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.
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 Kbp. The RNA contains a single long open reading frame which encodes three structural proteins (C, prM(M), 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 prM, 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 exantheme, 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-cytoploid 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 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
heterologous serotype. The antibody-dependent enhancement theory proposes that due to a secondary infection with a different serotype, which may lead to more severe disease.103

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.76;147;195 Moreover, cross-reactive T cells may also contribute to the immune-pathogenesis.38 Low-affinity T cells against the original infecting serotype dominate during secondary heterologous infection in a phenomenon termed ‘original antigenic sin’.194 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 cytokines10;15;24;36;59;82;122;164;263;264, including tumour necrosis factor-α4, interleukin (IL)-1 and -6121;130, 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.

4.2 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.241;276 The antibody-dependent enhancement theory proposes that due to circulating antibodies from a previous infection induce a complex reaction during a secondary infection with a different serotype, which may lead to more severe disease.103

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4.4 DENGUE SEROTYPES AND CLINICAL OUTCOME

DENV-2 viruses have most commonly been associated with DHF/DSS11;205;276, followed by DENV-1 and DENV-3 viruses81;116;184;191. DENV-4 appears to be the most clinically mildest, although it too can cause severe disease.103 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.11;236

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.90;233

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.68 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. Laterally, 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.1 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.