims to estimate the age-specific risks of clinical dengue attack (i.e. the risk of symptomatic dengue among the total number of DENV infections) during primary and secondary infections.

Methods: We analysed two pieces of epidemiological information in Binh Thuan province, southern Vietnam, i.e., age-specific seroprevalence and a community-wide longitudinal study of clinical dengue attack. The latter data set stratified febrile patients with DENV infection by age as well as infection parity. A simple modelling approach was employed to estimate the age-specific risks of clinical dengue attack during primary and secondary infections.

Results: Using the seroprevalence data, the force of infection was estimated to be 11.7% (95% confidence intervals (CI): 10.8-12.7) per year. Median age (and the 25-75 percentiles) of dengue fever patients during primary and secondary infections were 12 (9-20) and 20 (14-31) years, respectively. The estimated age-specific risk of clinical dengue increases as a function of age for both primary and secondary infections; the estimated proportion of symptomatic patients among the total number of infected individuals was estimated to be < 7% for those aged < 10 years for both primary and secondary infections, but increased as patients become older, reaching to 8-11% by the age of 20 years.

Conclusions/Significance: For both primary and secondary infections, higher age at DENV infection was shown to result in higher risk of clinical attack. Age as an important modulator of clinical dengue explains recent increase in dengue notifications in ageing countries in Southeast Asia, and moreover, poses a paradoxical problem of an increase in adult patients resulting from a decline in the force of infection, which may be caused by various factors including time-dependent variations in epidemiological, ecological and demographic dynamics.

1. INTRODUCTION

Dengue ranks among the most important infectious diseases with a major impact on public health in many countries in the tropics and subtropics. Estimates showed that approximately 3.5 billion people, ~55% of the world’s population live in countries at risk for dengue.14 The global incidence has increased steadily over the last six decades, simultaneously with an increase in geographic distribution and a transition from epidemic-type dengue with long interepidemic intervals to endemic-type with seasonal fluctuation.77,90 Dengue virus (DENV) transmission primarily takes place through bites by the principal mosquito vectors, Aedes aegypti, which feed preferentially on human blood, and are often found in and around human dwellings.112,245 Infection with any of the four dengue serotypes results in either asymptomatic infection, or a spectrum of clinically apparent disease ranging from mild undifferentiated febrile illness to severe dengue of which dengue shock syndrome (DSS) is the most common life threatening syndrome in children.109 The mechanisms for the variable clinical outcome are not completely elucidated, but genetic factors, race, maternal antibody, circulating serotype and infection with multiple serotypes are believed to play an important role in determining the disease severity.165

When it comes to the disease severity, a well-established epidemiological risk factor is the age at infection.27,30,94,110 It is known that differences in clinically apparent dengue vary by age; pre-school children and infants have rather more undifferentiated illnesses while pre-adolescent children often develop fever105, and moreover younger children with dengue haemorrhagic fever (DHF) is known to experience more severe clinical outcome (e.g. higher case fatality ratio) than adults.80 Dengue is a paediatric disease in Southeast Asia except for Singapore. A rigorous vector control program has substantially reduced the transmission in Singapore and, as a consequence, dengue patients are predominantly seen in adults. Nevertheless, with the elevation in patients’ age, the outcome of DENV infection may be more favourable since most dengue cases manifest as dengue fever (DF) instead of DHF.104

Apart from age, infection parity is known to be a critical factor of disease severity; primary infection with any of the four serotypes is believed to elicit lifelong immunity against that serotype, but confers partial or transient immunity against other serotypes.87,166 Cross-reactive, but sub-neutralizing DENV-reactive IgG, acquired of a previous heterotypic serotype may enhance DENV infectivity which may result in higher viral burden and contribute to induced disease severity. Heterologous secondary infections have been associated with large, clinical outbreaks of DHF/DSS where severe dengue occurs most frequently in children.93 In some rigorous observations, the age group with highest susceptibility to contracting DSS has been suggested to be pre-adolescent with a modal age of 8 to 11 years.241,276

Although age at infection and infection parity are the representative key modulators of clinical dengue and disease severity, their relationship has yet to be established and explicitly quantified. A previous study investigated the relationship between age at primary infection in Brazil and the risk of febrile illness, suggesting that adults are more likely than children to have clinical dengue.82 This result should ideally be validated in the Southeast Asian settings and, moreover, we have yet to understand the age-specific risk of symptomatic dengue during secondary infection. This present study tackles these issues by analysing epidemiological data sets in southern Vietnam, focusing specifically on the age-specific risk of symptomatic dengue given infection. That is, we do not consider age-specific severity of clinical dengue, and rather, focusing only on the conditional probability of illness given infection. Because of high transmission potential with co-circulating
multiple serotypes, dengue has been mainly a paediatric disease in Vietnam, and ironically, this provides us with an opportunity to investigate the age-specific risks of clinical attack both during primary and secondary infections. The present study aims to characterize a fundamental relationship between age at DENV infection and the risk of developing clinical attack.

2. METHODS

2.1 STUDY SITE

Our study rests on empirical observations in Binh Thuan province which is located along the south-eastern coast of Vietnam, 150 km northeast of Ho Chi Minh City, wedged between the Truong Son forested mountains (alt. 1100–1642 m) in the west and the South Chinese Sea in the east. It covers 7,828 km² and the estimated population was 1,140,429 inhabitants in 2004. The majority of the population lives in rural areas, with approximately 187,042 people in and around the capital, Phan Thiet City. Healthcare is provided by a provincial hospital in Phan Thiet city, nine district hospitals and 115 commune posts for primary healthcare (PHC) and disease control.

2.2 EPIDEMIOLOGICAL DATA

We examined two pieces of epidemiological information, (i) age-specific seroprevalence and (ii) age-specific frequency of clinical attack of dengue during primary and secondary infections, as determined by serological confirmation, in order to estimate the age-specific risk of clinical dengue attack. Supplementary figure S1 shows the participating PHCs and the villages in which the sero-survey were conducted. The mean distance between the source of seroprevalence data and all PHCs was 40.5 km (range 3-87 km).

The former data set, age stratified seroprevalence were examined through a cross-sectional survey among primary school children in two communes (Ham Kiem and Ham Hiep). This survey was conducted among 961 children, aged from 7 to 14 years, in 2003. Dengue serum specific IgG antibodies were measured by age group. The detailed results are given elsewhere.272-274 Approximately 1 ml of blood was collected by finger puncture in plain vials (Greiner, Minicollect), left to clot at ambient temperature, centrifuged at 1000 rpm for 15 minutes and serum was transferred to a sterile vial for storage at –20 °C until testing. All samples were tested for the presence of dengue specific serum antibodies against dengue virus using a commercial available indirect IgG enzyme-linked immunosorbent assay (ELISA). The indirect IgG ELISA was performed according to the manufactures instructions (Focus Technologies Inc., Cypress, CA, USA). Optical density (OD) values were measured at 450 nm with 620 nm as a reference with a Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were expressed as the ratio between the sample OD value and the OD value of the kit calibration serum (ODR), both after subtraction of the OD of an enclosed blank specimen. ODR values > 1 were considered positive. Seroprevalence (i.e. proportion positive) was stratified by age group.

Second, age-specific frequencies of clinical attack were derived from a prospective longitudinal observational study at 12 PHCs across Binh Thuan province and at the provincial malaria control center in Phan Thiet City. The study examines the aetiology of acute undifferentiated fever (AUF) from March 2001 to March 2006,218-220 All patients presenting with AUF were included. AUF was defined as any febrile illness of duration less than 14 days, confirmed by an axillary temperature of ≥38.0 °C, without any clinical indication for either severe systemic or organ specific disease. Malaria was excluded by microscopic examination of a thick blood smear. A standardized questionnaire was employed to collect demographic and clinical information. Serum samples were collected by venous puncture on presentation (acute sample; t0) and after 3 weeks (convalescence sample; t3). Serum samples were stored at -20°C at the study sites until monthly transfer to Cho Ray hospital (Ho Chi Minh City, Vietnam), where they were stored at -70°C. Complete sets of acute and convalescence samples were selected for dengue serology. In 2001 all collected paired sera were tested with dengue ELISA; afterwards paired samples were randomly selected as two patients per PHC and per month from the total dataset.219 Paired serum samples were tested for dengue with direct IgG ELISA and IgM-Capture ELISA (Focus Technologies Inc., Cypress, CA, USA). It should be noted that these serological tests did not distinguish between an infection with DENV from that with Japanese Encephalitis virus (JEV). Cross-reactivity with JEV antibodies may have occurred, potentially involving a small proportion of samples.271 Details regarding the ELISA and the interpretation of results were described previously.272 In brief, a fourfold increase of antibody concentrations between t0 and t3 was considered significant. The IgM concentration on t3, relative to the IgG concentration on t3 was also used as a criterion. Acute primary DENV infection was defined as positive IgM on t3 with an IgM/IgG ratio on t3 greater than one. A positive IgM on t3 with an IgM/IgG ratio on t3 less than one, or a negative IgM reaction on t3 but with a positive IgG t3 and a fourfold molar increase of IgG between t0 to t3 was classified as acute secondary dengue. A negative IgM reaction on t3, a positive IgG on t3 but without a fourfold increase between t0 and t3 was classified as “not acute dengue but past infection”, and a subject of both negative IgM and IgG on t3 was classified as “no dengue”. It could well be possible that patients with an immune response to secondary dengue infection have had a tertiary or even dengue infection with a fourth serotype. Because the immune response between sequential dengue infections was not explicitly distinguished only by using ELISA, we grouped all the individuals with a history of infections with multiple serotypes and define it as the secondary dengue infection in the present study. The epidemiological, virological and clinical features have been described elsewhere.273 All four serotypes have been circulating during the study period with a potential shift of the dominant DENV serotype over time. Whereas DENV-4 was the dominant serotype in 2001-2002, DENV-1 and DENV-2 later came to be most frequently isolated.
2.3 ETHICAL APPROVAL
The protocols for recruitment, testing and follow-up were approved by the Review Board of the Cho Ray Hospital, Ho Chi Minh City, Provincial Health Services and the community stations. The study was explained and discussed in meetings (e.g. with the People’s Committee of the villages, the PHC-staff and school teachers). All patients (or, for children, the parents or guardian) gave written informed consent.

2.4 MATHEMATICAL MODEL AND STATISTICAL ESTIMATION
Sequential transmission dynamics of DENV infection
A simple epidemiological model was developed to estimate the age-specific risks of clinical dengue attack during primary and secondary infections. The model was constructed to describe the age-specific frequencies of primary and secondary infections (which were used as the denominator population, representing infected individuals), and we combine it with our empirical data of the age-specific frequencies of individuals with symptomatic dengue during primary and secondary infections (which were dealt with as the numerator population, representing symptomatic dengue patients) in order to estimate the age-specific conditional risk of clinical dengue attack (given infection). Figure 1 illustrates the compartments of our model, describing the lifetime risks of primary, secondary and tertiary infections. The model accounts for the age-specificity of infection and acquisition of serotype-specific immunity over lifetime.

The similar sequential assumptions have been employed in previous studies\textsuperscript{50,70,71,198}, and the mathematical descriptions are given in the Appendix. Among the parameters in Figure 1, we estimate the force of infection (i.e., the rate at which susceptible individuals get infected), $\lambda$, from seroprevalence data, and the remaining parameters, i.e., the scaling factor representing the number of co-circulating serotypes ($\alpha$) and the loss rate of cross-protective immunity ($\delta$), were assumed known. All four serotypes have been observed in Binh Thuan province, but the relative frequency of serotype 3 has been smaller than other serotypes.\textsuperscript{228,275} Because of the irregularity in the serotype-specific frequency, which yields variations in the transmission potential between different serotypes, we therefore varied $\alpha$ from 2.5 to 4.0 with a default value 3.5. The length of cross-protective immunity in literature ranges from 1-2 weeks\textsuperscript{206} to 2-9 months.\textsuperscript{239} Although not based on an explicit statistical estimation, the latter study suggested that within 2 months after a primary attack offered full protection, and that within 9 months after the primary attack may still yield partial protective immunity to infection with heterologous strain (e.g. partial reductions in clinical symptoms).\textsuperscript{239} Accordingly, we varied $\delta$ from 10 days to 1 year with a default value of 1 month. Due to absence of strain information, we ignored potential variations in virulence between strains (see Discussion). The following assumptions were made to attain a simple statistical estimation; (i) the force of infection, $\lambda$, was age-independent (and we focused on relatively young age groups in the population), (ii) the transmission has reached to an endemic equilibrium so that the time-inhomogeneity can be ignored (as we focused on the datasets covering a short period of time), (iii) the transmission potential is identical among co-circulating serotypes (though we accounted for non-integer values for $\alpha$ as a theoretical adjustment), (iv) once an individual acquire infection with a single serotype, he/she becomes temporarily immune against infection with other heterologous serotypes (which is lost at a rate $\delta$) and permanently immune against further infections with the identical serotype, and (v) our empirical data represent the transmission dynamics for the entire population of Binh Thuan province. Although omitted from Figure 1 for simplicity, the natural death rate, $\mu$ (per year), occurs in each compartment. We obtained the population data from the Binh Thuan statistics office, Phan Thiet City, southern Vietnam, and estimated $\mu$, assuming that the population is stable and also that the survivorship in this rural population is sufficiently approximated by an exponential distribution.

![Figure 1. Sequential transmission dynamics of DENV infection.](image)
The age-specific proportion of those having experienced infection at least with one serotype is given by \(1 - \exp(-\alpha \lambda a)\), and the age-specific survivorship at age \(a\) is written as \(\exp(-\mu a)\).

Supposing that there were \(n_a\) seropositive and \(m_a\) seronegative results at age \(a\), the likelihood function to estimate \(\lambda\) is

\[
L(\lambda) = \prod_{a} (1 - \exp(-\alpha \lambda a))^n_a \exp(-\alpha \lambda a)^m_a.
\]

(1)

Exactly the same argument was made to estimate \(\mu\).

**Estimation of the age-specific risk of clinical attack**

Subsequently, we estimated the age-specific risks of clinical attack during primary and secondary infections, \(r_1(a)\) and \(r_2(a)\), respectively. Let \(S_0(a)\), \(S_1(a)\) and \(S_2(a)\) be the expected proportions of susceptible individuals (among a total population), based on our model, who are susceptible to the remaining \(\alpha\), \(\alpha-1\) and \(\alpha-2\) serotypes at age \(a\), respectively. The incidence of primary, secondary and tertiary infections at age \(a\) are then given by \(\alpha \lambda S_0(a)\), \((\alpha-1) \lambda S_1(a)\) and \((\alpha-2) \lambda S_2(a)\), respectively.

We ignored infection with fourth serotype assuming that it is rare, and we grouped the incidence of secondary and tertiary infections to adhere to our empirical observation of secondary infections in the longitudinal survey (and assumed that the risk of clinical attack between secondary and tertiary infections is identical). Accordingly, \(r_1(a)\) and \(r_2(a)\) were, respectively, conditioned on primary infection and secondary and tertiary infections. Observing a signature of an increase in \(r_1(a)\) and \(r_2(a)\) as a function of age in the empirical data, three statistical models, logit \((l)\), Weibull \((w)\) and exponential \((e)\) distributions, were employed for these functions, i.e.,

\[
\begin{align*}
r_{l,1}(a) &= \frac{kp_{10} \exp(ra)}{k + p_{10}(\exp(ra) - 1)}, \\
r_{l,2}(a) &= 1 - \exp(-ka), \\
r_{e,1}(a) &= 1 - \exp(-ka),
\end{align*}
\]

(2)

for \(i = 1\) or \(2\), where \(k\), \(p_{10}\) and \(r\) are the parameters. Given a data set of \(n\) patients during primary infection and \(m\) patients during secondary infection with their age at infection \(a\), the likelihood function to estimate parameters for \(r_{1}(a)\) and \(r_{2}(a)\) is

\[
L(\theta|a, \alpha, \lambda, \mu, \alpha-1, \alpha-2) = \prod_{i=1}^{n} \int_{a}^{\infty} r_{i,1}(a) S_i(a) S_{\alpha-1}(a) S_{\alpha-2}(a) da + \prod_{i=1}^{m} \int_{a}^{\infty} r_{i,2}(a) S_i(a) S_{\alpha-1}(a) S_{\alpha-2}(a) da
\]

(3)

for \(q = l, w\) or \(e\). It should be noted that \(S(a)\) were solved analytically and replaced by parameters \(\mu\), \(\lambda\), \(\alpha\) and \(\delta\) (see Appendix). Maximum likelihood estimates of parameters the were obtained by minimizing negative logarithm of (3). The lack-of-fit of three parametric models were compared by employing Akaike information criterion (AIC).

**3. RESULTS**

**3.1 DEMOGRAPHY AND THE FORCE OF INFECTION**

The age-specific population size of Binh Thuan province is shown in Figure 2A. Mean and median ages (and the 25-75 percentiles) were 24.7 and 20 (9-35) years, respectively. Employing an exponential approximation, the natural death rate \((\mu)\) was estimated to be \(4.05 \times 10^{-2}\) (95% confidence interval (CI): \(4.04 \times 10^{-2}\)–\(4.05 \times 10^{-2}\)) per year. Figure 2B shows the observed and predicted age-dependent seroprevalence. The force of infection of the total of, \(\alpha \lambda\), was estimated as 11.7% (95% CI: 10.8 - 12.7) per year.

**3.2 SYMPTOMATIC PRIMARY, SECONDARY OR TERTIARY DENGUE INFECTIONS**

A total of 14595 febrile patients were included in our longitudinal survey. Eighty-three patients were excluded as the inclusion criteria for AUF were not met. That is, eleven patients were afebrile (i.e. <38 °C), axillary temperatures of 19 patients were not documented, and 53 patients were diagnosed with an organ specific disease (e.g. pharyngitis) at presentation. Paired sera were collected from 8268 febrile patients; 1938 (23.4%) serum pairs were tested with dengue IgM- and IgG-ELISA. Of these, DENV infection was serologically confirmed in 382 patients (19.7%). Primary infection accounted for 76 confirmed patients (19.9%), and...
secondary infection for 306 patients. Their age-specific frequencies are shown in Figures 2C and 2D, respectively. Median age (and the 25-75 percentiles) of dengue fever patients during primary and secondary infections were 12 (9-20) and 20 (14-31) years, respectively, revealing that secondary infection occurs at significantly older ages (p < 0.01, Wilcoxon test). None of confirmed dengue infections were suggestive of severe clinical forms of dengue, DHF or DSS.

### 3.3 Epidemiological Dynamics of Dengue Infection

Using the maximum likelihood estimate of $\alpha \lambda = 0.117$ and the default values of $\alpha$ and $\lambda$, age-specific frequencies of primary, secondary and tertiary infections are shown in Figure 3A. As indicated by $1/\alpha \lambda$, the mean age at primary infection is 8.5 years, and secondary and tertiary infections occur at older ages. Figure 3B shows the estimated risks of symptomatic dengue during primary and secondary infections for Weibull and exponential assumptions (results with logit model is not shown as it yielded the similar qualitative pattern to Weibull). The conditional risks of clinical attack were shown to increase as a function of age during both primary and secondary infections. The estimated proportion of symptomatic subjects among the total number of infected individuals was below 7% for those aged younger than 10 years of age for both primary and secondary infections, but increased as patients become older, reaching to 8-11% by the age of 20 years. Within the age-band examined (< 60 years), both assumptions indicate that the risk of symptomatic dengue during secondary infection is higher than that during primary infection for all ages. The Weibull distributed age-specific risk of clinical dengue during primary and secondary infections plateau around the ages of 15 and 19 years, respectively. Figures 3C and 3D compares the observed and predicted age-specific numbers of symptomatic subjects during primary and secondary infections, respectively. Using default values of $\alpha$ and $\delta$, the AIC values were estimated to be 1461, 1460 and 1470 for logistic, Weibull and exponential assumptions, respectively. The preference of Weibull assumption did not change when we varied $\alpha$ from 2.5 to 4.0 and $\delta$ from 10 days to 12 months.

Figures 4A and 4B examine the univariate sensitivity of $r_2(\delta)$ to $\alpha$ for Weibull and exponential assumptions, respectively. The age-specific risk of clinical attack was the highest for all ages with $\alpha = 3$, but the overall difference in the conditional risk from those with other $\alpha$ remained within 5% for Weibull assumption. The Weibull assumption was always preferred in terms of AIC, but the difference in AIC between the logit and Weibull models remained <3 for the range of $\alpha$ that we examined. Similarly, Figures 4C and 4D show the univariate sensitivity of $r_2(\alpha)$ to $\delta$ for the Weibull and exponential assumptions, respectively. The age-specific risk of symptomatic secondary dengue infection with default $\delta$ (1 month) yielded the smallest estimates, but again the difference in the estimated risks of clinical attack remained within 5% for Weibull assumption. Again, AIC values indicated Weibull model as the best, but the differences in AIC values between logit and Weibull models remained <3 for the whole range of $\delta$. 

---

Figure 3. Epidemiological dynamics of dengue infection. (A) Age-specific frequency of primary (thin solid line), secondary (thick solid line) and tertiary infections (dashed line). The frequencies are characterized by the force of infection, the mean duration of cross-protective immunity and the number of co-circulating serotypes. (B) Age-specific probability of developing clinical attacks of dengue given primary (thick lines) and secondary or tertiary infection (thin lines). The continuous lines represent the results employing Weibull model, while broken lines are the estimates based on exponential model. (C & D) Comparisons between observed and expected age-specific numbers of clinical attacks during (C) primary and (D) secondary or tertiary infections. Three different models, logit (thin continuous lines; $\alpha=0.250$, $r=0.324\times10^{-4}$, $r=3.991$) and exponential (thick continuous lines; $\alpha=0.324\times10^{-4}$) approaches were employed.

Figure 4. Sensitivity of the conditional risk of symptomatic dengue disease given infection to uncertain parameters. All panels examine the age-specific risks of developing clinical attacks of dengue given secondary or tertiary infection. (A & C) Weibull model. (B & D) Exponential model. A & C examines the sensitivity of the risk to different number of co-circulating serotypes ($\alpha$, the range: 2.5 and 4.0). We assume that human hosts experience an identical risk of infection between serotypes. The baseline value of $\alpha$ was set at 3.5. C & D examines the sensitivity of the probability to different mean durations of cross-protective immunity following primary and secondary infections (1/\delta, the range: 10 days to 1 year). The duration of cross-protection following primary infection was assumed as identical to that following secondary infection. The baseline value of 1/\delta was set at 1 month. Results from the logit model are not shown, because the qualitative patterns are similar to those of Weibull model. Also, the sensitivity of the risk of symptomatic disease during primary infection is not shown, because the probability is not sensitive to variations in $\alpha$ and $\lambda$. 

---
Since our prospective study involved only 29 and 36 children aged 10 years or younger during primary and secondary infections, respectively, we also examined the effect of sample size on the age-specific conditional risk of illness given infection. Even when the absolute numbers of children ≤10 years was doubled, the qualitative patterns of risks (i.e., age-specific increase in the risk of disease, and higher risk during secondary infection than primary infection) remained the same. However, the age at which the risk of symptomatic dengue is saturated with logit and Weibull assumptions, was shifted to the left, approximately by 3-4 years younger as compared to the baseline. The AIC values were estimated to be 1664, 1664 and 1686 for logit, Weibull and exponential assumptions, respectively. These increases perhaps reflect a mismatch of the estimated force of infection with the incidence data.

4. DISCUSSION

We estimated the age-specific risks of clinical dengue attack, by combining two epidemiological data sets, (i) age-specific seroprevalence and (ii) age-specific frequency of symptomatic dengue during primary and secondary infections. The former data set was used to reconstruct the age-specific frequencies of primary and secondary infections (including those with and without symptoms), and subsequently, by taking the age-specific ratio of the latter dataset to the reconstructed infection frequency with an aid of modelling method, the age-specific conditional probability of disease given infection was estimated. Although our model required a number of simplifying assumptions, we have shown unambiguously that the conditional risks of clinical attack increased as a function of age for both primary and secondary infections. Thus, higher age-groups, e.g., adolescents and young adults are more likely to develop symptomatic dengue than younger individuals, e.g., primary school children. Moreover, Weibull model indicates that the age-specific risks of symptomatic disease in adults both during primary and secondary infection remain almost independent of age, perhaps reflecting greater variations in age-specific susceptibility to symptomatic disease among children and adolescents. To our knowledge, the present study is the first to characterize the age-specific risk of developing clinical attack during both primary and secondary dengue infection by means of epidemiological modeling method. Whereas the risk of severe complications given clinically apparent dengue (e.g., the risk of hospitalization and the case fatality ratio) in children is higher than in adults\(^ {85}\), the age-specific risk of disease itself is the other way around and increases with age.

Although pathogenesis of DENV infection and its severe complications involves many unanswered questions, and despite their multifactorial nature, our study emphasizes a critical importance of age as a key modulating factor of the risks of clinical attack during primary and secondary infections. Two important practical implications can be drawn from our results. First, as was shown in a case study in Thailand, a rapid demographic transition has taken place in many Southeast Asian countries where swift ageing, i.e., the shift of the age distribution of human population toward older ages, has been observed.\(^ {48}\) Our results support the notion of Cummings et al.\(^ {48}\) in that the ageing society is truly at risk of increase in dengue incidence. Indeed, Binh Thuan province yielded an average age at infection of 8.5 years, which is slightly greater than a previous published estimate (e.g. 5.2-6.1 years in Rayong, Thailand, 1980 \(^ {70}\)), indicating that the transmission is less intensive in Binh Thuan province in the 21st century than in Thailand, 1980. Second, while the incidence in Southeast Asian countries has been increasing, there has been a decline in the force of infection, resulting in a shift in the age distribution of DHF toward older age groups.\(^ {48,186}\) In addition to ageing, the decline might have reflected various factors including time-dependent variations in epidemiological, ecological and demographic dynamics, e.g. natural decline in the transmission, successful control of vectors and human migration. Our exercise suggests that such a decline in the force of infection could nevertheless result in an increase in older symptomatic individuals, thereby resulting in a paradoxical increase in the total number of symptomatic dengue patients (and thus, the incidence of symptomatic dengue individuals for the entire population). Although clinical outcome of severe DENV infection in adults may be more favourable than children, in terms of prognosis of clinically apparent dengue\(^ {93,304}\), the incidence of symptomatic cases may not decrease with a slight decline in the force of infection. Clarification on the population impact of age-specific risks of clinical attack on the total number of severe forms of dengue is the subject of our future studies.

Despite our successful estimation of the age-specific risk of clinical dengue attack, two limitations of the present study should be noted. First, the majority of DENV infections remain asymptomatic, and a very small amount of symptomatic patients (~5%) results in severe disease.\(^ {72}\) Our longitudinal survey data did not capture sub-clinical infections or very mild symptomatic patients, implying that our estimate of the risk of clinical attack may have been potentially underestimated. Nevertheless, our survey did not select for specific signs and symptoms of dengue, examining only AUF patients by laboratory testing, and thus, we believe that the age distributions (Figures 2C and 2D) reflected unbiased age-specific frequencies of all the symptomatic subjects during primary and secondary infections. Second, we assumed that the force of infection is time- and age-independent and the transmission potential is identical among all co-circulating serotypes. Ignorance on these realisms, e.g., seasonality, age-dependency and decline in \(\lambda\) over a long period of time, forces us to accentuate the lack of precision in our estimates of \(r_1(a)\) and \(r_2(a)\). For example, the lack of age-dependency may have led to slight underestimation in \(r_1(a)\) among small children and potentially an overestimation for both \(r_1(a)\) and \(r_2(a)\) among adults. Besides, whereas modelling approach such as ours certainly requires a number of unrealistic assumptions, we believe that our conclusion on the qualitative pattern, i.e., an increase in the risk of clinical attack with age, remains intact.
Of course, various other pre-infection factors other than age and infection parity contribute to the risk of disease severity, including the number of co-circulating serotypes and their pathogenicity. Indeed, our incidence data did not include any DHF patients among a total of 306 symptomatic patients during secondary infection. Although no direct comparison can be made, this 0% is significantly smaller than that estimated in a prospective study in Thailand. Possible explanations are that (a) our prospective study focused on the etiology of AUF, and the fraction of patients with severe dengue manifestations (e.g. who may not have presented at the PHCs or have sought care directly at higher medical level) might have been potentially disregarded, (b) the average age at infection in Binh Thuan province is higher than that in Bangkok during 1980s, and secondary infection at higher age can reduce the absolute number of DHF patients, and (c) not only serotype but also different strains could yield differential virulence given symptomatic infection. Moreover, molecular epidemiological studies have demonstrated that long-term expansion of dengue epidemic is regulated by selection-driven adaptive evolution of DENV strains. Since the absolute risk of symptomatic infection is vulnerable to the virus (strain-) specific virulence as well as our reliance on symptoms of patients and help seeking behavior, the estimate of absolute risk could potentially vary from one region to another. In that sense, our simple approach is regarded as a first step to characterize the epidemiological determinants of dengue in a rudimentary fashion, and our estimate at least confirmed age-specific increase in the risk of clinical dengue given infection, offering practically important implications. Modeling with sequential infection assumption still remains to be a common strategy to capture the serotypic sequential infection mechanisms, and future incorporation of strain-specificity needs to account for strain specific virulence as well as host-response (including cross immunity) to each strain, which will be far more complex than the simplistic sequential approach.

In conclusion, we examined the age-specific risks of clinical attack during primary and secondary DENV infections in Vietnam, showing that those at higher age-group are more likely to develop symptomatic disease than younger individuals. Age as an important modulator of clinical dengue attack explains recent epidemiological shift in dengue notification in ageing countries in Southeast Asia, and moreover, poses a paradoxical problem of an increase in adult patients resulting from a decline in the force of infection which may be caused by various factors including time-dependent variations in epidemiological, ecological and demographic dynamics.

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**SUPPLEMENTARY FIGURE**

Figure S1. Map of Vietnam (left) and Binh Thuan province (right).
APPENDIX

Here we describe the details of model that we employed for statistical inference (Figure 1).

Among the total population, the proportion of individuals who are susceptible to ($\alpha$-i) serotypes at age $a$ are denoted by $S_i(a)$ (for $i = 0, 1$ and $2$ with a default value $\alpha = 3.5$). Similarly, let $I_i(a)$ represent the proportion of those who have experienced infections with i-th serotype and remain still protected against the remaining heterologous serotypes at age $a$ due to short-lived cross-protective immunity. Supposing that the force of infection is age- and time-independent $\lambda$, the sequential transmission dynamics as a function of age $a$ are described by the following differential equations:

\[
\begin{align*}
\frac{dS_0(a)}{da} &= -(\alpha \lambda + \mu)S_0(a), \\
\frac{dI_0(a)}{da} &= \alpha \lambda S_0(a) - (\delta + \mu)I_0(a), \\
\frac{dS_1(a)}{da} &= \alpha \lambda S_1(a) - (\delta + \mu)S_1(a), \\
\frac{dI_1(a)}{da} &= (\alpha - 1)\lambda S_1(a) - (\delta + \mu)I_1(a), \\
\frac{dS_2(a)}{da} &= \alpha \lambda S_2(a) - (\delta + \mu)S_2(a), \\
\frac{dI_2(a)}{da} &= (\alpha - 2)\lambda S_2(a) - \mu I_2(a),
\end{align*}
\]

where $\alpha$ is a scaling factor of co-circulating serotypes (interpreted as the number of co-circulating serotypes) and $\delta$ is the rate at which $I_i(a)$ loses the cross-protective immunity against remaining heterologous serotypes. We estimate $\lambda$ from seroprevalence data, and assume that $\alpha$ and $\delta$ are known.

Our longitudinal survey included neither mild symptomatic dengue in infants nor vascular permeability syndrome among infants due to maternal antibody, and we ignore maternal antibody during first half year of life for simplicity. Accordingly, we assume that $S_i(0) = 1$ and other compartments are 0 at age 0. Accordingly, the age-specific incidence of primary, secondary and tertiary infections at age $a$ are given by $\alpha \lambda S_0(a)$, $(\alpha - 1)\lambda S_1(a)$ and $(\alpha - 2)\lambda S_2(a)$, respectively, and we analytically solved the equation system (4) to replace $S_i(a)$ by parameters $\mu$, $\lambda$, $\alpha$ and $\delta$. Supposing that the age-specific conditional probabilities of clinical dengue attack given primary and secondary infections are $r_1(a)$ and $r_2(a)$, respectively, the age-specific frequencies of primary infection and a combination of secondary and tertiary infections with clinical attack are expressed as $r_1(a)\alpha \lambda S_0(a)$ and $r_2(a)((\alpha - 1)\lambda S_1(a) + (\alpha - 2)\lambda S_2(a))$, respectively.

To estimate the parameters for $r_1(a)$ and $r_2(a)$, we normalized these frequencies, i.e.,

\[
\begin{align*}
\frac{f_1(a)}{f_0} &= \frac{\alpha \lambda r_1(a)S_0(a)}{\int_0^\infty \alpha \lambda r_2(s)S_0(s)ds} \frac{r_1(a)S_0(a)}{\int_0^\infty r_1(s)S_0(s)ds} \\
\frac{f_2(a)}{f_0} &= \frac{r_2(a)((\alpha - 1)\lambda S_1(a) + (\alpha - 2)\lambda S_2(a))}{\int_0^\infty r_2(s)((\alpha - 1)\lambda S_1(s) + (\alpha - 2)\lambda S_2(s))ds}.
\end{align*}
\]

We used (5) and (6) for the likelihood equation (3) in the main text.
DENGUE DYNAMICS IN BINH THUAN PROVINCE, SOUTHERN VIETNAM: PERIODICITY, SYNCHRONICITY AND CLIMATE VARIABILITY

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Background: Dengue is a major global public health problem with increasing incidence and geographic spread. The epidemiology is complex with long inter-epidemic intervals and endemic with seasonal fluctuations. This study was initiated to investigate dengue transmission dynamics in Binh Thuan province, southern Vietnam.

Methodology: Wavelet analyses were performed on time series of monthly notified dengue cases from January 1994 to June 2009 (i) to detect and quantify dengue periodicity, (ii) to describe synchrony patterns in both time and space, (iii) to investigate the spatio-temporal waves and (iv) to associate the relationship between dengue incidence and El Niño-Southern Oscillation (ENSO) indices in Binh Thuan province, southern Vietnam.

Principal findings: We demonstrate a continuous annual mode of oscillation and a multi-annual cycle of around 2–3-years was solely observed from 1996-2001. Synchrony in time and between districts was detected for both the annual and 2–3-year cycle. Phase differences used to describe the spatio-temporal patterns suggested that the seasonal wave of infection was either synchronous among all districts or moving away from Phan Thiet district. The 2–3-year periodic wave was moving towards, rather than away from Phan Thiet district. A strong non-stationary association between ENSO indices and climate variables with dengue incidence in the 2–3-year periodic band was found.

Conclusions: A multi-annual mode of oscillation was observed and these 2–3-year waves of infection probably started outside Binh Thuan province. Associations with climatic variables were observed with dengue incidence. Here, we have provided insight in dengue population transmission dynamics over the past 14.5 years. Further studies on an extensive time series dataset are needed to test the hypothesis that epidemics emanate from larger cities in southern Vietnam.

1. INTRODUCTION
Recent estimates indicate that approximately 3.5 billion people, about 55% of the world’s population, live in countries at risk for dengue infection.16 Dengue ranks among the most important infectious diseases in many countries in the tropics and subtropics. Dengue virus (DENV) transmission occurs primarily through bites by the mosquito vectors, Aedes aegypti, which feed preferentially on human blood, and are often found in and around human dwellings.112,245 Infection with any of the four dengue serotypes results in either asymptomatic infection, or a spectrum of clinically apparent disease ranging from mild undifferentiated febrile illness to severe dengue of which dengue shock syndrome (DSS) is the most common life threatening syndrome.245 Dengue has become a major international public health problem due to increasing geographic distribution and a transition from epidemic transmission with long inter-epidemic intervals to endemic with seasonal fluctuation.77,260 Seasonal and multi-annual cycles in dengue incidences vary over time and space.25 Multiple factors may influence the dynamics of dengue including environmental and climate factors, host-vector interactions and the population-wide immune landscape.161 Climate variability is postulated to be an important determinant of dengue epidemics.99,101 Meteorological conditions (temperature, humidity, wind and precipitation) may directly or indirectly affect vector survival, life-span, development and reproductive rates which could influence dengue spatio-temporal oscillations.107 Dengue incidence time series are characterized by nonlinear dynamics, with strong seasonality, multi-annual oscillations and non-stationary temporal variations (i.e. irregular temporal fluctuations in incidence).260 These features complicate the detection of both temporal and spatial patterns. Therefore, conventional methods such as Fourier analysis and generalized linear models (GLM) may be inadequate.

Wavelet analysis is suitable for investigating time series data from non-stationary systems and for inferring associations within such systems. Wavelet analysis is able to measure associations (coherence) between two time-series at any frequency (period) band and at every time-window period. Wavelet coherence analyses have been used to compare time series of disease incidence across localities and countries for the characterization of the evolution of epidemics periodicity and the identification of synchrony. Wavelet analyses have been used in analyzing various human infectious disease dynamics such as measles, influenza, leishmaniasis and dengue.30;31;39;83;139;314 Phase analysis completes wavelet analysis by allowing the investigation of phase shift between epidemics at different locations. For instance, phase differences at a given period and time window allowed the observations of spatio-temporal patterns of measles infections prior to measles vaccination.45 Phase angles illustrated spatio-temporal waves of measles and dengue infection which showed that waves originated from regional population centers.49,93 In this study, we performed wavelet analysis on time series of notified dengue cases (i) to detect and quantify variability in dengue incidence and how it progresses through time, (ii) to describe synchrony patterns in both time and space, (iii) to investigate the spatio-temporal waves of infection and (iv) to associate the relationship between dengue incidence and El Niño-Southern Oscillation (ENSO) and local climate variables in Binh Thuan province, southern Vietnam.

2. METHODS

2.1 EPIDEMIOLOGICAL AND CLIMATIC DATA
Binh Thuan Province is located along the south-eastern coast of Vietnam, 150 km northeast of Ho Chi Minh City. It covers 7,828 km² and the population from census data was 1,032,993 inhabitants in 1999.246 Binh Thuan Province is divided into 122 administrative units including 97 communities in semi-rural areas, 14 wards in Phan Thiet City (the capital), and 11 small towns and nine districts, including Phu Quy – an island off the coast.
Monthly notified dengue cases (i.e. clinically suspected cases of dengue without laboratory confirmation) in nine districts in Binh Thuan Province, southern Vietnam, were obtained from the provincial prevention department. Data include cases from January 1995 through June 2009. The provincial meteorological department provided historical monthly climatic data (i.e. mean temperature, humidity and rainfall). The ENSO indices (i.e. Multivariate El Niño-Southern Oscillation Index (MEI), Niño 1 + 2, Niño 3, Niño 4 and Niño 3.4) were extracted from http://www.cpc.ncep.noaa.gov/data/indices/.

2.2 WAVELET ANALYSIS – PERIODICITY OF DENGUE

Wavelet analysis was performed to explore the periodicity in the dengue incidence time series. Wavelet analysis provides the possibility of investigating and quantifying the temporal evolution of time series with different rhythmic components. In addition, wavelet analysis allows detection of changes in periodicity in time. The Morlet wavelet was used and all analyses were performed with Matlab software. All dengue incidence time series were square root transformed and all series were normalized and the trend was suppressed before analyses. The trend was suppressed before analysis by removing of the periodic components with period components greater than 8 years by using a classical low pass filter.

2.3 COHERENCY AND PHASE ANALYSES – SYNCHRONY BETWEEN DISTRICTS AND CLIMATE VARIABILITY

To quantify the dependencies between two time series, wavelet coherence analyses in neighbouring districts were performed. Wavelet coherence allows checking if different periodic modes of two time series tend to oscillate simultaneously, rising and falling together and quantifying the synchrony of these two time series. In addition, phase analysis was used to characterize the association between these two time series. All significance levels were based on 1000 bootstrapped series.

2.4 SPATIO-TEMPORAL PATTERNS

To investigate spatio-temporal patterns of dengue dynamics, phase difference was calculated between epidemics at different districts relative to Phan Thiet district, in which Phan Thiet City is the capital. Phase analyses generate a phase angle at each time step at a given periodic band. Further, we compute the phase difference to analyse the time delay between dengue incidences at different locations.

3. RESULTS

3.1 PERIODICITY OF DENGUE

Wavelet analyses of time series data from nine districts of Binh Thuan province are displayed in Figure 1. In general, the mean spectra show periodicity for all districts. More specifically, periodicities were detected in the 1-year and the 2–3-year bands. However, dengue dynamics showed different evolutions across the nine time series which can be divided into three groups based on wavelet cluster analysis: (i) the first group consists of three districts (Figure 1-F, H and I) in which a multi-annual cycle was more predominant. Phan Thiet and Tuy Phong district showed a constant cycle of between 2–3- years from 1996-2001 (Figure 1-F and H); (ii) in three districts, the annual cycle was predominant which represents the second group (Figure 1-D, E and G); (iii) a third group consists of districts in which no multi-annual cycle was visualized (Figure 1-A and C). The wavelet cluster tree is shown in Figure S1. When all time series were aggregated, the oscillation of dengue incidence showed a strong annual mode of oscillation with a significant transient multi-annual mode of oscillation around a 2–3-year periodic band between 1996-2001 (Figure 1-J).

3.2 SYNCHRONY BETWEEN DISTRICTS

We compared dengue dynamics between neighbouring districts when periodicities were detected in each district (Figure 2-A to L). High coherencies in all comparisons were detected for the 1-year periodicity (left). This annual mode of oscillations was also observed in the phase angle analyses (see Figure S2). Multi-annual oscillations at a 2–3-year period were identified, although these oscillations were transient and varied in time and space. There was a tendency that the multi-annual oscillation returned after 2006. Globally, coherency analysis between dengue incidence time series in Phan Thiet district and the eight other districts together shows two main regions of high significant coherence (Figure 2M). The first one is for the 1 year periodic band in 1995–2003 and in 2004–2008 and the second is for the 2–3-years bands in 1996–2001. Although there is a third significant coherence region (4 year periodic band, 2003-2004), it must be interpreted with caution due to the short length of the time series.

3.3 SPATIO-TEMPORAL PATTERNS IN DENGUE DYNAMICS

Spatio-temporal patterns were investigated by calculating phase differences as published previously. Based on the results in Figure 2M, phase differences were calculated for three periods: between 1996 and 2001, when the 2–3-year periodic band was pronounced; from 1995 to 2002 and from 2004 to 2008, when the annual periodic band was significant. The average phase difference relative to Phan Thiet is shown in Figure 3. Data indicate that the multi-annual (2–3-year period band) wave of dengue infection was moving away from another epicenter, rather than from Phan Thiet district as only three districts lagged behind (negative phase difference) or the time delay was greater than a half cycle. In contrast, the annual wave of dengue infection was either synchronous (1995-2002) with all districts or moving away (2004-2008) from Phan Thiet district.
Figure 1. Wavelet analyses of dengue time series with monthly data from 1994 to 2009 in 9 districts of Binh Thuan province. (A) Duc Linh, (B) Tinh Linh, (C) Ham Tan, (D) Ham Thuan Nam, (E) Ham Thuan Bac, (F) Phan Thiet, (G) Bac Binh, (H) Tuy Phong, (I) Phu Quy, (J) Binh Thuan province. For each district (i) left panel: the time series of cases in the district (ii) middle panel: the wavelet power spectrum of dengue cases (square rooted and normalized and trend suppressed); colors code for increasing spectrum intensity, from blue to red; dotted lines show statistically significant area (threshold of 5% confidence interval); the black curve delimits the cone of influence (region not influenced by edge effects) (iii) right panel: to the mean spectrum (solid line) with its threshold value of 5% (dotted line).

Figure 2. Wavelet coherence and phase analyses of dengue time series between neighboring districts in Binh Thuan province. The left panel represents the wavelet coherence. Blue, low coherence; red, high coherence. The dotted lines show $\alpha = 5\%$ significance level. The cone of influence (black curve) indicates the region not influenced by edge effects. The right panel represents the phase analysis between two districts (in blue and red), based on wavelets for 2–3-year periodic band. Green boxes represents the period of time where coherency is significant; when interpretation of analysis was possible. Red lines: first district; blue lines: second district; dashed black lines: phase difference between the two oscillating components.
3.4 ASSOCIATION BETWEEN DENGUE INCIDENCE AND CLIMATE VARIABILITY

For climate association analyses, the aggregated dengue incidence time series from all districts (Figure 1J, left) was used. Wavelet coherency between dengue incidence and ENSO indices and climate variables are shown in Figure 4 and 5, respectively. ENSO indices and climate variables were significantly associated with dengue incidence in the 2–3-year periodic band, although the associations were transient in time.

4. DISCUSSION

In this study, we have demonstrated annual modes of oscillation with a significant multi-annual cycle around a 2–3-year periodic band from 1996-2001. Synchrony in time and space for the annual cycle was detected and an overall synchronous 2–3-year cycle was quantified between Phan Thiet district and the other districts. We described the spatio-temporal patterns by using mean phase differences which suggest that the seasonal wave of infection was synchronous or moving away from Phan Thiet district. This contrasts the 2–3-year wave of infection, which was moving away from another epicenter towards Phan Thiet district. A significant non-stationary association between ENSO indices with dengue incidence in the multi-annual cycle was observed.
The ubiquitous periodicity of dengue incidence in Binh Thuan province is in accordance with dengue dynamics in Thailand. Annual periodic patterns are a common phenomenon in dengue transmission and have been reported in many tropical and subtropical countries, but the identification of a periodic multi-annual (e.g. 2-3-year) cycle differs between countries and in analyses used. A multi-annual periodicity of 3 years was detected in DHF data from Thailand using spectral density analysis, while recently, multi-annual dengue periodicity was not shown explicitly in reported dengue cases from Puerto Rico, Mexico and Thailand using wavelet analysis. Compared to our analyses, Johansson et al. have log-transformed their data and used a null hypothesis assuming that sequential observations are dependent and accounted for the potential influence of short-term autocorrelation on long-term time series. The use of log-transformation has homogenized the variance of the time series which provided more weight to the dominant annual component. Whether the multi-annual periodicity of dengue incidence exists or not, the detection of periodic cycles for dengue incidence itself provides no insights into the processes that cause dengue incidence oscillations (e.g. environmental and climate factors, host-vector interactions and population herd immunity). Interactions between intrinsic and extrinsic factors remain important to be elucidated and need further exploration.

Displacement of the predominant serotype has been associated with outbreaks and multi-annual fluctuations may be due to antibody-dependent enhancement (ADE) of infection with heterologous serotypes. Other factors that may be important for the occurrence of multi-annual cycles may be the impact of population movement, demographic change and climatic variables. Our findings provide evidence for a non-stationary relationship between ENSO indices and dengue incidence in the 2-3-year periodic cycle and associations with climate variables were significantly present. This suggests that El Niño affects local climatic variables which in turn affect dengue incidence in Binh Thuan province. However, it seems that large-scale indices seem to predict ecological processes better than local weather which has been addressed previously.

Our results also show a large decrease in the variability of dengue incidence after 2000-2001 corroborating the Thailand dataset. Possible explanations for the observed decrease could be a modification of the climatic conditions, a reduction in transmission due to declining mosquito populations, declining contact between human and mosquito populations, and/or modifications in diagnosis, classification and reporting dengue cases. A demographic shift to lower birth and death rates could induce modifications to dengue oscillations, a possible explanation which needs to be tested in Vietnam.

We have examined the spatio-temporal patterns of dengue infection, by computing the phase differences. Interestingly, mean phase differences suggested that the multi-annual wave of dengue infection was moving towards to Phan Thiet district and might originate from another, but nearby epicenter. In contrast, the annual wave of dengue infection was either synchronous with all districts or the wave of infection initiated in Phan Thiet district. Regional or local transmission in Phan Thiet district, probably influenced by climatic phenomena, modulated the annual cycles. Similar to the spatio-temporally travelling waves in incidence of DHF from Thailand, the multi-annual cycles in Vietnam may emanate from larger cities (e.g. Ho Chi Minh City which is located 200 km to the south west or Hanoi, the capital in the north of Vietnam). Further extensive analyses on surveillance data from other regions are needed to test this hypothesis.

Wavelet analyses have revealed interesting information on dengue transmission dynamics in Binh Thuan province. However, there was a limitation in the present study. Dengue data used in this study were based on notified clinically-suspected dengue cases from hospitals or clinics without laboratory confirmation. These numbers may be an underestimation of the true incidence. Notification behaviour and clinical suspicion are both subjective parameters which may be influenced by communications with colleagues, media attention, and history of clinicians on reporting. To mitigate these reasonable limitations, Vietnam has consistent reporting of dengue cases and clinicians are well trained in recognizing dengue, both of which indicate that surveillance data would be a good representation of symptomatic dengue patients. Another limiting factor is that the circulating serotypes were not known during the whole time period. Although, the dengue multi-annual cycle oscillates around a 2-3-year periodic band, the cycle for all serotypes may differ from each other resulting in different dengue serotype multi-annual cycles.

In conclusion, we observed a strong annual mode of oscillation with a significant multi-annual cycle around a 2-3-year periodic band from 1996-2001. Multi-annual waves of infection probably started outside Binh Thuan province. We report a significant non-stationary association between ENSO indices with dengue incidence for the multi-annual cycle. The existence of these plausible climatic determinants may be particularly interesting in the context of the development of early warning systems in which reliable predictors of dengue epidemics may be usefully employed. Nevertheless, this requires empirical proof and the understanding of the mechanisms linking climatic conditions and dengue propagation. Our findings provide insights into the long-term persistence and spatial spread of dengue throughout Binh Thuan province, southern Vietnam. Further studies on a more extensive time series dataset of a larger area could shed more light onto the spatio-temporal patterns in Vietnam.

5. ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Figure S1. Cluster tree of the dengue time series. The cluster tree was obtained by applying a classification method with a covariance threshold at $C = 99\%$ of the total covariance as described by Rouyer et al. (A) Duc Linh, (B) Tanh Linh, (C) Ham Tan, (D) Ham Thuan Nam, (E) Ham Thuan Bac, (F) Phan Thiet, (G) Bac Binh, (H) Tuy Phong, (I) Phu Quy.

Figure S2. Phase analyses of dengue time series between neighboring districts in Binh Thuan province. Phase analyses between two districts (in blue and red), based on wavelets for 1-y periodic band.
THE ROLE OF CLIMATE VARIABILITY AND CHANGE IN THE TRANSMISSION DYNAMICS AND GEOGRAPHIC DISTRIBUTION OF DENGUE

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The mounting evidence for anthropogenic changes in global climate raises many pressing questions about the potential effects on biological systems, and in particular the transmission of infectious diseases. Vector-borne diseases, such as dengue, may be particularly sensitive to both periodic fluctuations and sustained changes in global and local climates, because vector biology and viral replication are temperature and moisture dependent. This paper reviews the current state of knowledge on the associations between climate variability, climate change and dengue transmission, and the tools being used to quantify these associations. The underlying causes of dengue’s recent global expansion are multi-factorial and poorly understood, but climatic factors should be considered within the context of the socio-demographic, economic and immunological determinants that have contributed to dengue’s spread. These factors may mediate the direct effects of climate on dengue and many may operate at a very local level. Translating theoretical models of dengue transmission based on historical data into predictive models that can inform public health interventions is a critical next step and efforts should be focused on developing and refining models at smaller spatial scales to characterize the relationships between both climatic and non-climatic factors and dengue risk.

1. INTRODUCTION

Dengue is the most common vector-borne viral disease worldwide, and is ranked among the most important infectious diseases by the World Health Organization (WHO).295 Dengue poses a major challenge to international public health (World Health Assembly resolution, 2002). The disease burden and geographic range of dengue have expanded over the past 50 years, from approximately 15,000 cases reported annually to WHO from fewer than 10 countries during the 1960s to close to 1 million cases annually across more than 60 countries in 2000 – 2005.157 Recent estimates suggest that transmission occurs in up to 124 countries with at least 35 million symptomatic cases per year and around 20,000 deaths, and that approximately 3.5 billion people, or 55% of the world’s population, live in countries at risk for dengue.16 In Vietnam, dengue was first reported in 1959 and since then the number of reported cases has increased steadily, with major outbreaks in 1987 and 1998 throughout all provinces.96-98;109 Figure 1 shows the incidence of dengue and the case fatality rate in southern Vietnam since 1996.

In endemic areas of Southeast Asia dengue is mainly a childhood disease and children experience an annual exposure risk of ~10%.272,274 An upwards shift in the median age of dengue cases has recently been reported from Thailand46, however it is not yet clear whether this is replicated in other endemic settings.

Humans are the primary host of dengue virus (DENV) and transmission of DENV takes place through the bite of the principal mosquito vector Aedes aegypti, and to a lesser degree Aedes albopictus. Human infection by DENV can lead to a broad spectrum of outcomes. The majority of DENV infections are inapparent (50 – 90%)277,64; clinical manifestations range from dengue fever (DF), a self-limiting syndrome characterized by fever, malaise, arthralgia and myalgia, to severe disease that has been classically classified into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) and is characterized by vascular permeability, coagulopathy and, in the case of DSS, hypovolemic shock.87-235 Mortality rates vary from less than 1% to 10% in different settings, and depend critically on access to health care facilities and the degree of clinical experience in haematological monitoring and management of vascular leakage.30

The geographical distribution of dengue largely reflects the distribution of the principal vector Aedes aegypti. The expansion in the range and magnitude of the dengue disease burden over the past half-century has paralleled rapid population growth and urbanization, particularly in endemic areas of southeast Asia, and unprecedented increases in global human mobility and trade (reviewed in 300). All of these factors are conducive to an intensification of transmission in early endemic settings and the export of DENVs and their vectors from these early foci in Southeast Asia and parts of Central America into new areas, as occurred in the decades following the 1950s. In particular, many countries in the Americas experienced a re-emergence of dengue during the 1970s and 1980s after prior success in controlling Aedes aegypti through concerted campaigns to eliminate yellow fever, which shares the same primary vector.154 The rapid and continued expansion of dengue represents an increasing challenge to national and regional health authorities both in endemic settings and in currently unaffected or newly affected areas. The ability to accurately estimate the current disease burden and to predict

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**Figure 1. Dengue incidence and mortality in southern Vietnam, 1996 – 2009.** Bars show the incidence of dengue (DF and DHF) per 100,000 population in the southern 20 provinces of Vietnam between 1996 – 2009. The line shows the mortality rate among reported dengue cases over the same period. Data represents cases reported to the national dengue surveillance control program and includes hospitalized cases only. This data is reproduced with the permission of the Pasteur Institute, HCMC.
future trends is key to the planning of effective prevention and control interventions.

Climate is an important determinant of the spatial and temporal distribution of dengue and other vector-borne diseases. Variability and changes in climate are likely to affect the availability of these vector breeding sites, especially whether they are predominantly outdoor rain-filled objects or indoor containers that are filled manually. Humidity, or vapour pressure, is governed by a combination of rainfall and temperature and influences the lifespan of the mosquito and therefore the potential for transmission of the virus. Annual average vapour pressure has been suggested by some authors to be the most important climatic predictor of global dengue occurrence.

Temperature, rainfall and humidity are therefore important determinants of the geographic limits within which dengue transmission can be expected to be sustained, primarily through their effects on the survival and proliferation of the *Aedes* vector. Furthermore, within areas where minimum thresholds of these climate parameters are sufficient to sustain dengue virus transmission, seasonal fluctuations in these parameters will be important determinants of the duration and potentially the intensity of transmission.

**NON-CLIMATE DETERMINANTS OF DENGUE TRANSMISSION AND DISEASE SEVERITY**

Climatic conditions favourable for *Aedes* vectors are a necessary but insufficient criterion for dengue virus transmission to occur and to spread. The availability of susceptible human hosts is determined by local population density and levels of pre-existing immunity, and the presence of the dengue virus itself is the necessary third component of the transmission cycle.

Infection with any one of the four dengue virus serotypes is believed to elicit lifelong immunity against that serotype, but confers only partial or transient immunity against the other three serotypes. Secondary or subsequent infection with a heterologous virus is a well-established risk factor for severe dengue disease, a phenomenon that is thought to be mediated by a process of antibody-dependent enhancement (ADE) of DENV infection by cross-reactive non-neutralizing antibodies, resulting in a higher viral burden and an increase in the resultant pathogenic processes. Transient cross-protective immunity and ADE may also have implications for dengue transmission at a population level, as they may respectively reduce or increase the effective population of susceptible human hosts. Despite the importance of cross-protective immunity for understanding the epidemiological dynamics of dengue, little is known about the duration of cross-protection against a heterologous serotype. It is generally assumed that a first clinical dengue episode elicits cross-protective immunity that lasts 2-9 months, although a recent report estimates cross-protection to last as little as 1-2 weeks.

There is evidence that the infecting viral serotype, genotype and sequence of infection are also important determinants of disease severity, independent of infection parity. This may also influence dengue transmission dynamics if increased inherent virulence results in higher viral titres and increased infectiousness to mosquitoes. Although there is limited direct

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**2. EFFECTS OF CLIMATE ON DENGUE TRANSMISSION**

Climatic conditions play a key role in the biology and ecology of mosquito vectors and the viruses they transmit, and consequently also exert a strong influence on the risk of dengue transmission. Higher temperatures increase the rate of larval development and therefore emergence of adult vectors, increase the vector biting rate and reduce the time required for virus replication within the vector, known as the extrinsic incubation period (EIP). Mean vectors are infectious earlier and bite more frequently. Higher temperature may reduce vector survival time, which may offset the positive effect on vector abundance to some degree. Numerous studies have linked temperature to *Aedes aegypti* abundance and dengue incidence rates. These studies have used various statistical approaches considering different temperature parameters (e.g. mean, maximum, and minimum temperatures).

*Aedes* vectors breed predominantly in clean water-holding containers in close proximity to human dwellings. Variability in rainfall is likely to affect the availability of these vector breeding sites, and therefore influence vector abundance, however the magnitude and direction of this effect is uncertain and may differ between settings depending on the nature of local vector breeding sites, especially whether they are predominantly outdoor rain-filled
evidence for this as yet, particular genotypes of DENV serotype 2 (DENV-2) are known to have
replaced previously dominant strains in Vietnam, Thailand and Cambodia, suggestive of a
difference in viral fitness. Age, host genetic background, and the time interval between
sequential infections are other factors associated with the risk of developing severe
dengue disease.

Finally, factors which influence interactions between the virus, vector and host components
of the dengue epidemic will be critical in determining the overall effect on the establishment,
persistence and intensity of dengue transmission in a given setting. This includes factors that
determine the accessibility of vectors to human hosts, such as built environments that create
vector breeding sites in close proximity to humans, increases in population density associated
with urbanization, or the use of window screens and air-conditioning that reduce vector access
into human dwellings. As the Aedes vector has a restricted flight range, movement of dengue
infected humans is thought to play a major role in dengue transmission. Rabaa et al. have
demonstrated substantial DENV viral exchange between the urban centre of Ho Chi Minh City
and other provinces of southern Vietnam, suggesting that human movement between urban
and rural areas may play a central role in the rapid diffusion of DENV across southern Vietnam.

Movement and transport of humans or vectors at a national, regional or international scale
creates new opportunities for vectors to become established in permissive environments, and
for virus transmission to be established where competent vectors exist, and inadequate or
interrupted vector control activities determined by economic and political priorities will directly
affect individuals’ risk of exposure to infection in endemic settings. These social, demographic
and economic drivers are thought to be responsible for much of dengue’s expansion and
intensified transmission over recent decades.

3.1 TRANSMISSION DYNAMICS AND DISEASE MODELLING

3.2 TOOLS FOR MODELLING DENGUE TRANSMISSION DYNAMICS

Traditionally, Fourier analysis has been used to analyse relations between oscillating time
series. This technique decomposes time series into their different periodic components which
can then be compared between time series. Since Fourier analysis cannot take into account
temporal changes in the periodic behaviour of time series (i.e. their lack of stationarity), this
method, and others such as generalized linear models (GLM), may be inadequate for
investigating the determinants of dengue transmission dynamics. Wavelet analysis is suitable
for investigating time series data from non-stationary systems and for inferring associations
between such systems. This approach reveals how the different scales (i.e. the periodic
components) of the time series change over time. Wavelet analysis is able to measure
associations (coherency) between two time-series at any frequency (period) band and at every
time-window period.

Wavelet coherency analyses have been used to compare time series of disease incidence
across localities and countries for the characterization of the evolution of epidemic periodicity
with the spatial distribution of disease. Mathematical modelling of infectious disease dynamics
has two related main objectives: firstly to provide insights into the underlying biological and
ecological mechanisms that give rise to the dynamics observed historically and currently, and
secondly to make predictions about the future magnitude, timing and/or location of disease
transmission.

Seasonal and inter-annual climate variability is thought to be an important determinant of
dengue epidemic cycles. Numerous studies have examined the wave-like behaviour of
dengue transmission in different areas and have demonstrated various associations between
dengue incidence and climate variables. Table 1 summarizes published studies examining the relationship between extreme climatic events and dengue incidence time series. In particular, periodic dengue epidemics with multi-annual cycles appear to be associated with extreme weather events, and the El Niño Southern Oscillation (ENSO) has been suggested as a major indicator. The ENSO is a natural climate fluctuation and is probably the best understood systematic pattern of global climate variability. It affects rapid climatic changes in most countries bordering on or in the Pacific and Indian oceans. It is characterized by variations in the surface temperature of the tropical eastern Pacific Ocean, which consists of the warm El Niño event and the cold La Niña event. El Niño and La Niña events occur irregularly every 2-7 years and last for 12-18 months. Other indices for the quantification of ENSO events are the Southern Oscillation index (SOI) which compares the atmospheric pressure between Tahiti and Darwin, Australia, and the multivariate ENSO index (MEI) which combines the six main observed variables over the tropical Pacific.

Table 1 summaries published studies examining the relationship between extreme climatic events and dengue incidence time series.
and the identification of synchrony. Wavelet analyses have been used in analysing various human infectious disease dynamics such as measles, influenza, leishmaniasis and dengue.30,31,39,83,139,314

3.3 LIMITATIONS AND CONSIDERATIONS IN MODELLING DENGUE TRANSMISSION
Although annual periodic patterns are a common phenomenon in dengue endemic areas, the identification of a periodic multi-annual (e.g. 2–3-year) cycle differs between countries and in analyses used. Cazelles et al. used wavelet approaches to demonstrate a highly significant but discontinuous association between ENSO, precipitation and dengue epidemics in Thailand.31 Johansson et al. investigated the relationship between ENSO, local weather, and dengue incidence in Puerto Rico, Thailand and Mexico since the mid 1980s with an annual scale and multiyear cycles. Temperature, rainfall and dengue incidence were strongly associated in all three countries for the annual cycle. The associations with ENSO varied between countries in the multi-annual cycle. ENSO was associated with dengue incidence in Puerto Rico, but characterized with non-stationarity while there was no association in Mexico. In Thailand, ENSO was associated with climate and dengue incidence.139 Thai et al. provided insights into dengue transmission dynamics in Binh Thuan province, southern Vietnam, between 1996 – 2009. A continuous annual mode of oscillation with a non-stationary 2–3-year multi-annual cycle was observed (figure 2A) with strong irregular associations between dengue incidence and ENSO indices and climate variables (figure 2B). The dengue incidence time series for Phan Thiet City and eight neighbouring districts within Binh Thuan province are shown in figure 2C. Figure 2D shows the coherency between these time series which demonstrates high coherence in the 1 year period band and in the 2-3 year periodic band between 1996-2001. In addition, the spatio-temporal patterns seem to be different between the annual and multi-annual cycle.273

Although these wavelet analyses have provided important insights into the cyclical dynamics of dengue transmission, the associations found with ENSO have been irregular and transient, which reduces the potential for generating future predictions based on these climatic events. Furthermore, these analyses have tended to examine the association between climate variability and dengue incidence in isolation from other parameters likely to be relevant to the transmission dynamics of dengue, such as the host immune landscape and virus population dynamics. In contrast, others have modelled dengue incidence time series based on parameters related to host immune and viral factors, such as temporary cross-protective immunity, temporary ADE and variations in virus serotype virulence299 ADE alone69,208, or varying degrees of cross-protective immunity2 and have been able to reproduce the observed cyclical incidence trends and serotype oscillations based on assumptions around these parameters, in the absence of climate variability. Because climate, host immunity and viral factors are all likely to impact upon dengue transmission dynamics in a given setting, the interactions between, as well as independent effects of, these intrinsic (host and virus) and extrinsic (climate and environment) factors need further exploration in transmission models.

Aside from their contribution to a fundamental understanding of dengue cyclical dynamics, the public health relevance of transmission models lies in their potential to permit prediction of disease trends and therefore inform intervention. In Taiwan, the occurrence of local epidemics has been shown to be associated with imported dengue cases within a context of favourable
marginalising conditions of higher temperature and lower rainfall and humidity, which may inform prospective assessments of the risk of local transmission following the identification of an imported dengue case. To date there are few publications on the development and application of predictive models for dengue, however climate and environmental variables have been used to build risk assessment models for other vector-borne diseases such as Rift Valley fever, and to enact public health interventions on the basis of their predictions.9

The spatial scale of analysis is important when modelling the impact of various factors on dengue transmission. This is because dengue may be sensitive to differences in climatic, environmental, societal and demographic characteristics that occur at a local level, and the relative contribution of these factors may differ between settings. Even when considering more macro-climatic events such as ENSO, the spatial resolution of the dengue incidence data is important. Cummings et al. have demonstrated a spatiotemporal wave in the three-year dengue incidence cycle in Thailand, with each wave originating in Bangkok and radiating out to the provinces, which experience a lag in the peak incidence of up to 10 months.40 In contrast, our preliminary analysis of dengue surveillance data from southern Vietnam suggests that the annual peak incidence in Ho Chi Minh City occurs consistently at a 2 month lag after the peak incidence in the other 19 southern provinces combined. Such spatiotemporal observations may prove useful in informing risk assessments used by local or national dengue control programs to prepare for and respond to annual dengue epidemics in endemic settings, if consistent patterns can be identified in the spatial and temporal relationships between incidence trends in neighbouring geographic areas. This is particularly so if environmental or socio-demographic patterns to explain these relationships can be elucidated, providing potential targets for enhanced surveillance or interventions to prevent spread of the epidemic.

4. CLIMATE CHANGE AND Dengue

4.1 Evidence for a Changing Climate

The world climate is in a warming phase that began in the eighteenth century. The average temperature in Southeast Asia has increased between 0.1 - 0.3 °C per decade between 1951 and 2000 (81) and the average temperature will further increase by up to 2 – 4 °C by 2100, according to projections of the Intergovernmental Panel on Climate Change (IPCC).133,148 In particular, minimum temperatures have been rising faster than maximum or average temperatures. Changes in rainfall patterns have been geographically inconsistent, with some areas becoming wetter and others drier.156 Climate change represents these sustained changes in the mean state of the climate or in its variability, as compared with climate variability which encompasses seasonal and multiannual fluctuations in temperature, rainfall and humidity.

4.2 Modelling the Effect of Climate Change on Dengue

The innate sensitivity of vector-borne diseases, including dengue, to climatic conditions raises the question as to what effect, if any, we might expect sustained increases in temperature and variable changes in rainfall to have on the global disease burden. Again, two separate but related endpoints need to be considered: the geographic range of transmission, and the intensity and/or seasonality of transmission. Hales et al. modelled the global geographic range of dengue during the period 1975 – 1996 in relation to monthly averages of rainfall, vapour pressure (humidity) and maximum, minimum and mean temperatures, aggregated within each of 2000 cells that made up a grid covering the global land surface.95 Interestingly, annual average humidity was found to be the most important individual predictor of dengue distribution, and an estimated 30% of the global population (1.5 billion) were calculated to be at risk of dengue in 1990. On the basis of this finding and projected increases in population, the authors estimated that 34% of the world’s population (3.2 billion) will be at risk of dengue in 2055 if humidity remains at baseline values, compared with 44% (4.1 billion) if changes in humidity occur as predicted by IPCC. In contrast, more recent estimates of the current extent of dengue transmission, based on a review of published reports of dengue incidence, outbreaks, serosurveys and confirmed cases in travellers, suggest that up to 55% of the world’s population are already living in areas at risk of dengue during the first decade of the 21st century.16 Others have used a model based on a calculation of temperature-dependent vectorial capacity to estimate the effect of global temperature increases on dengue distribution and transmission intensity.138 This analysis indicated an expansion in the geographic range of dengue, to wider latitudes and higher altitudes, as well as an increased duration of the transmission season and higher transmission intensity in already affected areas. The largest effect of climate change on dengue transmission was expected where Aedes aegypti populations are already established but where virus replication is currently limited by the temperature.

4.3 Mediating Factors in the Effect of Climate Change on Dengue

These models describe the geographic and seasonal range which can be expected to sustain dengue, but do not account for factors that will determine how disease potential relates to disease occurrence. They also consider only the direct effects of climate change, and not indirect effects of climate change on socio-demographics, land use, changes in mode of urbanization (e.g. town planning) or other factors that may mitigate or exacerbate the direct effects of climate on vector-borne disease. There is little published evidence indicating a direct effect of climate change on the increased distribution and magnitude of dengue transmission, and the Aedes vectors, seen over the past decades. Rather this global resurgence has been attributed to rapid population growth and urban expansion in endemic areas of Southeast Asia, coincident with unprecedented connectedness between these areas and the rest of the world through trade and human movement. In the Americas, the re-establishment
of *Aedes aegypti* since the 1970s, following transnational efforts to eradicate the vector, can be attributed to declining political and financial commitment to resource intensive control efforts rather than any change in ecological suitability.\(^{165}\) Similarly the introduction of the secondary vector *Aedes albopictus* to North America, with subsequent spread to Central and South America, is thought to have occurred through shipments of used tires from Japan.\(^{117}\)

More recently, the *Ae. albopictus* mosquitoes were introduced into the Netherlands through the import of the ornamental plant Lucky bamboo (*Dracaena sanderiana*).\(^{123,243}\) To date, locally acquired DENV infections in non-endemic areas have been reported in Key West, Florida, USA and Nice, France.\(^{92,167}\) Although models have suggested that the vector can survive in the Netherlands, dengue virus transmission has not been demonstrated.\(^{267}\) Given this central role for demographic, economic and political influences in dengue’s recent resurgence, it seems reasonable to assume that these factors will continue to play a role in shaping the future burden and distribution of disease, and that any climate-related changes will not act independently of these factors.

Factors that will shape how changes in disease potential correspond to changes in actual disease occurrence include: the presence or introduction of the *Aedes* vector; availability of vector breeding sites; vector control activities; the circulation of one or more DENV serotypes; population demographics – in particular population density and movement; and the degree of contact between humans and vectors, which is influenced for example by housing design, availability of air-conditioning and the use of window and door screens. Many of these things may in fact be influenced by climate change indirectly. For example, changes in precipitation or rising inland water levels in endemic countries could potentially result in changed land use patterns, domestic or international migration and urbanization, all of which have implications for the risk of exposure of susceptible humans to infectious mosquitoes. Therefore when evaluating the likely impact of climate changes on dengue transmission, both direct and indirect effects should be considered.

Again, the spatial scale of analysis is paramount. At a global scale, climate-based models may be useful in indicating the broad geographic areas in which *Aedes* vectors, and potentially dengue transmission, could conceivably be sustained at present or in the future. However the relative effects of climate versus the range of non-climatic factors discussed above on the risk of dengue transmission is certain to depend on the local context. It has been demonstrated both in theoretical models at a global scale\(^{126}\) and in analysis of empirical data at a national level\(^{46}\) that local climate characteristics mediate the strength of association between climate variability and *Aedes* abundance or dengue incidence. These analyses showed a weaker association between monthly variations in temperature or precipitation and monthly variations in *Aedes* abundance or dengue cases in places with overall higher average temperatures or precipitation respectively. This demonstrates that the rate-limiting factors determining vector abundance and dengue incidence are likely to be locally defined. In an endemic setting such as southern Vietnam where current climatic conditions are already supportive of near year-round vector development and survival and virus replication, how big an effect is a sustained increase in temperature likely to have in a context of high population immunity and considering a potential life-shortening effect of higher temperatures on mosquito survival? Conditions that may mediate climate-related changes to vector density and disease incidence are also likely to operate at a local level, such as vector-control activities, water storage practices and characteristics of the built environment that determine micro-climates and the availability of breeding sites.

### 5. IMPACT OF CHANGES IN DENGUE TRANSMISSION RANGE AND INTENSITY ON CLINICAL OUTCOMES

Changes in the geographic distribution or intensity of dengue transmission will have implications for the clinical manifestation of disease. Two key risk factors for both symptomatic and severe dengue disease are the age at infection and the acquisition of secondary infections. Children with DENV infections are less likely than adults to experience clinical disease\(^{62,275,301}\), but are also less able to compensate for the increased vascular permeability that is characteristic of dengue pathogenesis, so are at higher risk than adults of severe outcomes including severe vascular leakage and hypovolemic shock (DSS).\(^{110,292,301}\) In sporadic dengue epidemics in Cuba and Taiwan clinical cases were more common in adults than children\(^{94,155,174,211}\), and in Singapore, where successful vector control has greatly reduced the force of infection and therefore the population immunity, adult cases of dengue fever represent the majority of clinical disease.\(^{63,202}\) In contrast, in the dengue endemic countries of southeast Asia and Central America dengue is primarily a disease of children and young adults, and by adulthood most individuals have partial or full immunity from multiple dengue virus exposures.\(^{12,27,272}\)

Repeated introductions of dengue virus to a non-endemic area have the potential to lead to sequential epidemics with heterologous DENV strains, with the associated increase in risk of DHF. An increased force of infection in endemic settings where multiple serotypes circulate could be expected to reduce the time between sequential infections, leading to a shift in the age distribution of disease towards younger age groups and potentially increasing the incidence of DHF/DSS in very young children. A recent analysis of hospitalized dengue patients in Ho Chi Minh City from 1996 – 2009 demonstrated that the highest risk of DSS was in paediatric dengue patients aged 6 – 10 years, but the youngest children (<5 years) were at significantly higher risk of mortality than older children.\(^{6}\) Efforts to model the epidemiology of dengue based on trends in climate, environmental and other factors should therefore consider also the implications of alterations in force of infection and the likelihood of sequential or co-circulation of heterologous serotypes on the age distribution of disease and therefore the clinical spectrum of dengue.
6. FURTHER ISSUES

Evaluating the effect of climate or other factors on the current or future dengue disease burden requires defined indicators of disease or infection. The most obvious indicator is clinical disease, however for dengue, perhaps more so than for many other infectious diseases of global importance, this is complicated in a number of ways. Firstly, national and international surveillance efforts for dengue generally rely on passively reported notifications of hospitalized cases, and therefore do not capture the full disease burden, with the ascertainment gap subject to considerable variation between settings and in time. Hospital-based data also carries inherent issues with determining the appropriate denominator for calculating burden of disease. Furthermore, dengue encompasses a spectrum of severities which may be variably included in the case definition for reporting. At the same time, surveillance in most endemic settings is based only on a clinical diagnosis with only small proportion of cases undergoing laboratory diagnostic testing, therefore over-reporting is an issue of variable magnitude within and between countries and over time. Furthermore, the quantitative relationship between clinical cases and total infections will vary substantially between epidemiological settings, depending on the intensity of transmission, age distribution of cases, population immunity and identity of circulating viruses. Indicators of vector abundance (i.e. house index, container index and Breteau index of pupae and larvae) have often been used as proxy measures for dengue risk, but their correlation with human disease is often poor and is likely to depend on local ecological and demographic characteristics. The lack of a robust and standardized indicator for dengue disease burden complicates efforts to predict and monitor future trends in dengue range and endemicity, which will rely on comparisons with a baseline, as well as models examining spatial patterns in dengue transmission, which may be confounded by geographic differences in clinical case ascertainment. Extensive work has been done to describe the geographic range and endemicity of malaria worldwide, using Plasmodium parasite prevalence as the index of endemicity. The existence of numerous published and unpublished parasite prevalence surveys from many locations and over a substantial time period made spatial and temporal comparisons of malaria endemicity valid and logistically feasible. An equivalent population-based virological marker for dengue burden is less obvious. Standard serological surveys give an indication of lifetime exposure risk, but must include a large age-stratified sample in order to be informative about historic and current transmission intensity. Serological markers of acute or recent DENV infection represent a more promising candidate for population-based assessments of dengue transmission risk.

We have discussed the limitations of transmission models or risk assessments that consider the climatic or immunological influences on dengue transmission in isolation from the multi-factorial environmental, social and demographic determinants that are accepted to have contributed to dengue’s recent resurgence. However, one explanation for the absence of these variables from models may be the difficulty in obtaining time series for socio-demographic and environmental factors that are comparable in time and geography to the case burden and climatic time series. Greater interdisciplinary collaboration between the ecology, social science and biomedical science fields would facilitate the incorporation of longitudinal data on relevant covariates as far as those data are available, or would at least allow for model findings to be considered within a broader environmental, social and demographic context.

7. CONCLUSIONS

Dengue, like all vector-borne diseases, is sensitive to climatic conditions that shape virus replication, vector development and survival, and therefore help define the geographical and seasonal limits within that can support dengue virus transmission. Climatic factors do not, however, act in isolation and non-climatic variables including population growth, human movement and environmental changes may have had far more to do with the global resurgence in dengue witnessed over recent decades than any direct effects of climate. The influences of climatic and non-climatic determinants on current and future dengue transmission are difficult to disentangle and therefore to quantitate independently. The challenge in refining models of dengue transmission to maximize their utility in predicting the location, magnitude and timing of future dengue epidemics or multiannual peaks in endemic cycles is to use data at appropriate spatial scales so that relevant ecological, social and demographic variables that operate at a local or regional scale can be incorporated into the model.

8. ACKNOWLEDGEMENTS

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Table 1. Relationship between extreme climate events and dengue incidence in time series.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study population</th>
<th>Study Period</th>
<th>Climate indicators</th>
<th>Data</th>
<th>Statistical Methods</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hales et al. (1999) 100</td>
<td>The South Pacific</td>
<td>1973-1994</td>
<td>Rainfall, temperature, SOI, ENSO</td>
<td>Monthly dengue cases</td>
<td>Correlation analysis</td>
<td>Positive correlations in various countries between dengue and SOI, temperature and rainfall.</td>
</tr>
<tr>
<td>Wu et al. (2010) 279</td>
<td>Taiwan</td>
<td>1998-2003</td>
<td>Temperature, humidity</td>
<td>Monthly incidence</td>
<td>Time-series analysis, ARIMA models</td>
<td>Negative association with temperature and humidity</td>
</tr>
</tbody>
</table>

ARIMA, Autoregressive Integrated Moving Average; ENSO, El Niño Southern Oscillation; SOI, Southern Oscillation index; SST, Sea Surface Temperature
Clinical observations: dengue manifestations and pathogenesis
PART II

CLINICAL OBSERVATIONS:
DENGUE MANIFESTATIONS AND PATHOGENESIS
Clinical, Epidemiological and Virological Features of Dengue Virus Infections in Vietnamese Patients Presenting to Primary Care Facilities with Acute Undifferentiated Fever

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**Objectives:** To explore clinical and virological characteristics and describe the epidemiology of dengue in patients who presented with acute undifferentiated fever (AUF) at primary health centers (PHCs) in Binh Thuan Province, Vietnam.

**Methods:** A prospective observational study was conducted from 2001 to 2006 to study the aetiology in AUF patients. Demographic and clinical information was obtained, and dengue polymerase chain reaction (RT-PCR) and serology were performed on a random selection of patients.

**Results:** 351 serologically confirmed dengue patients including 68 primary and 283 secondary infections were included in this study. In 25% (86/351) dengue virus (DENV) was detected by RT-PCR which 32 DENV-1, 16 DENV-2, 1 DENV-3 and 37 DENV-4 were identified. The predominant dengue serotype varied by year with seasonal fluctuation: DENV-4 in 2001-2002, DENV-1 and DENV-2 from 2003-2006. Primary dengue was more common in children. Higher viraemia levels (P = 0.010) were found in primary infections compared to secondary infections. DENV-1 infected patients had higher viraemia levels than DENV-2 (P = 0.003) and DENV-4 (P < 0.001) infected patients. Clinical symptoms were often seen in adults. Few differences in clinical symptoms were found between primary and secondary infection and no significant differences in clinical symptoms between the serotypes were observed.

**Conclusions:** Our data provide insight into the epidemiology, clinical profile and virological features of mild symptomatic dengue patients who presented to PHCs with AUF in Vietnam.

1. INTRODUCTION
Mosquito-borne flavivirus infections such as dengue have rapidly spread and are now one of the most important infectious diseases in the world, in terms of morbidity and mortality.88,307 It is a public health problem with growing global incidence and geographic distribution to almost all tropical and subtropical regions, and with a transition from epidemic to endemic transmission intensity. Recent estimates indicate that over 3.5 billion people (~55%) of the world population are living in areas at risk for dengue.16

Dengue is caused by an infection with a dengue virus (DENV) and transmitted primarily by Aedes spp. mosquito vectors.22 Any of the four distinct serotypes (DENV 1-4) can cause dengue fever (DF) or the more severe forms of the diseases; dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).235 The majority of DENV infections are probably asymptomatic, and only a small number of dengue infections (~5%) will result in severe forms of the disease.27 The mechanisms for the variable clinical course are not completely elucidated, but interactions between virus and host immunity and hyperendemicity of multiple serotypes are believed to play an important role in determining the outcome of disease.165

In Vietnam, dengue is not only an urban disease: the high population density and ecological conditions in the rural areas are also favourable for dengue transmission. Binh Thuan, a rural province in southern Vietnam, is highly endemic for dengue.14 Dengue usually presents as a nonspecific febrile illness and is rarely recognized as a clinical entity by physicians at primary health centres (PHC).218 However, recent studies have suggested that dengue is the most frequent cause of fever in patients who present to the PHCs and is responsible for approximately one-third of all patients with fever.219 The prevalence of dengue IgG antibodies among primary schoolchildren increased from 50% to 90% with increasing age, indicating high, relatively stable, transmission rates over many years. The annual sero-conversion rate among primary school children, corresponding to the annual incidence rate of primary dengue infections, ranged from 12-17%.272,274

The data presented here are derived from a prospective observational study from March 2001 to March 2006, with enrolment of acute undifferentiated fever (AUF) patients who presented to twelve PHCs and the provincial malaria control center in Binh Thuan province.218,219 One of the objectives was to describe the epidemiology and to detect outbreaks of dengue in Binh Thuan province. In dengue endemic regions, outbreaks often do not necessarily reflect an increase in transmission intensity but merely an increased number of patients with complicated dengue, mostly secondary infections after the (re-) introduction of a new serotype.200 During the study period no significant outbreaks of dengue were observed, other than the usual seasonal fluctuation. Here, we report PCR results for patients with serologically confirmed dengue and analyze the epidemiology and clinical and virological characteristics with respect to serotype, antibody response and vireamia.

2. METHODS

2.1 STUDY SITE AND POPULATION
The study site was described previously.218,219 Binh Thuan Province is located along the south-eastern coast of Vietnam, 150 km northeast of Ho Chi Minh City. It covers 7,828 km² and the estimated population was 1,140,429 inhabitants in 2004. A prospective observational study was conducted from March 2001 to March 2006. In this study, patients with AUF, who presented to the 12 study PHCs and at the provincial malaria control station center in Phan Thiet city, were included. Patients were invited to participate after giving informed consent. A standardized questionnaire was taken to collect demographic and clinical information. Serum samples were collected by venous puncture on presentation (acute sample; t0) and after 3 weeks (convalescent sample; t3). Serum samples were stored at -20°C at the study sites until monthly transfer to Cho Ray hospital (Ho Chi Minh City, Vietnam), where they were stored at -70°C.
2.2 Sampling Selection for Serology and PCR

Complete sets of acute and convalescent samples were collected for serology. In 2001 all collected paired sera were tested with dengue ELISA; from 2002 onwards paired sera were randomly selected as two patients per PHC and per month from the total dataset. Firstly, serum specific anti-dengue IgM and IgG ELISA were performed in sera patients with AUF. Based on the serological results, patients with DENV infection were included in this study. Secondly, RT-PCR was performed in the acute samples of patients with serologically confirmed dengue.

2.3 Dengue Diagnostics

Serology: Paired serum samples were tested for dengue with direct IgG ELISA and IgM-Capture ELISA (Focus Technologies Inc., Cypress, CA, USA). Details regarding the ELISA and the interpretation of results have been described previously. Briefly, a fourfold increase of antibody concentrations between t0 and t3 was considered significant. The IgM concentration on t3, relative to the IgG concentration on t3 was also used as a criterion. Acute primary dengue virus infection was defined as positive IgM on t3 with an IgM/IgG ratio on t3 greater than one. A positive IgM on t3 with an IgM/IgG ratio on t3 less than one, or a negative IgM reaction on t3 but with a positive IgG t3 and a fourfold molar increase of IgG between t0 to t3 were classified as acute secondary dengue. A negative IgM reaction on t3, a positive IgG on t3 but without a fourfold increase between t0 and t3 was classified as “not acute dengue but past infection”, and a case of both negative IgM and IgG on t3 was classified as “no dengue”. Dengue NS1 antigen was detected in a subset of serum samples using the Platelia™ Dengue NS1 Ag - ELISA (Bio-rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer’s instructions. Optical density (OD) was measured at 450/620 nm, using the Evolis™ absorbance reader. Results were expressed as the OD ratio (ODR) between the OD value of the sample and a calibrator sample that is enclosed with every test panel. An ODR ≥1.0 was interpreted as positive.

RNA was isolated from serum from the first serum as described elsewhere. RNA was reversely transcribed, and dengue virus vireaemia levels were assessed using an internally controlled, serotype specific, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay that has been described elsewhere; results were expressed as cDNA equivalents per ml of serum.

2.4 Ethical Considerations

The study was approved by the Review Board of Cho Ray Hospital, the provincial health services of Binh Thuan and the peoples committees of the participating communities. All patients (or, for children, the parents or guardian) gave written informed consent.

2.5 Statistical Analysis

All results were summarized in terms of medians and ranges for continuous data and non-parametric tests were used to compare within groups. For dichotomous variables, Fisher’s exact test was performed. Viraemia levels were expressed as the median and 25-75% interquartile (25-75 IR). All calculations were performed using SPSS (version 16.0, SPSS Inc. Illinois). A two-tailed p value of <0.05 was considered as statistically significant.

3. Results

3.1 Sample Population

A total of 14595 febrile patients were included. 83 patients did not meet the inclusion criteria and were excluded from further analysis. Paired sera were collected from 8268 febrile patients; 1938 (23.4%) serum pairs were tested with dengue an IgM- and IgG-ELISA. Dengue was serologically confirmed in 382 (19.7%) cases. Of these, RT-PCR was performed in 351 (91.9%) acute samples. DENV was detectable in 86 (24.5 %) samples among which 32 were DENV-1, 16 DENV-2, 1 DENV-3 and 37 DENV-4 were detected. Serologic testing by ELISA revealed 68 primary infections and 283 secondary infections. Demographic information on the study population is shown in table 1.

3.2 Epidemiologic Data

3.2.1 Occurrence of dengue serotypes

Dengue incidence in Binh Thuan peaks during the rainy season from May to October. DENV-4 was the dominant dengue serotype in 2001-2002. Thereafter, DENV-1 and DENV-2 became the most frequently isolated serotype (Figure 1).

<table>
<thead>
<tr>
<th>Patients with AUF (N = 14512)</th>
<th>Serologically confirmed/ Total tested (N = 351/1938)</th>
<th>Virologically confirmed/ Total tested (N = 86/351)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female (ratio)</td>
<td>1.28 (8139/6373)</td>
<td>1.76 (224/127)</td>
</tr>
<tr>
<td>Median age (yr) (range)</td>
<td>(0.1-95.1)</td>
<td>(4.2-73.7)</td>
</tr>
<tr>
<td>Child/Adult (ratio)</td>
<td>0.70</td>
<td>0.61</td>
</tr>
</tbody>
</table>

1 sex was unknown in 7 AUF patients 2 age was unknown in 8 AUF patients

Table 1. Demographic data of patients with acute undifferentiated fever and dengue
3.2.1 Distribution of primary and secondary infection by age group
When age groups were combined from patients selected for RT-PCR, acute secondary dengue \((n = 283)\) was four times as common as acute primary dengue \((n = 68)\). The acute primary/secondary dengue ratio in children (< 15 years of age) and adults were 0.49 and 0.12 \((P < 0.001\) by Fisher’s exact test), respectively. Figure 2 shows the serotypes which were found in 86 patients and the distribution of serologically confirmed primary and secondary DENV infections stratified by age group.

3.3 CLINICAL DATA
3.3.1 Differences in clinical presentation between primary and secondary infection
Patients with secondary infection were more likely to be older than those with primary infection \((P < 0.001)\). The median time between onset of fever and the first visit was 1 day (25-75 IR, 2 days) for primary and secondary dengue. Myalgia was more frequently reported in secondary infection, whereas gastrointestinal symptoms were more common in primary infections (table 2).

3.3.2 Differences in clinical presentation between primary and secondary dengue with different serotypes
Primary infection was diagnosed in 17 of 32 DENV-1 patients (53.3%) and 1 of 16 DENV-2 patients (6.3%). All DENV-4 patients \((n = 37)\) had an antibody response that was compatible
with secondary dengue. No significant differences between primary DENV-1 and DENV-2 were observed with respect to the clinical variables. Clinical variables were also not significantly different between secondary DENV-1, DENV-2 and DENV-4 infections.

### 3.3.3 Differences in clinical presentation between children and adults

Table 2 also shows the distribution of clinical variables between children and adults. The median time between onset of fever and the first visit was 1 day (25-75 IR, 1 days) for both children and adults. Symptoms and physical findings were more common in adult patients, such as myalgia, backache, arthralgia, and bruises.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Primary Infection (n = 67)</th>
<th>Secondary Infection (n = 283)</th>
<th>P</th>
<th>Children ≤ 15 years (n = 132)</th>
<th>Adults &gt; 15 years (n = 218)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>12.2 (5.3-15.1)</td>
<td>20.6 (5.5-75.0)</td>
<td>&lt; 0.001</td>
<td>11.2 (5.3-15.0)</td>
<td>28.1 (15.0-75.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Male/Female</td>
<td>44/23</td>
<td>180/103</td>
<td>80/52</td>
<td>144/74</td>
<td>40/50 (40.0-60.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>38.9 (38.0-39.9)</td>
<td>39.0 (38.0-42.0)</td>
<td>39.0 (38.0-42.0)</td>
<td>39.0 (38.0-42.0)</td>
<td>39.0 (38.0-42.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pulse pressure (mmhg)</td>
<td>30.0 (20.0-60.0)</td>
<td>30.0 (20.0-60.0)</td>
<td>30.0 (20.0-60.0)</td>
<td>30.0 (20.0-60.0)</td>
<td>30.0 (20.0-60.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>90.0 (70.0-130.0)</td>
<td>90.0 (70.0-130.0)</td>
<td>90.0 (70.0-130.0)</td>
<td>90.0 (70.0-130.0)</td>
<td>90.0 (70.0-130.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Symptoms (%)</td>
<td>(n = 132)</td>
<td>(n = 218)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
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<td>94.7</td>
<td>92.4</td>
<td>96.0</td>
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<td>44.7</td>
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<td>6.1</td>
<td>27.5</td>
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<tr>
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<td>31.8</td>
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<td>Arthralgia</td>
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<td>13.1</td>
<td>0.022</td>
<td>12.1</td>
<td>17.4</td>
<td>&lt; 0.001</td>
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</table>

*One child age < 4 years was excluded from analysis. Median range; differences between groups were analyzed with Mann-Whitney tests. Fisher's exact test were used in all dichotomous data. Gastrointestinal symptoms: abdominal pain, diarrhea, and constipation.
3.4 Virologic Data
Viraemia levels were measured in 71 of 86 RT-PCR positive patients (84%); 28 of 32 DENV-1, 11 of 16 DENV-2, 1 of 1 DENV-3 and 32 of 37 DENV-4. The viraemia levels in serum ranged from $1.5 \times 10^4$ to $2.7 \times 10^{11}$/ml, with a median of $1.1 \times 10^6$/ml (25–75 ir, $4.1 \times 10^6$/ml), and were sampled from day 0 to day 4 of fever.

3.4.1 Relationship between serum viraemia and antibody response
Viraemia levels were significantly higher in primary than in secondary dengue (4.2 $\times 10^6$/ml versus 8.8 $\times 10^5$/ml, respectively, $P = 0.010$, figure 3A). During the first two days of fever, viraemia levels were significantly higher in primary dengue than in secondary dengue (4.2 $\times 10^6$/ml versus 9.6 $\times 10^5$/ml, respectively, $P = 0.036$). The distribution of viraemia levels at different time points since illness days in primary and secondary dengue is shown in figure 3B.

3.4.2 Relationship between serum viraemia and the serotype
DENV-1 infected patients had higher median viraemia levels than DENV-4 infected patients. Viraemia levels in DENV-1 infected patients were higher than in DENV-2-infected patients. Viraemia levels in DENV-2 and DENV-4 infected patients were not significantly different (figure 3C). When immune status was taken into account, median viraemia levels remained higher in secondary DENV-1 infected patients than secondary DENV-4 (5.5 $\times 10^6$/ml versus 8.3 $\times 10^5$/ml, respectively, $P = 0.014$) and secondary DENV-2 (5.5 $\times 10^6$/ml versus 2.6 $\times 10^6$/ml, respectively, $P = 0.015$) infected patients. No association was observed between serum viraemia levels and sex and age.

3.4.3 Relationship between serum viraemia and NS1 antigen
NS1 antigen detection was performed in 100 (of 351) acute samples; 40 samples were NS1 antigen positive. DENV was detectable in 31 (78%) of these samples and viraemia levels were measured in 20 samples. DENV was demonstrated in 3 of 60 NS1 antigen negative samples (5.0%). Irrespective of immune status, viraemia levels were higher in NS1 antigen positive patients than NS1 antigen negative patients ($1.7 \times 10^6$/ml versus $8.6 \times 10^5$/ml, respectively) ($P = 0.016$, Mann-Whitney test). Figure 3D shows differences in viraemia levels among patients with and without NS1 antigen by immune status. The median viraemia levels in patients with secondary DENV infections with NS1 antigen were higher than those without NS1 antigen. Because of the small numbers, this did not reach significance ($P = 0.100$).

4. Discussion
In a previous study we showed that dengue is highly endemic in Binh Thuan province in southern Vietnam. Here we show that during a study period of five years, co-circulation of multiple DENV serotypes occurred in this region. Higher viraemia levels were found in primary infections in comparison to secondary infections. DENV-1 infected patients had higher viraemia levels than DENV-2 and DENV-4 infected patients. Clinical manifestations of infections with the different serotypes were similar but symptoms were more commonly observed in adults. Two symptoms differed significantly between primary and secondary infection which were myalgia and gastrointestinal symptoms.

The most prevalent serotype was DENV-4 during 2001 and 2002, followed by DENV-1 and DENV-2 in 2003. Although seasonal variations in virologically confirmed dengue cases were observed, these should be interpreted with caution, because only a proportion of AUF patients were tested by RT-PCR. A previous surveillance study showed that all four DENV serotypes co-circulated in southern Vietnam in 2001 with isolation of DENV-2 and DENV-3 in Binh Thuan province. This study shows that all DENV serotypes have been circulating in Binh Thuan province, probably with a shift of the dominant DENV serotype during the study period. Co-circulation of all DENV
serotypes is well described in Asia and indicates hyperendemicity. In discussing viraemia in relation to serotypes, it is important to point out that a primary immune response was biased towards DENV-1, which accounted for 87% of primary DENV infections. DENV-1 infections were characterized by significantly higher viraemia levels than DENV-4 infections and slightly higher than DENV-2 (figure 2D). DENV-1 infected patients are more likely to be younger than DENV-2 or DENV-4 infected patients. Age is an important risk factor for the development of severe dengue disease.

The relationship between viraemia and host antibody response is less clear and has been the topic of many studies. In DENV-1 infections, viraemia was higher than in secondary infections but this pattern was not found for DENV-2 infections for which lower viraemia was associated with higher anti-DENV antibody titres. Interestingly, primary immune status was found in half of the symptomatic DENV-1 cases whereas 100% DENV-4 and almost all DENV-2 infections exhibited a secondary response. Likewise, primary infections with DENV-1 were predominant compared to other serotypes in a retrospective study of Thai dengue cases. Analysis of a co-epidemic with DENV-2 and DENV-4 indicated that the vast majority of DENV-2 infections were associated with a secondary immune response. DENV-2 viruses have most commonly been associated with DHF/DSS, along with DENV-1 and DENV-3 viruses. DENV-2 and DENV-4 have been associated with increased disease severity as a secondary infection, whereas DENV-1 and DENV-3 seem to cause more severe disease in primary infection than do the other two serotypes. These data should be interpreted against their own history of DENV co-circulation and herd immunity, which may be different between regions. In our study, clinical manifestations did not differ significantly among patients infected with different serotypes but this was a study population selected with only mild disease in a highly endemic area with a history of circulation of all DENV serotypes. Our data showed sex differences with a male predominance. Reported sex differences are contradicting and differences in favour of males have been documented. The underlying causes of sex differences are not clear and multiple factors may play a role. A plausible explanation could be that there is a slight predominance in male births in Vietnam. A biased parents’ health seeking behaviours towards males, differences in susceptibility and clinical presentation are other plausible causes. The strength of this study is the prospective enrolment of AUF patients over a period of five years which provides a comprehensive overview of epidemiological pattern over time dengue at PHCs in Vietnam. Only AUF patients were tested for dengue and therefore the study population included mildly symptomatic patients. Interestingly, a considerable amount (20%) of AUF patients were seen with primary DENV infection at PHCs. Health seeking behaviour and the nature of study site (at PHCs) may have caused the identification of more symptomatic primary dengue infections. This study was conducted shortly after a period of time in which malaria was the main cause of fever. Public awareness that malaria causes fever was high. As results of which, febrile patients presented themselves very early at PHCs. We previously showed that patient delay was shorter for children, suggesting that parents are very concerned about the health of their children and take the opportunity to seek help as soon as possible. These patients are probably a true reflection of burden of symptomatic dengue in the general population.

A limitation of this study is that serotypes identification by RT-PCR showed a low yield. There are several possible explanations for this low detection rate. First, serum samples were aliquoted and stored at -20°C up to one month. Samples were collected for transportation to CRH hospital for storage at -70°C once monthly. DENV RNA could have degraded due to sub-optimal storage and transport conditions. Secondly, samples were used previously for other studies. It is possible that several freeze-thawing steps contributed to the degradation of DENV RNA. Thirdly, the majority of our study population presented very early in course of dengue and clinical manifestations were very mild. At this early stage of disease, viraemia levels may have been low.

In conclusion, our data confirm earlier findings that dengue is highly endemic in southern Vietnam and shows that all four serotypes are prevalent.

5. ACKNOWLEDGEMENT

This study was carried out with the support of the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) and the Wellcome Trust. Khoa T.D. Thai is supported by a ‘Mosaic’ fellowship from The Netherlands Organization for Scientific Research (NWO). This study would not have been possible without the contribution of many clinicians, nurses and co-workers at primary health centers across Binh Thuan Province. The authors thank Nghiern My Ngoc and Nguyen Thien Quy for their laboratory help.
HIGH INCIDENCE OF PERIPHERAL BLOOD PLASMACYTOSIS IN PATIENTS WITH DENGUE VIRUS INFECTION: A PROSPECTIVE STUDY

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**Background:** Polyclonal peripheral blood plasmacytosis has occasionally been described in dengue virus (DENV) infected patients. We initiated this prospective observational study to quantify and describe the kinetics and phenotype of peripheral blood plasma cells (PCs) in these patients.

**Methods:** Morphological examination and flow cytometric (FC) analysis for the characterization and immunophenotyping of lymphocyte subsets and PCs were performed in 35 and 31 patients suspected of DENV infection, respectively.

**Results:** Our results show that blood plasmacytosis is a very common haematological finding. Depending on the days of illness at presentation, blood plasmacytosis was observed in 64% to 73% of patients. Blood plasmacytosis was the most pronounced before 7 days of illness and declined rapidly thereafter, to completely disappear after 14 days of illness. Blood plasmacytosis was higher in secondary DENV infection. The majority of CD138⁺ PCs (89%) had a shared immunophenotype (CD45⁺/CD19⁻/CD56⁻) and in all cases the PCs were polyclonal.

**Conclusions:** Blood plasmacytosis, characterized by a transient presence of polyclonal PCs in the circulation, is a common event in DENV infection. Blood PCs may play a role in the humoral immune response to and pathogenesis of dengue.

1. **INTRODUCTION**

Dengue is the infectious disease which is caused by dengue virus (DENV). DENV transmission primarily takes place through bites by the mosquito vectors *Aedes aegypti* and *Aedes albopictus.* DENV is a single-stranded, positive-sense RNA virus. There are four DENV serotypes which, based on antigenic and genetic differences could be regarded as four distinct virus species (DENV1-4). All serotype are capable of infecting and replicating in numerous human cells, including dendritic cells, monocytes/macrophages, B cells, T cells, endothelial cells, hepatocytes and neuronal cells in vivo. Although a consensus that the mononuclear phagocyte lineage cells (monocytes and macrophages) constitute the primary targets for replication based on clinical and autopsy studies, there is still controversy about the primary target cell type(s) targeted in humans.

Infection by one of the four DENV serotypes cannot be distinguished on clinical grounds; all serotypes can cause a spectrum of clinical manifestations that varies from mild undifferentiated febrile illness to severe dengue, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) of which the latter is the most life threatening. The pathogenesis of severe DENV infection is poorly understood. One of the characteristic features of dengue is the occurrence of leucopenia and thrombocytopenia, probably resulting from virus induced bone marrow suppression. This is non-specific and not exclusively seen in dengue. Thrombocytopenia may worsen as a result of coagulopathy and vasculopathy in patients with severe disease. Despite the general bone marrow suppression, blood plasmacytosis has been reported in a few patients with DENV infection. However, the frequency of blood plasmacytosis in patients with dengue infection, the origin of these plasma cells (PCs) and the mechanisms by which they appear in the blood are not known.

Blood plasmacytosis is an unusual haematological finding that is most commonly seen in plasma cell leukaemia or advanced stage multiple myeloma, in which case the plasma cells are part of the malignant clone and thus are monoclonal. Non-malignant reactive peripheral plasmacytosis is occasionally found in a variety of diseases, such as tumours, autoimmune disorders, and infectious diseases including sepsis, primary infection and reactivation of Epstein-Barr virus, acute respiratory infections, parvovirus B19 infection, rubella and hepatitis virus A infection.

In this study, we prospectively quantified and described blood plasmacytosis in returned travellers with DENV infection. We also characterized the immunological phenotype of these PCs by flow cytometry and explored associations with viral load, the appearance of anti-DENV IgG antibodies, and the presence of primary or secondary DENV infection.

2. **MATERIALS AND METHODS**

2.1 **STUDY SITE AND STUDY POPULATION**

Returned travellers presenting with a history of less than 14 days of fever, clinically suspected of dengue, were included at the Department of Tropical Diseases, Academic Medical Center, Amsterdam, the Netherlands. Blood samples were collected from each patient for routine diagnostic procedures. All samples were tested by enzyme-linked immunosorbent assay (ELISA) for anti-DENV IgM and IgG antibodies and DENV real time reverse transcriptase polymerase chain reaction (RT-PCR). Morphological examination and flow cytometric (FC) analysis of blood were performed within 24 hours after the blood was drawn. All patients at this department are routinely informed that their data can be used for observational studies. All patients consented to participate in this study.

2.2 **DENGUE DIAGNOSTICS**

Serum samples were routinely tested for dengue antibodies with direct IgG enzyme-linked immunosorbent assay (ELISA) and IgM-Capture ELISA (Panbio Tech Co, Brisbane, Australia). For real time RT-PCR, RNA was isolated from blood plasma as described elsewhere. RNA was reverse transcribed and amplified using an internally controlled and quantitative serotype-specific, TaqMan-based assay as described elsewhere. Quantitative results were expressed as cDNA equivalents per ml. The RT-PCR and ELISA results were used for diagnosis and classification of dengue infection.
2.3 CLASSIFICATION OF PRIMARY AND SECONDARY DENGA

Acute primary DENV infections were confirmed by RNA detection and/or detection of dengue specific IgM antibodies in acute samples, in the absence of dengue serum specific IgG antibodies. A negative first acute sample for dengue serum specific IgM antibodies with seroconversion for IgM antibodies within 7 to 14 days was also considered as primary dengue. Acute secondary DENV infections were confirmed on acute samples (less than 14 days) with positive IgG results and detection of viral RNA, either with or without detectable dengue serum specific IgM antibodies.

For patients who presented with both positive IgM and IgG results in the first acute sample, but without detectable RNA by RT-PCR, the ratio between the IgM and IgG concentrations was used to distinguish primary and secondary infections. A ratio of anti-dengue IgM to IgG of greater than or equal to 1.8 was the criterion for primary dengue infection; values lower than 1.8 were considered to indicate secondary infections.132 The patients for whom the diagnosis DENV infection was not confirmed were grouped as other febrile illness (OFI) and included in this study as controls.

2.4 MORPHOLOGICAL EXAMINATION

A complete blood count with differential was performed on an automated cell counter (Sysmex XE-5000, Sysmex Corporation, Kobe, Japan). Blood smears were fixed and stained first with May-Grünwald eosine-methylene blue and then after rinsing with Giemsa’s azur eosine methylene blue solution (Merck). 100 white blood cells per slide were scored by one experienced technician blinded for disease outcome with a binocular microscope at x100 magnification. Lymphocytes were scored as normal lymphocytes, atypical lymphocytes (with abundant cytoplasm), plasmacytoid lymphocytes or PCs.

2.5 FLOW CYTOMETRY ANALYSIS OF PERIPHERAL BLOOD

Erythrocytes were lysed using ammoniumchloride and washed twice with phosphate-buffered saline. Thereafter, the cell suspension (100 μl) was mixed in tubes with 5 μl of undiluted monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), R-phycocerythrin coupled to the cyanine dye Cy5™ (PE Cy5), R-phycocerythrin coupled to the cyanine dye Cy7™ (PE Cy7), allophycocyanine (APC), allophycocyanine coupled to the cyanine dye H7 (APC-H7) and Horizon™ or Anemonia Majano cyanine (AmCyan) for the following cell surface markers: CD3, CD5, CD8, CD10, CD14, CD19, CD45, kappa and lambda Lights chains F(ab’)2 (DAKO A/S, Denmark), CD4 (Sanquin, the Netherlands) CD138, CD16/56 (Immuno Quality Products, the Netherlands) in a four-tube assay. After adding the mAbs, the cells were incubated in the dark for 15 minutes at room temperature. Fluorescence intensity was determined with an eight-color FACSCanto™ (BD Biosciences, USA) flow cytometer. Mononuclear cells were gated on the basis of forward and side light-scattering properties and subtyped on the basis of cluster of differentiation (CD) molecule expression. Isotype-matched control mAbs were used to define background fluorescence. On average, 1 x 10⁴ events were acquired and analyzed using CellQuest software (BD Biosciences, USA). For demonstration of PCs a lower limit of detection of 0.2 % of cells expressing the surface marker CD138 was applied.

2.6 STATISTICAL ANALYSIS

Statistical calculations were performed using SPSS (version 17.0, SPSS Inc. Illinois). Results were summarized in terms of medians and ranges for continuous data and non-parametric tests were used to compare within groups. For dichotomous variables, Fisher’s exact test was performed. The Mann-Whitney test was used for non-normally distributed variables. A two-tailed p-value of 0.05 was considered to be statistically significant.

3. RESULTS

3.1 STUDY POPULATION

Morphological examination of the blood smear was performed in 35 returned travellers with suspected DENV infection presenting at our hospital between September 2008 and June 2009, of whom FC analysis for immunophenotyping of PCs was done in 31 patients. Demographic information and travel history of the 35 returned travellers is shown in table 1. DENV infection was confirmed in 28 of 35 patients, of whom 15 had primary infection and 13 had secondary infection and seven patients were classified as OFI.
A high incidence of peripheral blood plasmacytosis was demonstrated in 16/28 (57%) returned travellers with confirmed DENV infection. The frequency of plasmacytosis was 73% (11/15) among patients from whom blood was collected during the first 7 days of illness. Among patients from whom a blood sample was collected during the first 14 days of illness (DOI), plasmacytosis occurred in 64% (16/25). Plasmacytosis was not observed in any of the 7 patients with OFI.

### 3.2 Kinetics of Plasmacytosis

During the first 7 days of illness, the median plasma cell count in DENV patients was 2.5% (25-75 interquartile range (IR): 0 - 8 %) of the total white blood cell count (figure 1A); the median absolute plasma cell concentration was 8.4 x10^7/L (25-75 IR: 0 - 3.5 x 10^7/L). The PC distribution by days of illness, including in eight patients with DENV infection for whom sequential samples were available, is shown in figure 1B and D. Figures 1C show a typical example of a PC and plasmacytoid lymphocyte in a patient with secondary DENV infection.

### 3.3 Immunophenotypes of CD138+ PCs in DENV Infection

The antigen profiles of lymphocytes and plasma cells (CD19, CD16/CD56, CD3, CD4, CD8, CD138) are presented in figure 2 for 25 patients with DENV infection and 6 patients with OFI. The lymphocyte subsets did not differ significantly between patients with DENV infection and patients with OFI, with the exception of PCs, characterized by being CD138+. In patients with DENV infection, PCs were detected during 15 days after onset of illness. The PCs were polyclonal in all cases with a mean kappa/lambda ratio of 1.3 (49.3:37.9). Subsequent immunophenotyping of PCs in the 19 patients with plasmacytosis revealed a shared phenotype: in 17/19 cases the majority of the PCs was CD138+/CD45+<br>CD19/CD56. A linear correlation was observed between the percentage of PCs as assessed by morphology and by flow cytometry (R^2 = 0.85).
3.4 ASSOCIATION BETWEEN PLASMACYTOSIS WITH VIRAL LOAD, ANTI-DENV IgG ANTIBODIES, PRIMARY AND SECONDARY DENV INFECTION

Viral RNA was detectable in 18 of 28 DENV patients, of whom 12 patients had primary and 6 had secondary DENV infection. The viral RNA levels ranged from $6.2 \times 10^2$ to $4.7 \times 10^9$/mL in DENV-1 infection and $8.9 \times 10^4$ to $1.6 \times 10^9$/mL in DENV-2 infection. There was no correlation between DENV load and the percentage of blood PCs or with blood PCs concentration (data not shown).

Morphological examination and anti-DENV IgG serology was performed on the same day in 12 samples from 10 patients with secondary DENV infection. Figure 3a shows percentages of PCs by anti-DENV IgG optical density ratios (ODR) stratified by days of illness. The anti-DENV IgG ODR increases beyond five days of illness, which is indicative of an increase of anti-DENV IgG concentration in serum. A negative correlation was observed between IgG ODR and the percentage of PCs, but this did not reach significance ($P = 0.053$).

The median percentage of PCs was significantly higher in secondary than in primary dengue (4.5% versus 1.0%, respectively, $P = 0.05$, figure 3B) when day of illness was not taken into account. The difference was even more striking during the first 7 days of illness (15% versus 0.0%, respectively, $P = 0.005$).

4. DISCUSSION

This is the first prospective study demonstrating that blood plasmacytosis, characterized by a transient increased amount of polyclonal PCs in the circulation, is a common hematological event in DENV infection. The plasmacytosis was most pronounced during the first week of disease and disappeared completely within two weeks. The PCs were all polyclonal by origin; the predominant immunophenotype was Cd138+/Cd45+/Cd19-/Cd56-.

Bone marrow PCs are long-lived non-proliferating cells generated as part of the humoral immune response to infections. Memory B cells that are activated by antigen in the secondary lymphoid organs differentiate into plasmablasts that migrate to the bone marrow where they develop into PCs. In contrast to bone marrow PCs, plasmablasts are short-lived proliferating cells and plasmablasts normally represent less than 0.1% of peripheral blood mononuclear cells (PBMCs). Also PCs are usually not observed in peripheral blood and non-malignant blood plasmacytosis in DENV infection thus far has only been described in two reports.

Circulating (CD138+) PCs can be regarded as PC progenitors or early or mature PCs. The differentiation of B cells into PCs is regulated by transcription factors such as BCL6, PAX5, Blimp-1, IRF-4 and XBP-1 and is characterized by a number of changes in B-cell surface molecules. CD19, CD20, CD21, CD22, and CD45 expression are down regulated, whereas...
CD38 and CD138 expression are upregulated. In fact, CD138 is the only antigen that discriminates plasmablasts (CD138+) from early and mature PCs (CD138-). The surface phenotypes of PCs in the majority of DENV infected patients (89%) were CD138+/CD45+/CD19+/CD56-. This differs from bone marrow and blood PCs from patients with reactive plasmacytosis, which are usually CD19-. Cd19 is a protein that is invariable present on B cell lineage cells but is lost during maturation to plasma cells. This could point at redistribution as the cause of increased number of plasma cells in peripheral blood. However, increased production may also play a role as CD45 are expressed. During DENV infection, virus and cytokines are detectable in blood and PBMCs, similar to what is seen in many other virus infections. Both B cells and monocytes can secrete IL-6. IL-6 has pro-and anti-inflammatory activities but is also growth, proliferation and survival factor for nonmalignant and malignant PCs. Polyclonal blood PCs have been observed in patients with IL-6 producing malignancies such cardiac myxoma and gastric carcinoma. IL-6 has the ability to prevent plasma cells from going into apoptosis and thus increases the survival of PCs. Other cytokines than IL-6 can also be involved in the development of plasmacytosis. The IL-6 receptor subunit glycoprotein 130 is shared with receptors of IL-11, oncostatin M, leukemia inhibitory factor, and ciliary neutrophic factor. IL-11 has been detected as a growth factor of IL-6-dependent plasmacytoma, and IL-10 has been reported to stimulate myeloma cells via oncostatin M. DENV infected monocytes produce IL-1, which is a known inducer of IL-6 and tumor necrosis factor-α and can induce an inflammatory response and the maturation of B cells into PCs. Moreover, increased levels of IL-6 have been associated with dengue disease severity, particularly in patients with DHF and DSS. Production of IL6 is also a common response in a variety of diseases that are not characterized by blood plasmacytosis. This implies that either IL6 production is not the sole explanation for blood plasmacytosis or that plasmacytosis does occur much more often than has been hitherto recognized.

Although our study provides insight into the biology and kinetics of blood plasmacytosis in DENV infection, the mechanism and possible role in dengue pathogenesis remain unclear. Important unanswered questions in this respect are whether active DENV replication takes place in peripheral PCs and whether PCs are redistributed and released from the bone marrow into the circulation or whether they represent an increased production and if this is virus or cytokine driven? Research into these questions can further address the contribution of peripheral PCs to dengue pathogenesis and expand our knowledge of DENV infection.

5. ACKNOWLEDGMENTS
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CHAPTER 11

HIGH-RESOLUTION ANALYSIS OF INTRA-HOST GENETIC DIVERSITY IN DENGUE VIRUS SEROTYPE 1 IDENTIFIES MIXED INFECTION

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* These authors contributed equally in this work.
Little is known about the rate at which genetic variation is generated within intra-host populations of dengue virus (DENV) and what implications this diversity has for dengue pathogenesis, disease severity, and host immunity. Previous studies of intra-host DENV variation have used a low frequency of sampling and/or experimental methods that do not fully account for errors generated through amplification and sequencing of viral RNAs. We investigated the extent and pattern of genetic diversity in sequence data in domain III (DIII) of the envelope (E) gene in serial plasma samples (n=49) taken from 17 patients infected with DENV-1, totaling some 8458 clones. Statistically rigorous approaches were employed to account for artifactual variants resulting from amplification and sequencing, and which we suggest have played a major role in previous studies of intra-host genetic variation. Accordingly, nucleotide sequence diversities of viral populations were very low, with conservative estimates of the average levels of genetic diversity ranging from 0 – 0.0013. Despite such sequence conservation, we observed clear evidence for mixed infection, with the presence of multiple phylogenetically distinct lineages present within the same host, while the presence of stop codon mutations in some samples suggests the action of complementation.

In contrast to some previous studies we observed no relationship between the extent and pattern of DENV-1 genetic diversity and disease severity, immune status, or level of viremia.

1. INTRODUCTION

DENV is a single-strand positive-sense RNA virus of the family Flaviviridae and exists as four closely related antigenically distinct serotypes, denoted DENV-1 to DENV-4. These serotypes differ at the consensus level by 25 to 40% at the amino acid (aa) level.126,285 Genetic variation within each of the four serotypes is defined as a series of “genotypes” (or “subtypes”) which can vary from one another by up to ~6-8% and 3% at the nucleotide and aa levels, respectively.126,232,285 For example, at least four major genotypes of DENV-1 exist each with different geographical distributions.55,318 The basis for the genetic diversity in DENV is its error-prone RNA polymerase58, such that mutations commonly occur during viral replication, and on which a combination of genetic drift and negative and/or positive natural selection is able to act. This high replication error results in DENV existing as a population of closely related variants within an individually infected host294,295 and this intra-host genetic diversity has been proposed to have implications for pathogenesis of DENV infection, variable disease outcomes, virus evolution, and host immunity.45

Several previous studies have confirmed that the population of DENV in humans and within individual Aedes mosquitoes contains measurable genetic variation.54,177,294,295 Levels of within-host genetic diversity have been previously shown to vary among patients. Reported levels of intra-host genetic diversity ranged from 0.21 to 1.67% for the E gene of DENV-3, with genome-defective DENVs observed in 3.9% - 5.8% of clones.294,295 Another similar study showed that the intra-host diversity for the C and NS2B genes ranged from 0.12 to 1.02% and 0.16 to 1.20%, respectively.285 Lin et al.177 showed that DENV exhibits substantial sequence diversity in humans and to a lesser extent in mosquitoes, with the major variant transmitted in both humans and mosquitoes. Intriguingly, Descloux et al. suggested that the level of intra-host genetic diversity was lower in patients suffering severe dengue disease – dengue hemorrhagic fever (DHF) and DDS – compared to those experiencing the milder dengue fever (DF), such that there is a direct link between viral genetic diversity and clinical outcome.54

All previous studies of intra-host DENV genetic diversity have utilized point measurements in which a limited set of clones (n = 10-50) containing short, amplified segments of the viral genome were sequenced. In most cases the proportion of mutations due to experimental (PCR/sequencing) error in these studies is uncertain, but likely an important contributor to the levels of diversity observed. As well as a limited sample of population diversity, it is unknown whether the extent of sequence variation changes during the course of infection, and the relationship between intra-host genetic variation and dengue severity is unclear. To address these issues we undertook an expansive study of intra-host DENV variation by sequencing a median of 155 high quality clones from serial plasma samples taken from 17 patients infected with DENV-1 and applying a rigorous quality control to exclude artifactual mutations. With these data we explored the relationship between intra-host genetic diversity and clinical outcome, focusing on the sequence encoding the domain III (DIII) of the envelope (E) gene. Importantly, DIII is involved in cell receptor binding and is the major target of virus neutralizing antibodies in humans18,145 and hence mutations within this region may have important functional consequences.

2. MATERIAL AND METHODS

2.1 STUDY POPULATION

Plasma samples from dengue patients included in the placebo arm of a clinical trial of chloroquine were used for this study.323 We selected seventeen patients for study based on the serotype of infection (i.e. DENV-1), serological response (i.e. primary or secondary), and disease severity (i.e. DF or DHF). Classification of disease severity was according to 1997 WHO classification criteria.307 For each patient, three sequential plasma samples, beginning with the enrolment plasma sample, were selected for analysis. Samples were selected to represent the breadth of viremia levels found in DENV-1 infected patients. Briefly, we selected three primary DF, seven secondary DF, and seven secondary DHF patients with a median age of 19, 19, and 20 years, respectively, with a male/female ratio of 1.3. The median day of illness at admission was 2.2 days (range: 0.6-2.8 days).
2.2 WHOLE GENOME (CONSENSUS) SEQUENCING OF DENV-1
Viral genomes in the enrolment samples were sequenced as part of the Broad Institute’s Genome Resources in Dengue project using a capillary sequencing directed amplification viral sequencing pipeline as previously reported.\textsuperscript{170} In short, isolated viral RNAs were reverse transcribed and then overlapping amplicons that span the complete genome were amplified using a high fidelity polymerase; resulting products were Sanger sequenced and resulting sequence coverage was \textasciitilde8-fold. Resulting sequence reads were assembled using the Broad Institute’s AV454 algorithm (Henn et al., in review). Consensus assemblies where used for alignment of clone reads as part of the variant calling process (see below)

2.3 RNA EXTRACTION, REAL-TIME PCR, CLONING AND SEQUENCING
Dengue viral RNA was isolated directly from plasma using the QIAamp viral RNA mini kit (Qiagen, Germany). RNA was reverse transcribed, and DENV-1 viremia levels were assessed using an internally controlled, serotype specific, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay that has been described elsewhere\textsuperscript{170}; results were expressed as cdNA equivalents per ml of serum.

Viral genomes in the enrolment samples were sequenced as part of the Broad Institute’s Genome Resources in Dengue project using a capillary sequencing directed amplification viral sequencing pipeline as previously reported.\textsuperscript{170} In short, isolated viral RNAs were reverse transcribed and then overlapping amplicons that span the complete genome were amplified using a high fidelity polymerase; resulting products were Sanger sequenced and resulting sequence coverage was \textasciitilde8-fold. Resulting sequence reads were assembled using the Broad Institute’s AV454 algorithm (Henn et al., in review). Consensus assemblies where used for alignment of clone reads as part of the variant calling process (see below).

The 462 nucleotide region encoding DIII of the E-gene was amplified using the primers: DIII-E P3: 5’- CAAGAGGACCACATTCC -3’ (corresponding to genome positions 1701 to 1720 of the DENV-1 reference strain (Hawaii, 1944)) and DIII-E P5: 5’- CCAAAGTCATCCGGTGTC - 3’ (positions 2182 to 2201). The PCR was performed with 5 µL 5X polymerase buffer (Roche), 1 µL 10 mM DIII-E P3 primer, 1 µL 10 mM DIII-E P5’ primer, 3.5 µL 25mM MgCl\textsubscript{2} (Roche), 1 µL 10 mM dNTPs (Invitrogen), 2.5 U Expand High Fidelity Plus\textsuperscript{TM} polymerase (Roche) and RNase-free water to a final volume of 25µl. The PCR conditions were 94°C for 2 min, followed by 45 cycles of 94°C 15 sec, 60°C 30 sec and 72°C 45 sec, and then 72°C 7 min. The resulting PCR product was visualized on a 1% agarose gel using ethidium bromide staining and UV light.

PCR amplimers were cloned into the T/A cloning vector, pCRII-TOPO, which was transformed into TOP10 competent cells (Invitrogen). Each transformation culture was plated out on Luria-Bertani (LB)/ampicillin/isopropylthiogalactoside (IPTG)/X-gal plates and grown overnight at 37°C. 382 white colonies (suggestive for amplicon insertion) were selected from each sample and sequenced using dye-terminator chemistry on an ABI 3730xl sequencer (Applied Biystems) from both ends to generate paired end reads and quality files.

2.4 VARIANT CALLING

2.4.1 Read Alignment and Merging.
Reads from each sample were aligned to the consensus genome sequence present in the enrolment plasma sample using the BLAST-Like Alignment Tool (BLAT) version 33.\textsuperscript{144} A custom script was used to merge overlapping forward and reverse reads, simultaneously assign appropriate base quality scores, and trim the resulting reads to the target amplicon sequence.

Overlapping forward and reverse reads were merged into a single contig and assigned quality scores. To control for poor alignment at the ends of reads, forward and reverse reads were required to have at least 5 bases aligning into the designed primer (i.e. DIII-E P3 and DIII-E P5) or were trimmed backwards 5 bases from the end of their alignment. The quality scores were assigned based on the agreement or disagreement of the bases between the forward and the reverse reads. The sum of quality scores were assigned for bases agreeing; bases disagreeing were assigned to the base with the highest quality score and quality score was assigned as the difference. Gaps were given quality equal to the lower quality of the adjacent base, or the lowest quality of any contiguous base of the same type (homopolymer adjustment); base(s) were discarded when the gap had higher quality than the inserted base(s) on the opposite strand and bases retained their quality scores if the quality of bases were higher. Indels of the same length in both reads were retained as real. Complex events (e.g. inserts relative to reference opposite deletions, or insertions or deletions of different length) were replaced with a number of Ns equal to the length of the consensus between the two flanking consistent alignments and quality score=0. When the overlapping region (of the forward or reverse) read did not extend to the designed primer, the merged read was extended to include whichever read had the largest number of aligning bases on that side of the overlap, and assigned the raw quality for those bases. In cases where both complement forward or reverse reads did not align, we trimmed the single read to the target amplicon region and retained it for variant calling.

2.4.2 Base variant calling.
To reduce false positive base variant calls we employed a Neighborhood Quality Standard (NQS) algorithm\textsuperscript{7} to filter bases used for variant calling. Bases not meeting a NQS condition over those regions were excluded, i.e. a base satisfies the NQS condition if the base has PHRED score ≥20, and the neighboring five bases on each side have PHRED scores ≥15. Two variant base data sets were generated for downstream analysis. In the first, highest quality, data set defined as VP base variants were called using the V-Phaser algorithm (Macalalad et al., in review). In short, V-Phaser applies an error probability model defined by a process read error rate, and refined by the inclusion of variant nucleotide phasing information, to define the frequency at which a nucleotide polymorphism needs to be observed to be a true variant given the observed sequence coverage. In general, for the data sets analyzed as part of this study variants were identified as real if they were observed on two or more reads. To explore how erroneous PCR and sequencing may have contributed to the observed levels of genetic diversity we generated a second variant data set, defined as 1HQ that included variants that
were seen only once (i.e. singletons). In both the 1HQ and VP data sets only high quality bases that passed NQS were used for base variant calling.

2.4.3 Variant Haplotype Calling.
For each aligned read (see Read Alignment and Merging) we computed a vector of valid base variant calls (see Base Variant Calling). The minimal set of such vectors required to explain all reads was collected using a custom haplotype calling algorithm. For each sample, the algorithm was seeded with a single read and then reads were assigned a haplotype. If a read matched unambiguously based on the variant positions to an existing haplotype group (in first iteration match is to seed read) it was assigned that haplotype, otherwise it was assigned as a new haplotype. This process was iterated until all reads were grouped into defined haplotypes defined by variant vectors. We assigned reads that have variant vectors with missing data (e.g. due to failure to align or presence of a call which is not considered valid) by a similar process. For reads that the partial vector maps unambiguously to a complete haplotype the missing information is “corrected” based on the complete vector; those reads that do not map unambiguously are assigned as “incomplete” haplotypes.

Nucleotide sequence accession numbers.
All nucleotide sequences generated here have been submitted to GenBank and assigned accession numbers 2262271431-2299350311 (Supplementary table S2).

2.5 EVOLUTIONARY ANALYSIS

2.5.1 Measurements of genetic variation.
Alignments of full length pseudo-reads (i.e. all valid variants) from the haplotypes were generated with the MUSCLE software (version 3.7)\(^1\), using default settings. Because the very low numbers of mutations observed, the mean pairwise genetic diversity within each sample was calculated from the uncorrected pairwise distance matrix (p-distance) between taxa and the population standard error (SE) was estimated with 1000 bootstrap replicates using the MEGA5 program.\(^2\) To estimate the mean numbers of synonymous (d\(_S\)) and nonsynonymous (d\(_N\)) substitutions per site (ratio d\(_N\)/d\(_S\)) in each sample we utilized the Jukes-Cantor substitution model within MEGAS5.\(^2\) Mutations detected within each sample were further characterized as to their frequency and presence in other samples and were mapped to inferred amino acid (aa) sequences.

2.5.2 Pattern of intra-host evolution.
The evolutionary relationships among the DENV-1 sequences from each sample were inferred through the construction of minimum spanning networks, utilizing the program TCS 1.21\(^4\) and following the algorithm of Templeton et al.\(^2\) Inferences from this method depend on the chosen probability of parsimony and we chose a value of 99% (i.e. a 99% connection limit). This number of mutational differences associated with the probability just before the (99%) cut-off is the maximum number of mutational connections between pairs of sequences. Networks that are unconnected at the 99% probability of parsimony were linked by decreasing the connection probability. The power of this approach is that it allows the population frequency of each mutation to be assessed, and parsimony-based approach is justified by the small number of total mutations observed.

2.5.3 Global DENV-1 phylogenetic inference.
To determine the frequency of mixed infections in our data sets, the sequences of each individual patient were aligned together with 1390 previously published DENV-1 E gene sequences (i.e. ‘background data set’), which combines subsets of genotype I (n=1111), II (n=91) and III (n=188). Phylogenetic trees for these data were then estimated using the maximum-likelihood (ML) method available in the RAxML package (version 7.0.4).\(^2\) In all cases we used GTR+G model of nucleotide substitution, as determined by ModelTest v3.7.\(^2\) The reliability of specific groupings on the trees was estimated using bootstrap with 1000 pseudoreplicates.

3. RESULTS

3.1 EXTENT AND PATTERN OF INTRA-HOST GENETIC VARIATION

The clinical, serological, and demographic features of the 17 DENV-1 infected patients that participated in this study are shown in supplementary Table 1. To determine the intra-host evolutionary dynamics of DENV-1 in these patients, we studied genetic diversity in 49 serial plasma samples collected during the course of their illness. Overall, we sequenced 8458 clones of the 463 nucleotide region encoding DIII of the E-gene derived from 49 serial plasma samples collected during the course of infection. In the VP data set, 8458 clones were assigned into complete haplotypes with a median of 155 (range: 4 - 362) clones analyzed at each time point (Table 2); these data excluded singleton mutations and included only high quality variant positions that were seen frequently enough at a given sequence coverage to be unlikely to occur as a result of error alone (i.e. typically observed at least twice). In the 1HQ data set which contains all variants observed including singletons that may be artefacts resulting from process errors, 8315 and 143 clones were assigned to complete and incomplete haplotypes, respectively. A median of 155 (range: 4 - 361) clones were analyzed at each time point (Table 3). In the VP data set which included only highly confident variant calls, but which may have excluded some bona fide mutations at low frequency, we identified a total of 281 nt mutations
across the 8458 clones of the 463 nt region (Table 2), corresponding to a mutational frequency of $7.2 \times 10^{-5}$ (95% confidence interval (CI): 6.4 - 8.1 $\times 10^{-5}$) mutations per nt site. Across all patients and time points, these mutations where observed at 43 residues (Supplementary Table S1). In all patients, the majority of sequences (65-100%, mean 97%) recovered were identical to the consensus. A measure of selection pressure could be calculated in 18 samples, with mean values of pairwise distance ranging from 0.00005 to 0.00130 (mean, 0.00034, Table 2). There was no significant difference in the mean pairwise distance between patients with DHF and DF (0.00030 vs. 0.00041, Table 2). To determine the selection pressure affecting DENV within each patient, we estimated the mean value of $d_n/d_s$ for each sample. Mean $d_n/d_s$ values varied between 0.13 and 1.9 with an average value of 0.23. Of the 8458 clones sequenced, 4 clones contained a total of 6 stop codons (0.05%) (Table 2). In sum, these stringently filtered data provided a conservative picture of the level of genetic diversity in these samples, but those that are very likely to be real biological variants and suggest that sequence diversities in viral populations may be very low.

### 3.2 INTRA-HOST PHYLOGENETIC RELATIONSHIPS

To infer the evolutionary history of mutations in each sample we inferred minimum spanning networks (Figure 1 and 2). In five patients (i.e. 49, 121, 154, 323 and 391), the viral population harbored only the consensus sequence. Six patients (i.e. 59, 82, 107, 336, 349 and 376) contained haplotypes that are multiple mutational steps ($\geq 2$) away from the consensus sequence, such that longer branches stem from the consensus sequence. In addition, two patients (82 and 162) harbored multiple phylogenetically distinct viral lineages (i.e. haplotypes) across multiple time points (figure 1A and 1B). A third patient (336) also supported multiple haplotypes when the parsimony probability was reduced to 97% (Figure 1E); in this network, hap 1 ($n = 2$) required seven additional mutational steps, which was suggestive for mixed infections.

### 3.3 EVIDENCE OF MIXED INFECTIONS

Notably, one sample (G2542, patient 336) contained two identical clones that differed seven nt (1.5%) from the consensus sequence, and hence far greater than that observed in the majority of other patients (mean = 0.1%). This prompted us to determine whether the high level of genetic diversity in patient 336 was due to mixed infections from different origins within the global diversity of DENV-1. Phylogenetic analyses of the alignment of all haplotypes of each patient with the ‘background data set’ (a global samples of DENV-1 E DIII sequences from GenBank) provided strong evidence for multiple infections, all involving genotype 1 viruses (Figure 3). Specifically, patient 336 harbored a mixed infection with viruses from clade 1 and 5 (clades as described by Raghwani et al.227).

### 3.4 ANALYSIS OF THE 1HQA DATA SET

As a comparison with the high quality but conservative VP data set, and to assess the likely extent of sequencing error, we performed an additional analysis of the 1HQA data set. Among the 8315 clones of the VP data set, 2936 nt mutations were observed, corresponding to a mutation frequency of $7.6 \times 10^{-4}$ (95% CI: 7.4-7.9 $\times 10^{-4}$) mutations per nt site (Table 3). A total of 1922 aa mutations were observed. The majority of clones ($n = 1434, 17.4\%$) harbored a single aa mutation while 2.8% carried multiple aa mutations. Mean estimates of pairwise genetic diversity varied from 0.00048 to 0.00360 (mean, 0.00164) and the mean values of $d_n/d_s$ ranged from 0 to 1.6 (mean, 0.58). These $d_n/d_s$ values are much higher than those seen between patients, which are normally <0.1, suggesting that intra-host variation is characterized by transient deleterious mutations or caused by the experimental procedure, which results in an elevation of $d_n/d_s$ values 124. In addition, 36 in-frame stop codons in 32 clones were identified.
in 17 of the 49 samples studied (Table 3). Genome-defective DENVs observed in 0.38% of clones. All mutations in the VP data set (n = 43) were also observed in sequential samples and/or across multiple patients in the 1HQ data set (supplementary Table S1, left). Many mutation positions (n = 625 of 845, 74%) were observed in sequential samples and/or across multiple patients in the 1HQ data set, but these mutations lacked statistical rigor to be called a valid variant in the VP data set.

4. DISCUSSION

The intra-host population genetic structure of DENV has previously been described as a population of closely related sequences.1,47,125,177,294,295 Our study, which comprises the largest series of samples and patients as well as stringent filtering of sequence quality, confirms these observations, but shows that the occurrence of mutations in the virus population are much lower than previously reported. The mean pairwise genetic diversity varied between 0.00048 to 0.00360 and 0.00005 to 0.00130 in the 1HQ and VP data sets, respectively, with no significant difference in the mean pairwise distance between patients with DHF and DF. The substantially higher sequence variation in our 1HQ data set resembled that described in previous reports.54,177,294,295 However, given that the 1HQ data set undoubtedly includes a significant number of artifactual mutations, the high sequence variation in this data set should
be regarded as the upper bound of DENV genetic diversity. As a consequence, it is likely that previous estimates of intra-host genetic diversity in DENV have been inflated by the erroneous inclusion of PCR and sequencing errors in the diversity calculations and hence should be treated with caution.

It is important to note that accurate estimations of intra-host sequence variability depend largely on the accuracy of the experimental procedure, particularly the fidelity of RT-PCR and sequencing. However, distinguishing bona fide from artifactual mutations is not a trivial exercise. Our rigorous approach to error correction relies on: (i) the alignment of clonal sequences to a reference sequence for haplotype calling, (ii) the identification of unambiguous mutations with high quality score of base(s) and (iii) whether mutations were seen once (1hQ data set) or frequently enough at a given sequence coverage to be unlikely to be from error (VP data set). Indeed, the 1hQ data set must harbor a high, but underestimated number of artifactual mutations which were likely introduced during reverse transcription, PCR amplification or sequencing. The process error rate (i.e. RT-PCR + cloning + Sanger sequencing) can be expected to be on the order of 2-8 x 10^-6/nt/cycle when a proofreading polymerase is used as reported by Malet et al.182 which corresponds to an expectation that ~0.036% of the observed mutations could be errors in our experimental system (% mutations in 1hQ data set 0.024-0.269%). Conversely, the VP data set undoubtedly represents biological variants, but may underestimate the true intra-host sequence variation, as the variant calling algorithm will call singletons as errors despite that some of these mutations possibly representing true biological variants. Notably, the probability of a mutation occurring independently at random across multiple sequential samples is very low, hence singleton variants observed in multiple samples may have a higher likelihood of being true biological variants than those observed in a single patient. Indeed, although RT, PCR and sequencing errors likely contribute to the majority of variants observed in the 1hQ data set, we were able to identify mutations that occurred in multiple patients and time points (supplementary Table S1), suggesting that they are biological variants even though they are at low frequency within individual patients and hence excluded from the VP data set.

Overall, our VP data set indicates that the DIII segment of the E gene in DENV-1 exhibits limited sequence variation during the course of infection. In addition, it is striking that in both the VP and H01 data sets, we found no clear evidence for adaptive evolution in the DIII region, either in the form of consistently high dS/dN ratios and/or mutations that exhibited a steady increase in frequency, even though it is thought to be the principle target for neutralizing antibodies.185,195 The lack of positive selection in this case is likely to be a function of the fact that dengue is a self-limiting infection in which innate, humoral and cellular immune mechanisms removes the virus population before evidence of positive selection can be detected.192,196,286

The relationship between viral genetic variation and disease severity has been well documented in human immunodeficiency virus 1 (HIV-1) and hepatitis C virus (HCV).196,286 For example, higher HIV-1 sequence diversity has been shown to be associated with slower disease progression.196 Similarly, disease progression in HCV infection was associated with measurable genetic evolution, while resolving hepatitis correlated with evolutionary stasis in the acute phase of HCV infection.196 Because our analysis considered a relatively large number of sequences per patients and and these patients likely harbored differences in immunological responses, we were able to look for associations between the intra-host diversity of DENV-1 and disease outcome, immune status or viremia. Notably, we observed no clear evolutionary patterns in relation to any of these variables. These results sit in contrast to results reported by Desclaux et al.54 who showed higher intra-host sequence variation in patients with DHF/ DSS than those with DF. The basis for the differences in results between our studies are unknown, but could be related to the methods used to filter sequence quality or to sample size. In addition, Desclaux et al. assessed a much smaller number of clones (662 clones from 16 sera samples at a single time point), increasing the chance of stochastic effects.

Finally, one of the most striking observations from this study was the presence within some patients of phylogenetically distinct lineages or subtypes of genotype 1 DENV-1, indicative of mixed infection. That these mixed infection events were also observed within the high quality VP data sets indicates that they are bona fide. This is the first time that intra-serotype mixed infection has been reported in DENV-1, and we likely greatly underestimate its true frequency as we are only able to infer the occurrence of mixed infection when it involves lineages that fall into topologically distinct places on phylogenetic trees (i.e. we cannot identify mixed infection among very closely related viral lineages). Intriguingly, a previous study of DENV-2 evolution also revealed the presence of mixed infection, such that individual patients harbored multiple phylogenetically distinct lineages.1 We therefore conclude that mixed infection is a potentially important contributor to intra-host virus genetic and phenotypic diversity, and provides the raw material for intra-serotype recombination.144,311 However, we were unable to determine whether these mixed infections represent the simultaneous infection (i.e. co-infection) or superinfection of multiple viral lineages in humans. This is clearly an area that requires additional study.

5. ACKNOWLEDGEMENT
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### Table 1. Summary characteristics of the seventeen patients with DENV-1 infections.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Disease outcome</th>
<th>Immune status (Pri/Sec)</th>
<th>Clade (within genotype 1)*</th>
<th>Age</th>
<th>Sex</th>
<th>GenBank accession number</th>
<th>DENV-1 RNA (copies/mL)</th>
<th>Day of illness</th>
<th>Broad ID</th>
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<td>3</td>
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* Clades of genotype 1 as designated by Raghvani, et al. DF, dengue fever; DHF I and II, dengue hemorrhagic fever grade 1 and 2; pri, primary dengue; sec, secondary dengue; M, male; F, female
Table 2. Analysis of intra-host variation in DENV sequences in sequential samples from 17 patients in the VP dataset.

<table>
<thead>
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<th>Study ID</th>
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<th>% of nt mutations</th>
<th>Mean pairwise distance</th>
<th>SE</th>
<th>Global $d_{n}/d_{s}$</th>
<th>Haplotypes (n)</th>
<th>Total number of aa mutations</th>
<th>no. of stop codons</th>
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**Table 3.** Analysis of intra-host variation in DENV sequences in sequential samples from 17 patients in the HQ dataset.
Supplementary table S1. Intra-host DENV-1 nucleotide (nt) mutation positions present in sequential samples and across multiple patients in the 1HQ data set (left) and all valid mutation positions present in the VP data set (right). Numbers across the top are patient and sample numbers. (http://jvi.asm.org/content/suppl/2011/12/16/86.2.835.DC1/TableS1.xls)

Supplementary table S2. List of nucleotide sequence accession numbers with its corresponding sample numbers. (http://jvi.asm.org/content/suppl/2011/12/16/86.2.835.DC1/TableS2.xls)
Discussion

Fisher’s boats in lien huong, Binh thuan
Dengue is the most common vector-borne viral disease worldwide, and is ranked among the most important infectious diseases by the World Health Organization (WHO). Also in Vietnam, dengue poses a major challenge to public health. The studies presented in this thesis addressed the epidemiology and disease transmission of dengue (chapter 2-8), and the clinical and virological pathogenesis (chapter 9-11).

**THE PROBLEM OF DENGUE**

Humans are the primary host of dengue virus (DENV) and transmission of DENV takes place through the bite of the principal mosquito vector *Aedes aegypti*. Dengue is difficult to grasp as a clinical entity. It usually presents as a highly unspecific illness and is hardly recognized as a clinical entity by primary health care physicians. The large burden of dengue (chapter 2, 3, 4), the relatively low complication rate (chapter 6 and 9) and the difficulties in predicting severe disease make it almost impossible for the primary health services in developing countries to tailor the care to those who need it, while giving care to all is unaffordable. Inabilities to make an early diagnosis and to identify those who are at highest risk to progress to severe complications in the early phase have impeded novel treatment interventions. Advancement in understanding of dengue pathogenesis (e.g. antibody dependent enhancement, differences of serotypes or genotypes, viral load, antigenemia and cytokine profile) and perhaps the identification of new prognostic markers (biomarkers, host genetics) may improve case management and treatment intervention strategies. For the near future, health care providers will have to rely on predictive algorithms which include a combination of clinical symptoms, signs, and laboratory parameters such as haematological profile, viremia, NS1 antigenemia to identify severe dengue.

Disease control is mainly based on tackling the vector and elimination of environmental risk factors. Vector control strategies include environmental changes (improved water supply, emptying or covering tanks, and underground reservoirs), personal protection (protective clothing, mats, and nets), biological control (fish, bacteria) and chemical control (insecticide). However, these tools have not been implemented vigorously and are unsatisfactory for reduction of transmission. More systematic insight is needed in the strengths and weaknesses, opportunities and threats of the implementation of the dengue control program at provincial, district and community level is needed. Although some environmental risk factors (i.e. use of a pit latrine and littering with discarded cans and having pigs on domestic setting) were identified to be associated with dengue antibody prevalence in children in Binh Thuan province and described in chapter 3, disease control by means of sanitary measures may be extremely difficult, and it may even increase the risk of major epidemics by decreasing basic reproduction number ($R_0$).

**DENGE EPIDEMIOLOGY, BURDEN OF DISEASE AND TRANSMISSION**

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. Dengue fever can easily be confused with other infectious diseases, especially in the early phase of disease. Communicable diseases, including dengue constitute a substantial part of the health problems in Vietnam. Dengue among AUF was a very common disease, with major impact on public health in Binh Thuan province, affecting mainly children and young adolescents (chapter 2 and 9). The majority of patients with (uncomplicated) dengue were not recognised as such, which lead to substantial under-reporting of dengue in the health information system, as reporting is usually based on clinical signs and symptoms. Patients with severe symptomatic dengue are routinely reported to the Provincial Center for Preventive Medicine, mostly without laboratory confirmation. Because routine notification data grossly underestimated the true incidence of dengue, chapter 3 and 4 described two methods to estimate the sero-conversion rate, which corresponds to the true incidence rate of first infections. The incidence of first infections is a good indicator of the real infection pressure in the community. Similar to other dengue endemic areas of Southeast Asia, dengue is mainly a childhood disease and children experience an annual exposure risk (incidence or $R_0$) of ~10%. The relatively high estimates of $R_0$ and the gradual increase in sero-prevalence with age, suggest that dengue in in Binh Thuan province is already hyper-endemic.

**LOCALLY ACQUIRED DENGUE**

Despite the wealth of knowledge on many aspects of the biology of dengue, patterns of dengue transmission at the village (chapter 5) and provincial level (chapter 7) remains scarce. It is known that children and young adolescent are most affected (also chapter 2, 3, 4, 6 and 9), but it is uncertain if these infections occur at home, at school, or elsewhere. In chapter 5, we attempt to address this issue by demonstrating that new DENV infections occurred near places where sero-prevalence was highest at baseline. This point at important spatial heterogeneity in the transmission of dengue. Undiagnosed asymptomatic DENV infections or unrecognized dengue patients with mild symptoms may be more important for the spread of DENV than symptomatic and notified cases. Undetected persistence of local transmission may have implications for future deployment of prevention strategies.

Furthermore, movement of dengue infected humans is thought to play one of the most important role in dengue transmission, since the *Aedes* vector has a restricted flight range (<150 m). Movement and transport of humans or vectors at a national, regional or international scale creates new opportunities for vectors to establish in permissive environments, and for virus to be transmitted where competent vectors exist. Inadequate or interrupted vector control activities, determined by economic and political priorities will directly affect individuals’ risk of exposure to infection in endemic settings. These social,
demographic and economic drivers that are thought to be responsible for much of dengue’s expansion and intensified transmission over recent decades, are not easily to overcome. Among all the barriers that obstruct reduction of transmission, an underestimated issue is the socio-cultural context of dengue. Risk perceptions among people remain unclear and people are reluctant to change behaviour. Since dengue transmission is such a multifactorial problem, multifaceted research approaches are probably the most effective. Participatory research could gain insight in the missing link between human risk perceptions, attitude and behaviour and the poor results of dengue transmission reduction.

TRANSMISSION DYNAMICS

Dengue transmission in endemic settings is characterized by nonlinear dynamics over time, with strong seasonality, multi-annual oscillations and irregular temporal fluctuations in incidence. Besides the seasonality of dengue transmission, periodic epidemics and more irregular intervals of outbreaks are commonly observed. Multiple factors may influence the dynamics of dengue including environmental and climate factors, host-vector interactions and the population-wide immune landscape (reviewed in chapter 8). Climate variability is postulated to be an important determinant of dengue epidemics. Wavelet analysis has been demonstrated to be suitable for investigating time series data from non-stationary systems and for inferring associations within such systems. In chapter 7, we provide insights into the persistence and spatial spread of dengue throughout Binh Thuan province, southern Vietnam. We found that the multi-annual wave of dengue infection was moving towards Phan Thiet district and might originate from another, but nearby epicenter. Whether the multi-annual periodicity of dengue incidence exists or not, the detection of periodic cycles for dengue incidence itself provides no insights into the processes that cause dengue incidence oscillations (e.g. environmental and climate factors, host-vector interactions and population herd immunity). Further extensive collection of and analyses on surveillance data from other provinces in southern Vietnam are pending to test this hypothesis.

HOST DETERMINANT OF CLINICAL OUTCOMES

Two key risk factors for both symptomatic and severe dengue disease are 1) the age at infection and 2) the acquisition of secondary infections. A recent study in Brazil investigating the relationship between age at primary infection and the risk of febrile illness, suggested that adults are more likely than children to have clinical dengue. Although age at infection and infection parity are the representative key modulators of clinical dengue and disease severity, their relationship was established and explicitly quantified in chapter 6, by analysing epidemiological data sets in southern Vietnam. For both primary and secondary infections, higher age at DENV infection was shown to result in higher risk of clinical attack. Age as an important modulator of clinical dengue explains the recent increases in dengue notifications rates in ageing countries in Southeast Asia, and moreover, poses a paradoxical problem of an increase in the incidence among adult patients resulting from a decline in the force of infection. It should be noted that only a small proportion of patients with secondary DENV infection (and an even smaller proportion with primary infection) develop severe dengue. Our current knowledge on the relationship between age and symptomatic dengue merits additional studies on the clarification on the population impact of age-specific risks of clinical attack on the total number of severe forms of dengue.

PLASMACYTOSIS IN DENGUE

One of the characteristic features of dengue is the occurrence of leucopenia and thrombocytopenia, probably resulting from virus induced bone marrow suppression. Despite the general bone marrow suppression, blood plasmacytosis has been reported in a few patients with DENV infection. Blood plasmacytosis is an unusual haematological finding that is most commonly seen in plasma cell leukaemia or advanced stage multiple myeloma, in which case the plasma cells are part of the malignant clone and thus are monoclonal. Non-malignant reactive peripheral plasmacytosis is occasionally found in a variety of diseases. Polyclonal peripheral blood plasmacytosis has occasionally been described in dengue virus (DENV) infected patients. In chapter 10, we showed that blood plasmacytosis is a common event in DENV infection, which is characterized by a transient presence of polyclonal PCs in the circulation. In this specific context, an associated plasmacytosis will be seen more frequently in travelers with dengue and in dengue endemic areas. In resource poor settings, peripheral blood smears (performed for malaria diagnosis or other conditions) may demonstrate extreme blood smears. Peripheral plasmacytosis will resolve quickly (< 14 days), if dengue is the underlying illness. It should be emphasized that recognizing this self-limited phenomenon could obviates extensive clinical evaluation.

Indeed, the mechanism, contribution and possible role of peripheral plasmacytosis in dengue pathogenesis remain unclear and deserve future investigation. Important questions are whether active DENV replication takes place in peripheral PCs and whether PCs are redistributed and released from the bone marrow into the circulation or whether they represent an increased production and if this is virus or cytokine driven?

VIRAL DIVERSITY IN DISEASE SEVERITY

The basis for the genetic diversity in DENV is its error-prone RNA polymerase, such that mutations commonly occur during viral replication. DENV therefore exists as a population of closely related sequences and this degree of intra-host genetic diversity has been proposed to have implications for pathogenesis of DENV infection, disease outcome, virus evolution, and host immunity.
In contrast to some previous studies we observed no relationship between the extent and pattern of DENV-1 genetic diversity and disease severity, immune status, or level of viremia (chapter 11). We also showed that nucleotide sequence diversities of viral populations were very low. Despite such sequence conservation, we observed clear evidence for mixed infection, with the presence of multiple phylogenetically distinct lineages present within the same host and we likely greatly underestimate its true frequency as we cannot identify mixed infection among very closely related viral lineages. Mixed infection is a potentially important contributor to intra-host virus genetic and phenotypic diversity, and provides the raw material for intra-serotype recombination.\textsuperscript{144,311} However, we were unable to determine whether these mixed infections represent the simultaneous infection (i.e. co-infection) or superinfection of multiple viral lineages in humans. This is clearly an area that requires additional study.

**FUTURE PERSPECTIVE**

Current research priorities for dengue are to improve case-management and prevent progress to severe disease and mortality, to enhance understanding of dengue pathogenesis and to reduce dengue virus transmission and to conduct policy research that contributes to an adequate public health response. This thesis contribute to advancing the understanding of different aspects of the burden of disease, its epidemiology and disease dissemination/transmission and described clinical observational studies for a better understanding of dengue pathogenesis. However, many questions remain unanswered and merit further investigation. Table 1 summarizes questions derived from this thesis for future research.

**CONCLUSION**

Dengue remains an enormous public health threat globally and in Vietnam which will remain a major public health issue for the coming decades. The disease burden poses major pressure on health care services and has social and economic implications. Development of public health intervention tools are needed and may require community participation for containment of dengue transmission. Knowledge of the virulence of the virus including sequential infection, host factors, host immune response on dengue pathogenesis may promote treatment and intervention strategies.

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**Table 1. Research questions derived from this thesis.**

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<td>How and to what extent do asymptomatic infected individuals and patients with mild dengue contribute to transmission patterns? How can asymptomatic infected individuals or patients with mild dengue be identified?</td>
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<td>What is the age-specific proportion of severe dengue patients among the proportion with symptomatic dengue?</td>
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<td>What are the dengue transmission dynamics in southern Vietnam at district and provincial level?</td>
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<td>Does the multi-annual transmission cycle eminates from Ho Chi Minh City? What is the influence of Thailand and Cambodia on dengue transmission in southern Vietnam? What is the influence of local climate on transmission dynamics?</td>
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<td>What is the contribution of virus virulence (serotype, genotype or lineages) to disease severity? What is there impact on age at infection?</td>
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<td>Does DENV replication takes place in peripheral PCs? Are PCs redistributed and released from the bone marrow into the circulation or increased production? Is this phenomenon virus or cytokine driven?</td>
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<td>What is the extent of genetic diversity of DENV in the vector (A. aegypti and A albopictus)? What are the statistical methods to determine whether a mixed infection of multiple viral lineages represent co-infection or superinfection?</td>
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</table>

**CONCLUSION**

Dengue remains an enormous public health threat globally and in Vietnam which will remain a major public health issue for the coming decades. The disease burden poses major pressure on health care services and has social and economic implications. Development of public health intervention tools are needed and may require community participation for containment of dengue transmission. Knowledge of the virulence of the virus including sequential infection, host factors, host immune response on dengue pathogenesis may promote treatment and intervention strategies.
Dengue incidence and its geographical distribution have increased dramatically in the past six decades. Chapter 1 is an introduction to the current knowledge of dengue epidemiology and describes the pathogenic concepts of dengue disease. It is estimated that, globally, 1 out of 100 people are infected each year. Dengue is not one disease entity, it is a spectrum of diseases caused by four serotypes of Dengue virus (DENV). It is the most prevalent arthropod-borne virus affecting humans in the tropics and subtropics.

Dengue fever (DF)/dengue haemorrhagic fever (DHF) is a growing public health problem in Vietnam. Chapters 2-8 elaborate the disease burden of dengue, its epidemiology including its transmission, in southern Vietnam. In chapter 2, we quantify the dengue-attributable disease burden in Binh Thuan province. Based on surveillance data of one year, we showed that 12.6% of all patients who consulted a primary health facility due to fever. Serological studies showed that dengue contributed to approximately one quarter of all presented acute undifferentiated fevers (AUf). In contrast, the number of notified dengue cases was far less than 1% of the annual dengue incidence. This substantial under-reporting of dengue in the health information systems in Binh Thuan province, is due to reporting that is based on symptomatology of dengue and this is highly non-specific.

We determined age specific prevalence of dengue and associated risk factors, by conducting a sero-survey at two primary schools and combined that with a household survey. In chapter 3 we show antibody prevalence is 65.7% which increase with age. Using the age dependant sero-prevalence we estimated the annual incidence with binary regression at 11.7%.

The prevalence of dengue IgG antibodies was significantly higher in children who (i) confirmed using a pit latrine, (ii) whose domestic environment was littered with discarded cans and (iii) who had pigs. In chapter 4, we further investigated the incidence of dengue by sero-conversion in order to validate the findings in chapter 3 and evaluate the possibility of cross-reactivity against other flaviviruses. We followed a cohort of dengue sero-negative children for 23 months. Sero-conversion was observed in 66 children which corresponds with an annual incidence of 17.3%. Cross-reactivity with Japanese Encephalitis virus (JEV) specific IgG antibodies was considered in 4.6%. Data highlight the high incidence of DENV infection among Vietnamese children and JEV infections are rare. We concluded that the annual incidence of dengue can be estimated with a single cross-sectional sero-prevalence survey in children. In Binh Thuan DENV transmission is stable with a constantly high annual incidence of first infections that occur mainly peri-domestic. The data acquired in chapter 3 and 4 were well suited to identify incident infections and to relate these to prevalence at baseline and thus assess geographical heterogeneity, i.e. clustering, in dengue transmission (chapter 5). Geographical heterogeneity of dengue transmission was explored using a permutation null distribution test. This showed for the first time that clustering of DENV transmission occurs at household level among asymptomatic children. Risk areas could be identified by sero-prevalence surveys combined with mapping. Control of dengue virus transmission could be supported by identification and control of hotspots.

To unravel the contribution of age at DENV infections, we estimated the conditional probability of clinical dengue attacks given primary and secondary infections in chapter 6. We developed a compartmental model and applied it to the observed age-specific frequency of clinical attacks of dengue. We used (i) age-specific sero-prevalence (chapter 3) and (ii) age-specific frequency of clinical attack of dengue during primary and secondary infections as determined by serological confirmation (chapter 9). We showed that the probability of a clinical attack increases as a function of age for both primary and secondary infections. We showed empirically that the probability of clinical dengue during secondary infection is higher than during primary infection. In Chapter 7, we studied dengue dynamics at population level by performing wavelet analyses on time series of monthly notified dengue cases. A continuous annual mode of oscillation was found with a non-stationary 2–3-year multi-annual cycle. Phase differences suggested that the seasonal wave of infection was either synchronous with all districts or moving away from Phan Thiet district, while the multi-annual wave of infection was moving towards Phan Thiet district. We also found a strong non-stationary association between ENSO indices and climate variables with dengue incidence. Climatic factors should be considered within the context of the socio-demographic, economic and immunological determinants that contribute to the spread of dengue. These factors are reviewed in the last chapter of the first part, Chapter 8.

Part 2 of this thesis describes clinical observations that expand our knowledge on clinical features of dengue in both patients from endemic settings and travellers. In chapter 9, we explore clinical and virological characteristics and described the epidemiology of dengue using clinical data and acute and convalescent sera from patients who presented with AUF in Binh Thuan province. We used available data from a prospective observational study which was conducted from 2001 to 2006 and detected DENV in 25% of serologically confirmed dengue cases. The predominant dengue serotype varied by year with seasonal fluctuation: DENV-1 and DENV-2 from 2003-2006, DENV-4 in 2001-2002. Primary dengue was more common in children. Higher virus blood concentrations levels were found in primary infections compared to secondary infections. Clinical symptoms were often seen in adults. Few differences in clinical symptoms were found between primary and secondary infections and no significant differences in clinical symptoms between the serotypes were observed.

We also studied a hitherto neglected sign of dengue, plasmocytosis. We prospectively quantified and described the kinetics and phenotype of peripheral blood plasma cells (PCs)
in DENV infected travellers presenting at the Academic Medical Center in Amsterdam, the Netherlands in chapter 10. Blood plasmacytosis, characterized by a transient presence of polyclonal PCs in the circulation, is a common event in DENV infection. Blood plasmacytosis was observed in 64-73% of patients, depending on the duration of illness at presentation. Blood plasmacytosis was higher in secondary DENV infection and 89% had a shared immunophenotype (CD45+/CD19-/CD56-). The mechanism for the plasmacytosis is yet unresolved. In chapter 11, we cloned and sequenced the main antigenic determinants of DENV-1 in serial samples from patients with primary, secondary DF and/or DHF to explore the intra-host diversity and intra-host evolution of DENV-1. Result showed that the majority of detected mutations were transient, where the consensus sequences remain invariant and only a minority of mutations persists during the course of infection. We also found evidence of mixed genotypic infection in several patients. No clear relationship between dengue disease severity, immune status, viremia and the extent of DENV-1 sequence diversity or evolutionary patterns was found.

SAMENVATTING IN HET NEDERLANDS EN VIETNAMEES

1. NEDERLANDSE SAMENVATTING

Dengue (knokkelkoorts) incidentie en verspreidingsgebied is sterk toegenomen in de laatste zes decennia. Hoofdstuk 1 is een introductie over de huidige kennis van dengue epidemiologie en transmissie en beschrijft de huidige opvattingen over de pathogenese van dengue. Er wordt geschat dat, wereldwijd, 1 op de 100 mensen jaarlijks geïnfecteerd wordt met Dengue Virus (DENV). Dengue heeft een brede klinische presentatie en wordt veroorzaakt door vier serotypen. Het is een veel voorkomende virusinfectie die door een vector wordt overgedragen op mensen in tropische en subtropische landen. Tot op heden is de pathogenese niet volledig bekend; waarschijnlijk is het multifactorieel.

Dengue koorts (DF)/dengue hemoragische koorts is een groeiend probleem voor de volksgezondheid in Vietnam. Hoofdstukken 2-8 bespreken uitvoerig de dengue ziektelast, de epidemiologie inclusief dengue transmissie in zuid Vietnam. In hoofdstuk 2, kwantificeren we de dengue ziektelast in Binh Thuan, door resultaten te presenteren als een piramide. Op basis van surveillantie data laten we zien dat 12.6% van alle patiënten die medische hulp zoeken bij eerstelijnsgezondheidsvoorzieningen dat deden vanwege koorts. Dengue is de veroorzaak van een kwart van alle ongediagnosticeerde koortsen. De aangiftecijfers van dengue zijn echter heel anders. Minder dan 1% van de jaarlijkse incidentie van dengue wordt gemeld. Deze substantiële onderrapportage van dengue in het gezondheidsinformatiesysteem in Binh Thuan wordt veroorzaakt doordat patiënten worden gerapporteerd op basis van klinische verschijnselen; deze blijken erg aspecifiek te zijn.

Ook onderzochten we de leeftijd specifieke dengue sero-prevalentie en gerelateerde risicofactoren. We deden daarvoor een serologie survey op twee basisscholen en vulden dat aan met een survey onder huishoudens te verrichten Dit wordt beschreven in hoofdstuk 3. We toonden hierbij aan dat de prevalentie van aantoonbare antilichamen 65.7% was en dat deze toenam met de leeftijd. Met een binair regressie model hebben we de incidentie van dengue berekend op 11.7%. De prevalentie van dengue IgG antilichamen was significant hoger in kinderen die (i) thuis gebruik maakten van een pit latrine, (ii) woonden op een erf bezaaid met plastic zwerfafval (iii) en die varkens op het erf hadden. In hoofdstuk 4 beschrijven we hoe we de dengue incidentie op een andere manier hebben gemeten, gebruik makend van sero-conversie data. We keken naar de mogelijkheid van kruisreactiviteit tegen andere flavivirussen en we vergeleken de bevindingen met die in hoofdstuk 3. We vervolgen dengue seronegatieve kinderen over een periode van 23 maanden. Sero-conversie werd opgemerkt in 66 kinderen, wat overeen komt met een jaarlijks incidentie van 17.3%. Kruisreactiviteit met Japanese Encefałitis virus (JEV) specifieke IgG antilichamen werd gevonden bij 4.6% van de kinderen.
De resultaten bevestigen de hoge incidentie van DENV infectie in Vietnamese kinderen; JEV infecties zijn zeldzaam. We concluderen dat de jaarlijkse incidentie van dengue berekend kan worden met een dwarsdoorsnede sero-prevalentie survey. Dengue transmissie in Binh Thuan is stabiel met een constante hoge jaarlijkse incidentie en infecties worden opgelopen in en rond woningen. De data die zijn beschreven in hoofdstukken 3 en 4 bleken geschikt om het optreden van dengue in kinderen in verband te brengen met de sero-prevalentie ter plekke. Daarmee konden we geografische heterogeniteit beoordelen met de vraag of DENV transmissie zich concentratie in bepaalde huishoudens. (hoofdstuk 5). Geografische heterogeniteit van dengue transmissie werd onderzocht met een permutatie-nul-distributietest. Dit toonde voor het eerst aan dat dengue virus transmissie in asymptomatische kinderen zich concentreert op het niveau van de woning. In het vervolg kunnen risicogebieden dus worden geïdentificeerd met een sero-prevalentie survey gecombineerd met een geografische indeling. De bestrijding van DENV transmissie kan hiermee worden verrijkt met de identificatie en controle van hotspots.

Om de bijdrage van leeftijd op de presentatie van DENV infectie te onderzoeken berekenden we de conditionele waarschijnlijkheid van klinische dengue bij primaire en secundaire infectie. Dit wordt beschreven in hoofdstuk 6. We ontwikkelden een compartiment model voor de geobserveerde leeftijdspecifieke frequentie van symptomatic dengue. We gebruikten daarvoor (i) de leeftijdspecifieke sero-prevalentie (hoofdstuk 3) en (ii) de leeftijdspecifieke frequentie van klinische dengue bij primaire en secundaire DENV infecties (hoofdstuk 10). Dit toonde aan dat de waarschijnlijkheid op symptomatic dengue toeneemt als een functie van leeftijd voor zowel primaire en secundaire DENV infecties. We hebben empirisch aangetoond dat de kans op symptomatic dengue hoger is bij secundaire infecties dan bij primaire infecties. In hoofdstuk 7, beschrijven we de dynamiek van dengue transmissie op populatie niveau. We hebben “wavelet” analyse uitgevoerd op tijd series van maandelijks aangegeven gevallen van dengue in Binh Thuan provincie, zuid Vietnam. We vonden een jaarlijkse oscillatie zien met regulaire meerjarige cycli van 2-3-jaar. Analyse van faseverschillen toonde aan dat de jaarlijkse golf van infecties synchroniseerde met alle districten en vanuit Phan Thiet district bewoog en dat de meerjarige golf van infecties naar Phan Thiet district toe bewoog. We vonden ook een zwak verband tussen incidentie en ENSO data en lokale weersomstandigheden. Deze klimatologische factoren moeten worden beschouwd in de context van sociaaldemographische, economische en immunologische determinanten die allemaal bijdragen aan de verspreiding van dengue. Deze factoren werden besproken in het laatste hoofdstuk van het eerste deel, hoofdstuk 8.

Vervolgens werd een kenmerk van dengue bestudeerd dat tot dan weinig aandacht kreeg: plasmocytose. The kinetiek en fenotypering van perifere bloed plasmacellen (PCs) bij DENV patiënten werden beschreven in hoofdstuk 10. Bloed plasmacytose, gekarakteriseerd door een voorbijgaande stijging van het aantal circulerende polyklonale PCs, bleek frequent voor te komen bij DENV infecties, bij 64-73% van de patiënten, afhankelijk van de ziekteduur bij eerste presentatie. Bloed plasmacytose was hoger in secundaire DENV infecties dan in primaire infecties en 89% van de cellen hadden een gemeenschappelijk immunofenotype (CD45+/CD19-/CD56-). In hoofdstuk 11, beschrijven we gedetailleerd de diversiteit van DENV-1 genoomsequenties. We vonden dat de meerderheid van mutaties kortstondig aanwezig was, waarbij de consensus-sequentie onveranderd bleef; een minderheid van de mutaties in het genoomsequentie bleef aanwezig gedurende de gehele acute fase van dengue (<72 uur) en we tonen klaarblijkelijk gemengde genotype infecties aan. Er werd geen duidelijk verband gevonden tussen de ernst van de ziekte de immunologische status, de hoogte van de viremie en de mate van DENV-1 sequentie diversiteit of virus-evolutie-patroon.
2. VIETNAMESE SAMENVATTING

Trong sâu thẳm niềm vui qua, đã có sự gia tăng đáng kể về tỷ lệ nhiễm DENV và phân bố địa lý của nhiễm DENV. **Chương 1** giới thiệu những hiểu biết hiện nay về dịch tễ học và môi trường bệnh sinh của DENV. Hàng năm, trên toàn thế giới ước tính có 100 người có 1 người bị nhiễm DENV. Sốt dengue không phải đơn thư lãng một bệnh mà là biểu hiện của nhiều dịch cảm giác gây ra do bệnh chủng khác nhau của DENV. DENV là virus phổ biến nhất của nhóm arbovirus gây bệnh trên người ở vùng nhiệt đới và cận nhiệt đới.

Sốt dengue/sốt xuất huyết dengue là một vấn đề sức khỏe cộng đồng ngày càng quan tâm ở Việt Nam. Từ **Chương 2 đến Chương 8** phần tich chia tiệt giảm nặng bệnh tật gây ra do DENV, dịch tễ học, bao gồm sự lan truyền bệnh ở các tình phân nam Việt Nam. Trong **Chương 2**, chúng tôi tính toán mức độ ảnh hưởng của DENV ở tỉnh Bình Thuận. Theo số liệu khảo sát trong một năm, 12.6% bệnh nhân đến khám tại các cơ sở y tế là do sốt. Nghiên cứu xuất huyết thanh cơ thể thấy DENV góp phần vào gần một phần tư nguyên nhân của các ca bệnh sốt cấp tính chủ yếu do nhiễm nguyễn nhân (acute undifferentiated fevers). Mặc dù vậy, số ca bệnh sốt xuất huyết được báo cáo chỉ khoảng 1% trong số ca nhiễm hàng năm. Sự chênh lệch dễ dàng thấy trong hệ thống thông tin y tế ở Bình Thuận là do các ca sốt xuất huyết chỉ được báo cáo khi có triệu chứng lâm sàng diện hình và điều này là rất không dễ hiểu.

Tần số nhiễm DENV theo tuổi và các yếu tố nguy cơ được xác định qua khảo sát xuất huyết thanh cơ thể tại hai trường tiểu học, phổ biến với khả năng có ít các hệ gây. **Chương 3**, chúng tôi báo cáo tỷ lệ mắc bệnh có khoảng 6.7% và gia tăng theo tuổi. Sử dụng phân tích hồi quy phân tích, tỷ lệ nhiễm DENV hơn tăng, đặc biệt ở tuổi 11.7%. Sự khác biệt này có thể do ở mức độ nhiễm bệnh của các bộ phận của bệnh nhân. **Chương 4**, tỷ lệ nhiễm DENV qua khảo sát biến đổi tỷ lệ xuất huyết thanh cơ thể và các yếu tố nguy cơ được xác định qua khảo sát xuất huyết thanh cơ thể tại hai trường tiểu học, phổ biến với khả năng có ít các hệ gây. **Chương 5**, chúng tôi tính toán tỷ lệ mắc bệnh của DENV trong một năm, 12.6% bệnh nhân đến khám tại các cơ sở y tế là do sốt. Nghiên cứu xuất huyết thanh cơ thể thấy DENV góp phần vào gần một phần tư nguyên nhân của các ca bệnh sốt cấp tính chủ yếu do nhiễm nguyễn nhân (acute undifferentiated fevers). Mặc dù vậy, số ca bệnh sốt xuất huyết được báo cáo chỉ khoảng 1% trong số ca nhiễm hàng năm. Sự chênh lệch dễ dàng thấy trong hệ thống thông tin y tế ở Bình Thuận là do các ca sốt xuất huyết chỉ được báo cáo khi có triệu chứng lâm sàng diện hình và điều này là rất không dễ hiểu.

Những hiểu biết hiện nay về dịch tễ học và mô tả cơ chế bệnh sinh của DENV. Từ **Chương 6**, cắt ngang tỷ lệ DENV hàng năm cao ở những ca bệnh đầu tiên xuất hiện trong các khu dân cư (pre-domestic). Số liệu thu được ở **Chương 3** và chương 4 phù hợp để xác định tỷ lệ nhiễm DENV và tính toán số liệu với phần số báo cáo, từ đó đánh giá sự khác nhau về phần bò địa lý, vì dự sự phần nhân, trong quá trình truyền bệnh (chương 5). Sự khác nhau về phần bò địa lý của quá trình lan truyền DENV được xác định bằng kiểm định hoan toàn về phân bò (a permutation null distribution test). Kết quả cho thấy, lần đầu tiên phân nhóm của quá trình truyền bệnh do DENV xảy ra ở mức độ cộng đồng trong số các trường nhiễm DENV không có triệu chứng. Khu vực nguy cơ có thể xác định bằng các khảo sát xuất huyết thanh cơ thể phù hợp với phương pháp phân tích vi. Kiểm soát lan truyền bệnh có thể được hỗ trợ thông qua việc xác định và kiểm soát các điểm nóng của bệnh.

**Chương 6**, để làm sáng tỏ sự số đông góp của lựa chọn đối với nhiễm DENV, chúng tôi tính toán khả năng có điều kiện của bệnh có ở mức độ lâm sàng qua lãnh nhiễm đầu tiên (sơ nhiễm) và tái nhiễm. Chúng tôi đã xây dựng mô hình phân bố và sử dụng mô hình này để nghiên cứu sự ảnh hưởng của mức độ lâm sàng theo tuổi. Thống qua phương pháp xuất huyết thanh cơ thể, sử dụng (i) tỷ lệ lâm sàng nhiễm DENV theo tuổi (chương 3) và (ii) sự ảnh hưởng của DENV ở mức độ lâm sàng theo tuổi trong quá trình nhiễm bệnh (chương 9). Kết quả cho thấy khả năng ảnh hưởng của mức độ lâm sàng gia tăng theo tuổi đối với cả cơ sở, nhiễm, cũng như tái nhiễm DENV và biểu hiện lâm sàng trong lần tái nhiễm là cao hơn trong lần nhiễm đầu. **Chương 7**, chúng tôi nghiên cứu sự biến đổi của bệnh ở trong dân chúng thông qua các phân tích theo thời gian các ca bệnh được ghi nhận hàng tháng. Kiểu biến đổi có tính liên tục hàng năm được tìm thấy là dao động theo chu kỳ từ 2 – 3 năm. Sự khác nhau giữa các giai đoạn cho thấy sự nhiễm bệnh thay đổi theo mùa hoặc là động nhất ở tất cả các huyện hay có sự di chuyển ra khỏi thành phố Phan Thiết, trong khi đó kiểu biểu hiện bệnh biến đổi trong nhiều năm lại có xu hướng chuyển về Phan Thiết. Chúng tôi cũng tìm thấy mối tương quan đồng hồ giữa hiện tượng ENSO và sự biến đổi của thời tiết với tỷ lệ nhiễm DENV. Ý nghĩa thời tiết nên được xem xét cũng với các yếu tố khác có ảnh hưởng đến quá trình truyền bệnh của DENV như nằm trong miền nhiệt đới và miền núi (chương 8).

Phần 2 của luận văn mở tả các đặc điểm lâm sàng của bệnh do DENV, từ đó mở rộng các hiểu biết về lâm sàng của DENV trên các bệnh nhân thuộc khu vực dịch để cũng như các bệnh nhân bị nhiễm DENV trong quá trình điều trị và vận dịch tế.

Chúng tôi cũng nghiên cứu sự gia tăng của tế bào bạch cầu trong máu (plasmocytosis) của bệnh nhân nhiễm DENV, một dấu hiệu chưa được chú ý cho đến nay. Đây là một nghiên cứu tiền cứu nhằm định lượng và mô tả động học và các kiểu hình của tế bào bạch cầu trong máu của bệnh nhân bị nhiễm DENV sau khi đi du lịch đến khám tại trung tâm Y khoa hàm lâm (AMC), Amsterdam, Hà Lan (chương 10). Sự gia tăng tế bào bạch cầu máu với đặc điểm là sự hiện diện thoáng qua của tế bào đa dòng trong hệ tuần hoàn là một hiện tượng phổ biến trong nhiễm DENV. Sự gia tăng tế bào bạch cầu máu được thấy trong 64 – 73% bệnh nhân, tùy thuộc vào thời gian từ lúc nhiễm đến lúc khám khám. Sự gia tăng tế bào bạch cầu máu cao hơn ở các bệnh nhân bị tái nhiễm và 89% các bệnh nhân có sự tương đồng về kiểu tế bào miễn dịch (CD45+/CD19-/CD56-). Cơ chế của sự gia tăng tế bào bạch cầu máu vẫn chưa được hiểu rõ. Ở chương 11, chúng tôi đã nhân bản và giải mã vùng gene quyết định đặc tính kháng nguyên của DENV-1 từ các mẫu bệnh phẩm thu nhận theo thời gian tại bệnh nhân sốt DEN hay sốt xuất huyết DEN ở nhóm hay tái nhiễm để tìm hiểu về các biến động và tiến hóa của DENV-1 bên trong cơ thể người bệnh. Kết quả cho thấy phần lớn đột biến xuất hiện chỉ có tính chất thoáng qua, trong đó các chuỗi báo tồn và nhóm đột biến diễn biến trong suốt quá trình bệnh. Chúng tôi cũng tìm thấy bằng chứng của phức hợp nhiều kiểu gene khác nhau của DENV-1 trong nhiễm bệnh nhân. Chúng tôi không tìm thấy mối liên hệ giữa mức độ nặng của bệnh với tình trạng miễn dịch, nồng độ virus trong máu và mức độ biến động di truyền hay tiến hóa của DENV-1.


14. Thai KD, Wolthers KC, de Vries PJ. Epidemiology and diagnostics of dengue virus infection in Dutch travellers. (Submitted)


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Figure 1. Wavelet analyses of dengue time series with monthly data from 1994 to 2009 in 9 districts of Binh Thuan province. (A) Duc Linh, (B) Tanh Linh, (C) Ham Tan, (D) Ham Thuan Nam, (E) Ham Thuan Bac, (F) Phan Thiet, (G) Tac Binh, (H) Tuy Phong, (I) Phu Gai. (i) Binh Thuan province. For each district (i) left panel: the time series of cases in the district (ii) middle panel: the wavelet power spectrum of dengue cases (square rooted and normalized and trend suppressed); colors code for increasing spectrum intensity, from blue to red; dotted lines show statistically significant area (threshold of 5% confidence interval); the black curve delimits the cone of influence (region not influenced by edge effects) (iii) right panel: to the mean spectrum (solid line) with its threshold value of 5% (dotted line).

Figure 3. Phase differences from Phan Thiet district.
(A) Map of Vietnam (B) Mean phase differences for 1996-2001 data at the 2-3-year period band. (C) Mean phase differences for 1995-2002 data at the 1-year period band. (D) as in C, but for 2004-2008 data.
Figure 2. Wavelet coherence and phase analyses of dengue time series between neighboring districts in Binh Thuan province. The left panel represents the wavelet coherence. Blue, low coherence; red, high coherence. The dotted lines show $\alpha = 5\%$ significance level. The cone of influence (black curve) indicates the region not influenced by edge effects. The right panels represent the phase analyses between two districts (in blue and red), based on wavelets for 2–3-year periodic band. Green boxes represent the period of time where coherency is significant, when interpretation of analysis was possible. Red lines: first district; blue lines: second district; dashed black lines: phase difference between the two oscillating components.

Figure 4. Wavelet coherence analyses of dengue incidence with ENSO indices. (A) MEI, (B) Niño 1+2, (C) Niño 3, (D) Niño 4 and (E) Niño 3.4. Blue, low coherence; red, high coherence. The dotted lines show $\alpha = 5\%$ and $10\%$ significance levels. The cone of influence (black curve) indicates the region not influenced by edge effects.

Figure 5. Wavelet coherence analyses of dengue incidence with local climate variables. (A) mean temperature, (B) humidity and (C) rainfall. Blue, low coherence; red, high coherence. The dotted lines show $\alpha = 5\%$ significance levels. The cone of influence (black curve) indicates the region not influenced by edge effects.
Figure S2. Phase analyses of dengue time series between neighboring districts in Binh Thuan province. Phase analyses between two districts (in blue and red), based on wavelets for 1-yr periodic band.
Figure 2. Wavelet analysis and wavelet coherence analysis with dengue incidence time series and with an ENSO index in Binh Thuan province, Southern Vietnam. (A) Wavelet analysis of the dengue incidence time series with monthly data. Color scheme shows increasing intensity, from blue to red; dotted lines show statistically significant area; the black curve delimits the cone of influence. (B) Wavelet coherence analysis of the dengue incidence time series with the ENSO index Niño 4. Dark blue and dark red indicates low coherence and high coherence, respectively. The dotted lines show $\alpha = 5\%$ and $10\%$ significance levels. The cone of influence (black curve) indicates the region not influenced by edge effects. (C) Time series of dengue incidence in Phan Thiet City (solid red line) and the eight neighboring districts combined (dotted blue line). (D) Wavelet coherence analysis of time series in panel C showing a continuous annual mode of oscillation with a non-stationary 2-3 year multi-annual cycle. Dark blue indicates low coherence and dark red indicates high coherence. The dotted-dashed lines show $\alpha = 5\%$ significance levels computed based on 1000 bootstrapped series. The cone of influence (black curve) indicates the region not influenced by edge effects. This figure has been published previously\textsuperscript{273} and is reproduced with the permission of the authors.

Figure 1. White blood cell subset and the kinetics of peripheral blood plasma cells in DENV infected patients. (A) Leukocyte profile, determined by MGG staining, in patients with DENV infection stratified by days of illness. Data indicate median and 25-75\% IR. L, lymphocyte; AL, atypical lymphocyte; PL, plasmacytoid lymphocyte; PC, plasma cell; NG, neutrophil granulocyte; M, monocytes. No plasma cells were found in patients with OI. (B & D) Distribution of percentage (B) and absolute (D) plasma cells (of total WBC) by days of illness in patients with DENV infection. The kinetics of sequential plasma cells results in 8 patients with DENV infection are indicated by connected lines. (C) Plasma cell (left) and plasmacytoid lymphocyte (right) in peripheral blood of a patient with secondary DENV infection. (magnification $\times 100$).
Figure 1. Minimum spanning networks of intra-host DENV-1 sequence data (VP data set). Each network was inferred by compiling sequences from multiple days. Numbers in the upper left corner correspond to the patient number; percentages indicate the probability of parsimony used to construct the network. Haplotypes with the high ancestral probability are displayed as circles. Circles sizes are proportional to the number of sequences that exhibit each variant, and the pie chart in each circle indicate the percentage of each variant at different timepoints. Connecting lines indicate a single mutation shared among haplotypes. (A and B) Minimum spanning network in which multiple viral lineages were observed across time-points (patient 82 and 162). (C) Minimum spanning network in which one mutation was shared between haplotypes (patient 309). (D) Minimum spanning network with star-like typology (patient 59). (E) Minimum spanning network with reduced parsimony probability (patient 336).

Figure 2. Minimum spanning networks of intra-host DENV sequence data (VP data set) in which one (B, E, G, J and K) and/or two (C, I and J) mutations were shared between haplotypes. All sequences were identical to the consensus in A, D, F, H and L.

Refer to Figure 1 for more information.
Figure 3. Maximum likelihood (ML) phylogenetic tree for all \( n = 89 \) consensus sequences derived from clones in the VP data set in relation to 1390 equivalent background DENV-1 sequences collected from GenBank. Red colored lines represent clones from sample G2542 and red arrow bars signify mixed infection. Clades are indicated as numbers. Horizontal branches are drawn to a scale of nucleotide substitutions per site, and the tree is midpoint rooted, nodes are ordered increasingly and presented as a polar tree.


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