Early diagnosis and prognosis of severe dengue

Thesis

EARLY DIAGNOSIS AND PROGNOSIS OF SEVERE DENGUE

by

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ABSTRACT

Dengue is considered as an emerging infectious disease with a wide expansion to more than 100 countries and territories in the world. Dengue has great social, economic and health impacts on endemic regions causing the disease burden to the community and becoming one of the most serious public health concerns.

We described clinical and virological features of paediatric dengue cases in southern Vietnam in a large prospective cohort study. We used the new classification of WHO 2009 to include dengue shock syndrome, severe bleeding and organ failure in the category of severe dengue in data analysis. Of 7544 patients enrolled into the study with complete haemobiochemical results available, 2060 patients (27.3%) had laboratory-confirmed diagnosis of dengue. The number of cases with severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 117 (5.7%), 156 (7.6%) and 1787 (86.7%) respectively. Proportions of RT-PCR positivity in the groups of severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 115/117 (98.3%), 149/156 (95.5%) and 1690/1787 (94.6%) respectively.

It is a challenge for attending physicians in the outpatient settings to recognize early dengue patients. A simple tool, called Early Dengue Classifier (EDC), including information of age, white blood cell and platelet counts can be used alone or in combination with the NS1 rapid test to make early diagnosis of dengue within 72 hours of onset illness. We demonstrate that the early diagnosis of dengue can be enhanced beyond the current standard of care using a simple evidence-based algorithm.

A prognostic algorithm using vomiting, platelet count, aspartate aminotransferase and NS1 rapid test result, called Early Severe Dengue Identifier (ESDI), can predict severe complications. Though the positive predictive value of the ESDI was low as seen with common prognostic models for a rare outcome, the results should support patient management and clinical trials of specific therapies.

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ABBREVIATIONS

ALB	albumin.
AIC	Akaike information criterion.
ALT	alanine aminotransferase.
AST	aspartate aminotransferase.
AUC	area under the ROC curve.
BDH	Binh Duong Provincial Hospital
BIC	Bayesian information criterion.
BMI	body mass index.
CART	classification and regression trees.
CH1	Children's Hospital 1.
CH2	Children's Hospital 2.
CI	confidence interval.
CK	creatine kinase.
CRF	case report form.
DENV	dengue virus.
DF	dengue fever.
DHF	dengue haemorrhagic fever.
DSS	dengue shock syndrome.
DNH	Dong Nai Children's Hospital.
ELISA	enzyme-linked immunosorbent assay.
GAM	generalized additive models.
GI	gastrointestinal bleeding.
Hct	haematocrit.
HTD	Hospital for Tropical Diseases.
ICU	intensive care unit.
IgA	immunoglobulin A.
IgG	immunoglobulin G.
IgM	immunoglobulin M.
IQR	interquartile range.
IV	intravenous.
JE	Japanese encephalitis.
LAH	Long An Provincial Hospital.
OFI	other febrile illness.
OR	odds ratio.
OUCRU	Oxford University Clinical Research Unit.
PCR	polymerase chain reaction.
PLT	platelet count.
PRNT	plaque reduction neutralization test.
RCT	randomised controlled trial.
RF	random forests.
RNA	ribonucleic acid.
RT-PCR	reverse transcriptase polymerase chain reaction.
TGH	Tien Giang Provincial Hospital.
WBC	white blood cell count.
WHO	World Health Organization.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1 DENGUE EPIDEMIOLOGY

1.1.1 DENGUE EPIDEMIOLOGY IN THE WORLD

Dengue is the most prevalent mosquito-borne viral disease in the world. Dengue incidence has increased 30-fold in the recent decades with a global expansion to new geographic regions [1]. Nowadays, dengue is endemic in more than 100 countries where 70 to 500 million infections are estimated to occur each year, resulting in 36 million clinically apparent cases and 21,000 deaths. Approximately 3.6 billion people are living in at-risk areas [2]. The American, South-east Asia and the Western Pacific regions are the most seriously affected (Figure 1-1). More than 70% of these people live in the Asia-Pacific Region [3]. Travellers are at significant risk of acquiring the disease and also contribute to its spread to nonendemic regions. Population growth, urbanization and modern transportation contribute to the rapid increase in the number of epidemics and circulating viruses [4, 5]. At present, although there are several clinical trials of vaccine against dengue, the only method to control or prevent the transmission of virus is to combat vector mosquitoes [2, 6-11] The leading dengue vaccine was developed by Sanofi Pasteur and results from two phase III clinical trials with their tetravalent live-attenuated chimeric yellow-fever dengue (CYD) vaccine, have recently been reported [6, 12]. For each of the four dengue serotypes the pre-membrane and envelope proteins from a wild type dengue virus were substituted into the yellow fever 17D vaccine backbone [13]. The results from these trials showed that this CYD vaccine candidate provided up to 60% protection against dengue of any severity, and up to 80% against hospitalizations due to dengue in the first year of follow up after the primary vaccination series. Subsequent follow up studies have demonstrated the CYD-TDV vaccination was associated with an elevated risk of hospitalization for dengue in year 3 among children younger than 9 years of age. Therefore, it is necessary to have more studies on long-term vaccine efficacy for vaccines developed from different technologies to achieve a better result in dengue prevention [14, 15].

Numerous vector control measures such as environmental management, larvicide and adulticide chemicals and human protection have had limited efficacy in stopping dengue transmission. The novel dengue control method using *Wolbachia* infected mosquitoes is currently being evaluated in Vietnam, Indonesia, Brazil and Columbia and is a promising tool for dengue control and prevention [14, 15]. *Wolbachia* are introduced into *Ae. aegypti* to reduce the lifespan of female mosquitoes and inhibit the replication of dengue virus in mosquito tissues. These properties make *Wolbachia*-infected *Ae. aegypti* less likely to transmit dengue. Another method called RIDL (Release of Insects with Dominant Lethality) has been developed by the British company Oxitec to produce fertile male adult mosquitoes that induce a high mortality in the descendants. The adults generated with this technique and released in the environment are not sterile but their descendants have a survival rate of 0% (this lethality can be switched off by introducing the antibiotic, tetracycline, into their diet). Repeated releases of sufficient numbers of sterile males will result in a reduction in the target mosquito population below the minimum level needed to support dengue transmission.

Dengue has been designated a major international public health concern by the World Health Organization, posing great social and economic burden for the population in endemic regions (Figure 1-1 and 1-2).

Figure 1-1. Countries/areas at risk of dengue transmission, 2008 *(Taken from [1])*

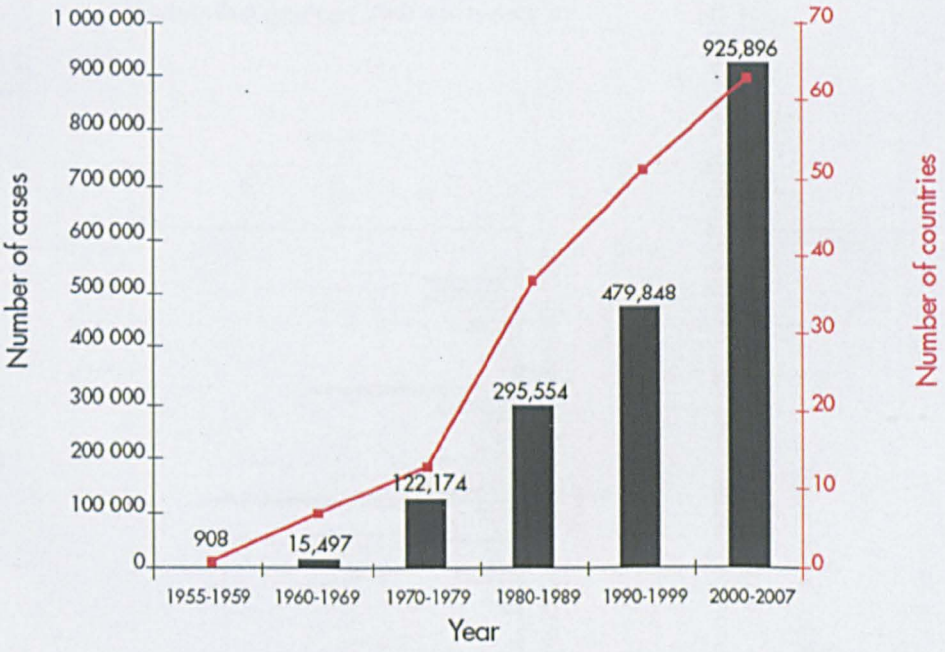


Figure 1-2. Average annual number of dengue fever (DF) and dengue haemorrhagic fever (DHF) cases reported to WHO, and of countries reporting dengue, 1955–2007 *(Taken from [1])*

1.1.2 DENGUE EPIDEMIOLOGY IN VIETNAM

Vietnam is a sub-tropical country where dengue is endemic, with an estimated tens of thousands of cases reported every year. In 1913 a dengue-like illness was first recorded in north and central Vietnam [16]. In 1929 the first of many dengue fever (DF) epidemics took place in the south of Vietnam. However, it was not until 1963 that the first dengue haemorrhagic fever (DHF) outbreak in the country was officially reported in the Mekong Delta region of southern Vietnam [17]. From the year 1963 to 1995, there were 1,518,808 DHF cases and 14,133 deaths reported. Between 1975 and 2000, epidemic peaks of increasing magnitude occurred approximately every three to five years. The largest nationwide outbreak occurred in 1987 with 354,517 DHF cases and 1,566 deaths. Another large epidemic involved 119,429 DHF cases and 342 fatalities in 1998 [18]. Important features of dengue epidemiology in Vietnam from 1960 to 2010 are shown in Figure 1-3.

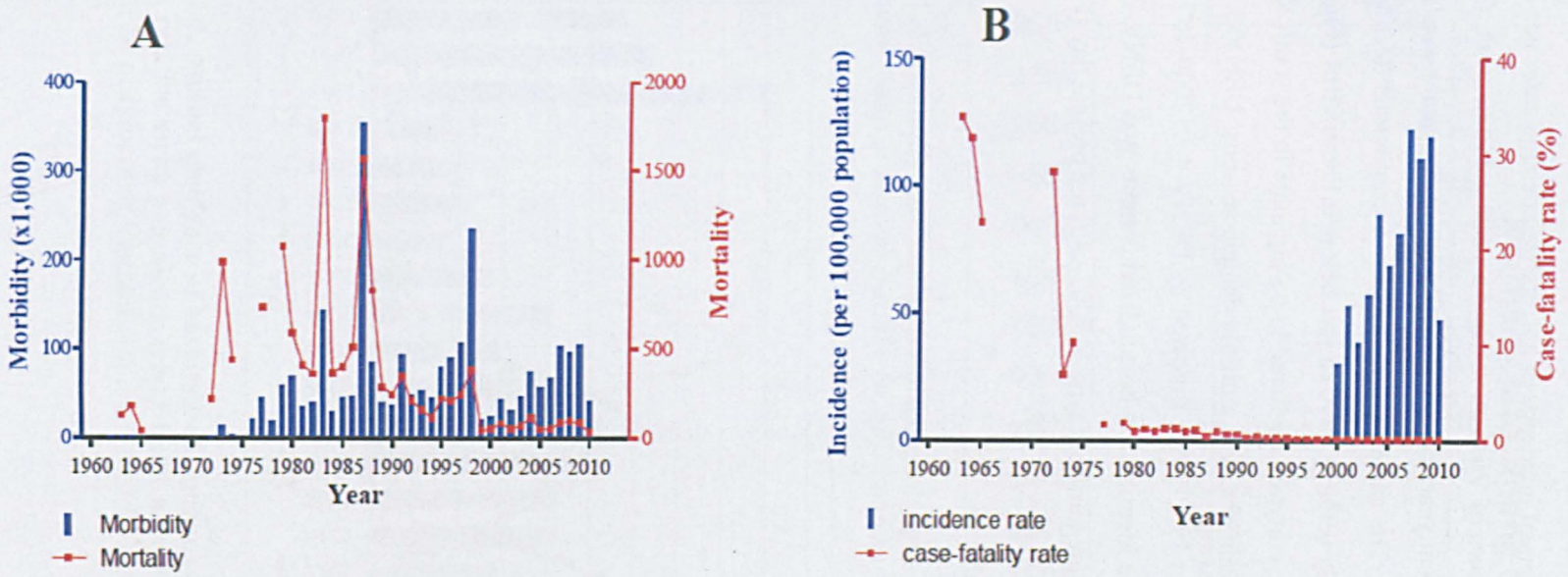


Figure 1-3. Vietnamese dengue cases and deaths officially reported from 1960-2010. *(Source: WHO, Dengue Net [19]).*
(A) Number of dengue (DF/DHF/DSS) cases and deaths in Vietnam. (B) Case-fatality rate and incidence rate in Vietnam. Incidence data was not available prior 2000

From 1996 to 2014, the annual incidence of hospitalized dengue cases per 100,000 population in 20 southern provinces of Vietnam reached a peak at 450 in 1998, followed by a sudden drop in the subsequent years before gradually increasing again over the next decade to 263/100,000 in 2007. The same period also saw a continuous decline in the mortality rate from approximately 0.3% in 1996 to just below 0.1% in 2014 (Figure 1-4, 1-5) [20]. All four dengue virus serotypes are found to co-circulate in the south Vietnam, but usually a single serotype dominates over multiple dengue seasons. DENV-3 was the predominant serotype reported in 1997-1999, followed by DENV-4 in 2000-2002. From 2003 to 2006, the major serotype identified in hospitalized patients was DENV-2, which was replaced by DENV-1 as the predominant type in 2007 until the present day (Figure 1-5) [20].

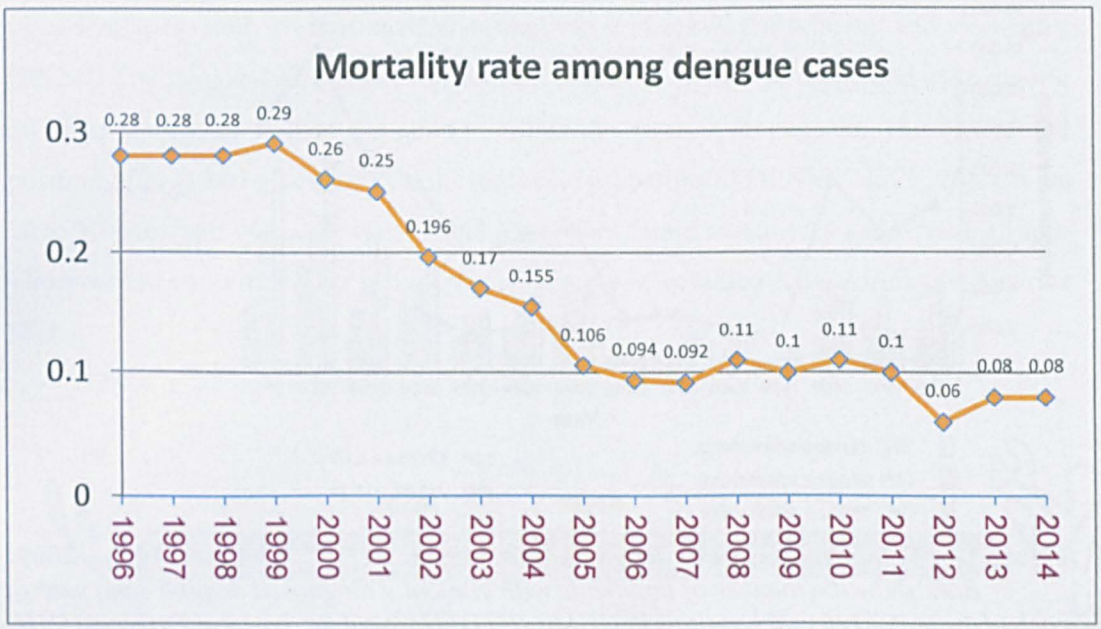


Figure 1-4. Mortality rate among hospitalized dengue cases in 20 southern provinces of Vietnam. *(Source: Pasteur Institute HCMC, Vietnam [20])*

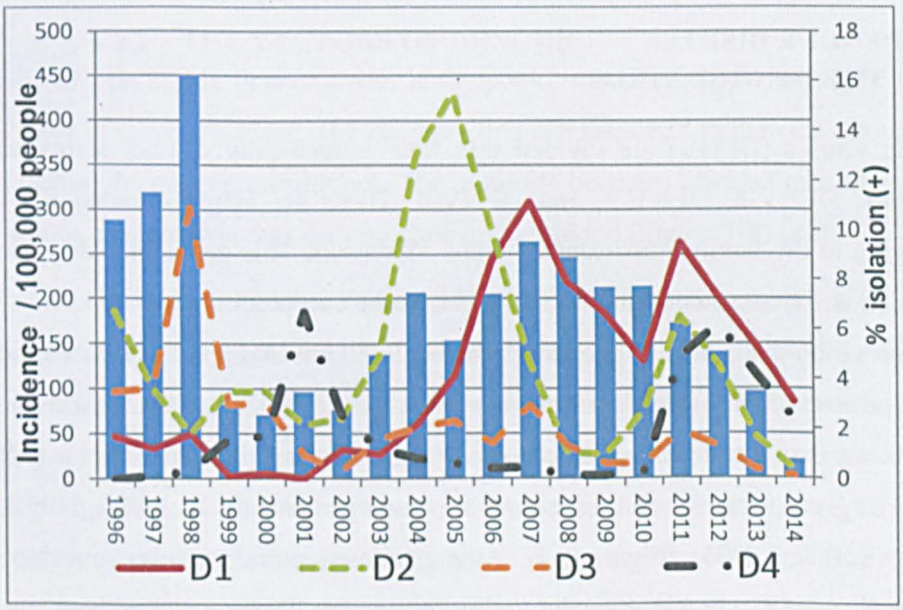


Figure 1-5. Annual incidence of hospitalized dengue cases (left-hand axis, solid bars) and the relative virus prevalence (right hand axis) between 1996 and 2014 in the southern 20 provinces of Vietnam. *(Source: Pasteur Institute HCMC, Vietnam [20])*

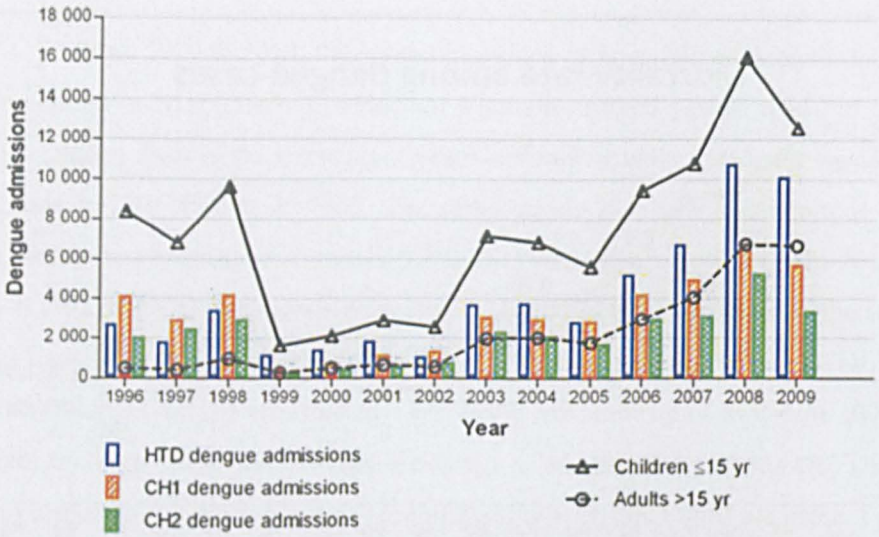


Figure 1-6. Temporal trends in dengue admissions in HCMC, Vietnam, 1996 – 2009.

Bars show the number of inpatients with clinically diagnosed dengue each year at each of the major referral hospitals in HCMC (HTD: Hospital for Tropical Diseases, CH1: Children's Hospital 1 and CH2: Children's Hospital 2). Lines show the number of dengue cases in children aged 15 years or less (triangles) and in adults over 15 years old (circles), combined across the study sites. (Reproduced from [21]).

1.2 THE DENGUE DISEASE

1.2.1 THE DENGUE VIRUS

Dengue viruses (DENV) are divided into four antigenically distinct serotypes named DENV-1, DENV-2, DENV-3, and DENV-4. These are single-stranded RNA viruses belonging to the family *Flaviviridae*, genus *Flavivirus*. This genus includes also the West Nile virus, Japanese encephalitis virus, tick-borne encephalitis virus and yellow fever virus. The virion comprises a spherical particle, 40–50 nm in diameter, with a lipid envelope. The positive single-strand RNA genome, which is 10.7 kb in length, has a single open reading frame that encodes three structural proteins - the capsid (C), membrane (M) and envelope (E) glycoproteins - and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Figure 1-7). Among non-structural proteins, envelope glycoprotein, NS1, is of diagnostic and pathological importance. Dengue viruses have two conserved *N*-linked glycosylation sites at Asn-67 and Asn-153. The glycosylation site at Asn-153 is conserved in most flaviviruses, while the glycosylation site at Asn-67 appears to be unique to dengue viruses and is absent in other class 2 enveloped viruses. The loop bearing the glycan on Asn-153 is two residues shorter in DENV-3 than in the other dengue

virus serotypes. Both glycans have been implicated in cellular attachment and viral entry [22-24]. The glycosylation pattern differs according to DENV serotype and even among different strains, as well as the cells in which the virus is propagated. The degree and position of N-linked glycans affect the antigenic properties of DENV [23, 25, 26]. Unlike 20 to 30 years ago when serotypes 3 and 4 were not found in America and Africa, all four serotypes are now circulating in hyperendemic regions including Asia, Africa and America [27].

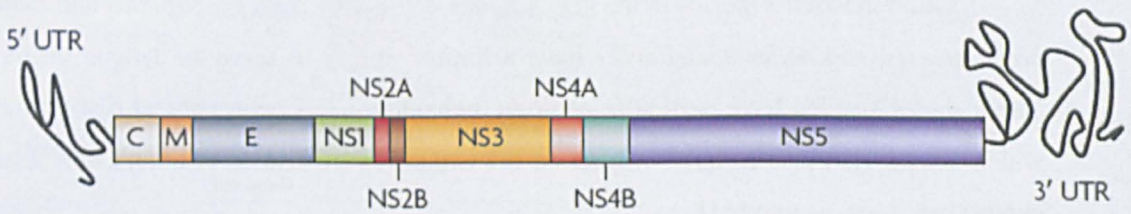


Figure 1-7. The dengue virus genome. *(Reproduced from [27])*

1.2.2 THE VECTORS OF DENGUE

The highly domesticated, anthropophilic *Aedes aegypti* mosquito is the primary vector of dengue viruses. The dengue virus is transmitted to humans through the bites of infected *Ae. aegypti* mosquitoes. The mosquito becomes infected with dengue virus when it bites a person who has dengue virus in their blood (Figure 1-8) [28].

This mosquito inhabits tropical and subtropical areas and is widely spread around the world, mostly between latitudes 35°N and 35°S. High seasonal rainfall and high environmental temperature, the latter more important than the former, are the two main factors contributing to expansion of the *Aedes aegypti* mosquito population and habitat, consequently resulting in increased dengue transmission. Because of low temperature on highlands or mountains, *Aedes aegypti* is rarely found above 1,000 metres. When the temperature increases, all stages of the *Aedes* life cycle become shorter - leading to a larger mosquito population, increased feeding rates and shorter extrinsic incubation periods, i.e. reducing the time that dengue viruses disseminate to salivary glands in infected adult mosquitoes.

Male and female adult mosquitoes feed on nectar of plants; however, female mosquitoes need blood in order to produce eggs, and are active in the daytime. Eggs have the ability to survive drying for long periods of time, allowing them to be easily spread to new locations. Artificial or natural water containers such as flower pots, discarded tires, plates under potted plants, cemetery vases, buckets, tin cans, clogged rain gutters, etc. that are within or close to places where humans live are ideal larval habitats for *Aedes aegypti* mosquito.

Other mosquito species in the genus *Aedes* - including *Aedes albopictus* and *Aedes polynesiensis*, and *Aedes scutellaris* - have a limited ability to serve as dengue vectors. These *Aedes* species have particular ecology, behaviours and geographical distribution, which are thought also to have influenced the expansion of disease although to a lesser extent than *Aedes aegypti* [1].

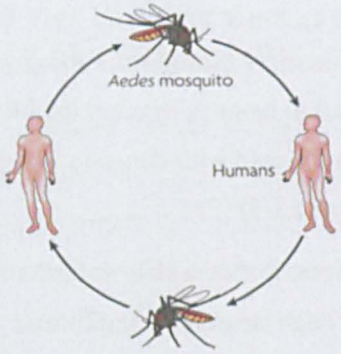


Figure 1-8. Dengue transmission [28]
The dengue virus is spread through a human-to-mosquito-to-human cycle of transmission.

1.2.3 CLINICAL MANIFESTATIONS

The majority of DENV infections are asymptomatic. Symptomatic DENV infection has a broad clinical spectrum, ranging from a non-severe acute febrile illness sometimes with evidence of mild vascular leakage and haemostatic derangements, to a severe form of illness with hypovolemic shock due to plasma leakage, severe bleeding

and/or severe organ dysfunction [1, 29]. After an average incubation period of 4–10 days (range 3 - 14 days), the illness begins abruptly and is followed by the three phase - febrile, critical and recovery (Figure 1-9).

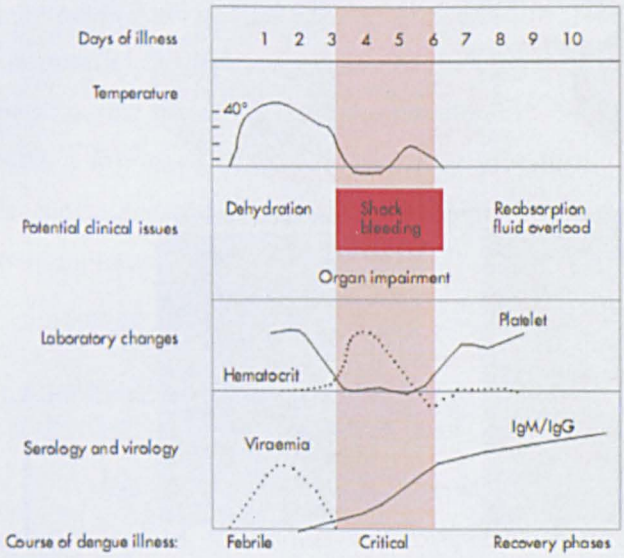


Figure 1-9. The course of dengue illness

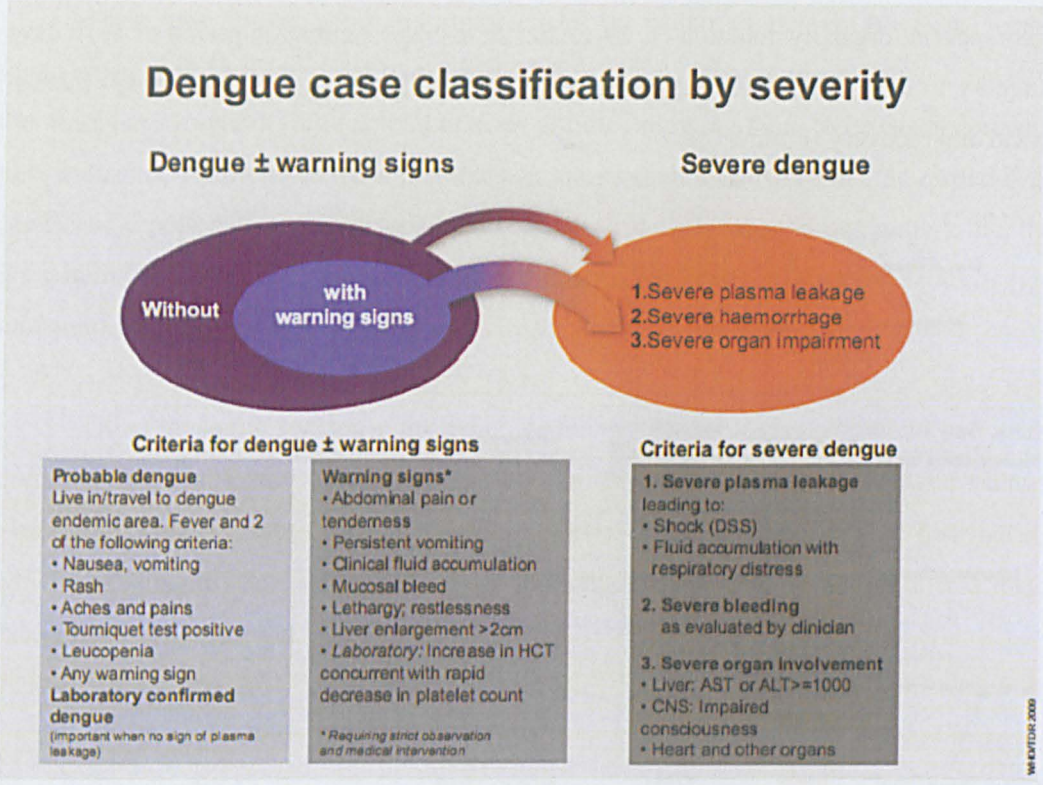


Figure 1-10. Suggested dengue case classification and levels of severity. *(Taken from WHO Guideline, 2009)*

1.2.3.1 Febrile phase

Patients typically develop a sudden rise in temperature, usually lasting 2 - 7 days, and is frequently associated with a flushed face, skin erythema, generalized body ache, myalgia, arthralgia and headache [30]. A faint macular or maculopapular rash is present in some cases, but is evanescent and easily miss. Some other manifestations include sore throat, injected pharynx and conjunctival injection. It is often for the patients to have anorexia, nausea and vomiting. These often non-specific signs and symptoms, particularly in the first few days of fever, can make it difficult to clinically distinguish dengue from non-dengue illnesses such as influenza, measles, infectious mononucleosis, scarlet fever, meningitis, acute appendicitis [1]. In addition, clinical experience suggests that early clinical signs and symptoms are indistinguishable between patients with severe and uncomplicated disease evolutions [27, 31, 32]. A positive tourniquet test in this phase

increases the probability of dengue. This test has been considered as supportive evidence for a clinical diagnosis of dengue, but since spontaneous petechiae are often present anyway and the test can be positive in other infections, the sensitivity, specificity and diagnostic utility are limited [32, 33]. Mild haemorrhagic manifestations like petechiae and mucosal membrane bleeding (e.g. nose and gums) may be seen. Massive uterine bleeding (in pubertal females and women of childbearing age) and gastrointestinal bleeding may occur during this phase but is not common [32, 34]. The liver is often enlarged and tender after a few days of fever. Laboratory investigations show a progressive decrease in leucocyte and platelet counts in this period, sometimes with mild increases in hepatic transaminases [1, 32].

1.2.3.2 Critical phase

Most patients begin to recover when the fever subsides but in a small number of cases, estimated to be less than 5%, the patient deteriorates around the period of defervescence, which is usually between days 3-7 of illness. This critical stage coincides with the leakage of plasma when there is the increase in the permeability of the capillary system [35]. Clinical signs of plasma leakage include ascites, pleural effusions and haemoconcentration. Severe plasma leakage can cause cardiovascular collapse, the so-called dengue shock syndrome (DSS), which is usually imminently preceded by warning signs such as abdominal pain, persistent vomiting and hepatomegaly. On clinical examination, DSS presents with cold or clammy skin, restlessness, rapid and weak pulse, narrow pulse pressure or hypotension [1]. Haemorrhagic manifestations are seen more frequently in this period since thrombocytopenia and coagulopathy may become more prominent. However, most of the bleeding features are mild either on the skin or superficial mucosa such as petechiae, easy bruising, epistaxis and gingival bleeding. In some circumstances, severe haemorrhage like gastrointestinal bleeding and large hematoma at venipuncture sites may occur especially for patients with prolonged shock. Of special importance is that severe bleeding can occur in adults even without significant plasma leakage [36, 37]. Inappropriate management of DSS can result in prolonged shock, metabolic acidosis, coagulopathy, liver impairment, multiple organ failure and death. The

critical phase with clinically significant plasma leakage usually lasts 24-48 hours then patients enter the recovery phase.

1.2.3.3 Recovery phase

Surviving patients recover within 2-3 days with the normalization of vascular permeability and re-absorption of extravascular compartment fluid. Patients feel better, resume appetite with stable hemodynamic status and diuresis. A second skin rash often appears during the transition from critical to recovery phases. This so-called recovery rash ranges from a mild maculopapular rash to a very florid appearance with intense erythema and dense petechiae interspersed with islands of pale skin. Sinus bradycardia or arrhythmia can sometimes occur in this stage but does not affect the general well-being. The haematocrit returns to normal though it may temporarily decrease below the baseline values due to the dilutional effect of the re-absorbed fluid. The white blood cell and platelet count increase soon after defervescence with a faster recovery of white blood cell count compared to that of the platelet count.

In addition to severe ascites and/or pleural effusions, especially in case with a large amount of intravenous fluid given during the critical phase, overload of re-absorbed fluid can cause respiratory distress [1]. A rare complication in the convalescent period is pulmonary oedema or congestive heart failure due to excessive fluid therapy.

1.3 DENGUE CASE CLASSIFICATION

The traditional World Health Organization (WHO) dengue classification and management scheme in 1997 divided symptomatic dengue into three groups: undifferentiated fever, dengue fever and dengue haemorrhagic fever. Dengue haemorrhagic fever was again classified into four grades of severity, where grades III and IV were defined as dengue shock syndrome [38]. In clinical practice, this classification system has shown many drawbacks because of the changes in dengue epidemiology with the expansion to many new geographical regions and older age groups. Also, the old classification might make clinicians incorrectly define severe dengue as dengue fever especially in adults and did not pay attention to organ failure in the case definition [39-41]. As a result, a revised WHO dengue case classification was issued in 2009 after an extensive

prospective study was conducted across many Southeast Asian and Latin American countries, dividing dengue into dengue without warning signs, dengue with warning signs and severe dengue [1, 42]. Severe dengue presents one or more of the following complications: (a) plasma leakage that may lead to shock and/or fluid accumulation with respiratory distress, (b) severe bleeding, and/or (c) severe organ impairment (hepatic dysfunction, renal failure, encephalopathy or encephalitis, cardiomyopathy and other organs) (Figure 1-10).

1.4 CLINICAL MANAGEMENT

There has been no specific antiviral medicine or any vaccine available for the treatment of dengue to date. Appropriate management for patients with dengue is supportive treatment by antipyretics and oral fluid intake to prevent dehydration due to plasma leakage [1, 43]. Aspirin and other nonsteroidal anti-inflammatory drugs including ibuprofen should be avoided due to the possibility of worsening bleeding complications [44]. It is possible to manage most patients at home if there are no warning signs. Patients are required to come back for re-examination and taken blood to check the haematocrit and platelet count on a daily basis if a clinically suspected dengue is diagnosed [45, 46]. If the disease progresses unfavourably with the presence of warning signs or a rapid increase in haemoconcentration and/or severe decrease in platelet count, patients must be hospitalized for close observation. Volume replacement by intravenous therapy with isotonic crystalloid solutions is indicated for those patients with persistent vomiting, rapidly continuous elevation of the haematocrit or any signs of cardiovascular compromise [47-49]. Adjust the volume and rate of fluid infused to maintain the hemodynamic status, especially the tissue perfusion and diuresis (at least 0.5ml/kg/hour) [1, 50]. In case of shock, resuscitation with the intravenous fluid therapy is much more aggressive under careful monitoring the vital signs, time of capillary filling, haematocrit and diuresis. If the patient's state does not show improvement, it is then necessary to switch to using colloids and measuring the central venous pressure as a guide for fluid replacement [1, 49, 51]. The time for fluid infusion usually lasts 24-48 hours corresponding to the critical phase. Patients with respiratory distress can be managed by oxygen support via nasal cannula or continuous positive airway pressure [52]. Fresh whole blood or packed red cells can be given to patients with severe

bleeding, which sometimes manifests discreetly only by the state of shock while the haematocrit decreases [50, 53, 54].

1.5 HOST COMPONENTS AFFECTING DENGUE DISEASE MANIFESTATION AND PROGRESSION

Many factors influence the susceptibility of the human to and/or the severity of disease including age, sex, immune status, genetic predisposition, nutritional status and co-morbidities.

1.5.1 Immune status

Infection with one serotype of DENV provides lifelong immunity to that serotype, but results only in partial and transient protection against subsequent infection by the other three serotypes. Importantly, many studies have shown that secondary infection with a different DENV type is a major risk factor for DHF/DSS, possibly due to antibody-dependent enhancement [55-58]. In this mechanism, non-neutralising antibodies increase the infected cell mass and virus production. The large infected cell mass and the high viral load are a prelude for severe disease development. The binding of residual heterotypic nonneutralizing antibodies from the earlier infection with the new virus is hypothesized to facilitate viral entry and replication. The resulting increased viral load triggers an immune cascade that alters microvascular function and causes a transient increase in vascular permeability that may be severe enough to cause DSS. DENV-specific antibodies also exist in newborn infants; when these passively transferred maternal antibody levels wane, the increase in DHF occurring during the first infection is held as evidence for the importance of ADE in the pathogenesis of severe dengue [59-62].

1.5.2 Age

A majority of cases occur in children, especially in the school age range. However, dengue can occur at all ages. Infants under one year may experience symptomatic disease with high risk of severe dengue and death [21, 63]. Overall, mortality rate was highest in young children with DSS and decreased with increasing age [21]. During recent years, the

incidence of dengue in adults has increased rapidly, often accompanied with underlying chronic disorders that can cause significant morbidity and mortality [64, 65]. A plausible explanation for this trend could be a reduction in the force of infection due to socioeconomic development and/or improved vector control such that fewer people are exposed during childhood leaving a larger reservoir of susceptible adults [66]. A second explanation could be the movement of adults into endemic regions where there is a risk of exposure to infected mosquitoes. Alternatively the flow of susceptible individuals into the population decreases due to lower birth and death rates in transitioning economies, which increase the lifespan of immune individuals and the likelihood that an infectious mosquito will feed on an immune individual [67].

1.5.3 Sex

A higher proportion of dengue cases are reported to occur in male than female patients [21, 68]. However, it has been shown that the risk for DSS and death from this complication is higher in females [21, 68]. The hypothesis for this is either a more robust cellular immune response or a higher intrinsic susceptibility to capillary permeability in females than males [16].

1.5.4 Race

Race has been identified as an individual risk factor since DHF/DSS is more prevalent in white compared to black persons. Dengue outbreaks in Cuba have provided evidence of a reduced risk of people of African descent for DHF/DSS compared to those of Caucasoid race. These observations from Cuban dengue outbreaks have significant epidemiological interest, as the differences in susceptibility to DHF/DSS among racial groups in Cuba coincide with that reported in African and Black Caribbean populations [69, 70]. Serologic studies showed that a high proportion of Africans and those who are genetically of African origin in Haiti had the presence of antibody to dengue virus, but the number of symptomatic dengue cases reported among these groups was small [71, 72]. One of the plausible explanations is that there could be the presence of a disease "resistance " gene in black persons.

1.5.5 Host genetics

Several host genetic factors such as some human leukocyte antigen (HLA) class I alleles, a promoter variant of CD209 (DCSIGN1-336), some tumour necrosis factor-α and lymphotoxin-α polymorphism genes and AB blood group have been identified as risk factors for severe dengue [5, 73-77]. However, most of the studies to date have been small and the results have not been replicated in second populations so the significance remains uncertain. Recently however a large genome-wide association study from Vietnam has identified susceptibility loci for DSS at MICB and PLCE1, and this has since been validated in Thai children [78, 79].

1.5.6 Nutritional status

It has been shown that malnourished children had a lower risk of contracting dengue infection while obese children had a greater risk of infection with dengue viruses [73-75]. Although some studies about nutritional status and dengue severity have reported inconclusive results, most studies conclude that obesity is a risk factor for developing DHF/DSS and children with malnutrition had a reduced risk of severe disease [73, 76]. A plausible explanation for this is based on the host immune response. Malnourished children are spared from severe DHF/DSS because they have a suppressed cellular immune response. On the contrary, obese children are expected to have a stronger immune response than normal children, so they are at higher risk of developing severe dengue [16, 76, 80]. Complications of fluid overload were found more in obese patients compared to normal and malnourished patients [75]. In obese children, it is more difficult to estimate fluid replacement and intravenous fluid based on body weight may be too much resulting a higher complication rate for overhydration among these patients. In contrast, Kalayanarooj reported in a review of 4,532 cases with dengue infection that malnourished children had a higher risk of developing shock than normal and obese patients [75]. Other studies suggested that the nutritional status does not influence the course of dengue infection, i.e, excess nutrition does not appear to be a risk factor for severe dengue, nor does malnutrition appear to be predictive of good outcomes [74, 81-83].

1.5.7 Co-morbidities

It has been reported that patients with certain co-morbidities like diabetes mellitus, hypertension, chronic renal failure, asthma, and allergies are at high risk of developing DHF/DSS [5, 77]. However, there is little knowledge about this field. Changes of the vascular endothelium occurring in diabetes mellitus and allergies might trigger biological changes resulting in increased capillary fragility and vessel permeability observed in DHF patients. Patients with history of allergies often have constantly activated immune system and there is liberation of proinflammatory cytokines in tissues, particularly in endothelium [83]. Similarly, in type-2 diabetes mellitus, the activation of T-lymphocytes and release of cytokines like gamma interferon and tumour necrosis factor alpha ultimately increase the capillary fragility and permeability [84]. Similar physiopathological mechanism has been observed in development of DHF/DSS.

1.6 DIAGNOSIS OF DENGUE

1.6.1 DIFFERENTIAL DIAGNOSIS OF DENGUE

Dengue and severe dengue can be misdiagnosed with other diseases, especially during the early febrile phase because of non-specific signs or symptoms [1]. It is extremely important to know the regional epidemiology of febrile diseases and the different stages of dengue infection to make the differential diagnosis. Many severe infectious diseases such as malaria, leptospirosis and typhoid fever are sometimes very similar to dengue. During the febrile phase, dengue must be differentiated from flu-like syndromes (influenza, measles, chikungunya, etc.), or various conditions with rashes and fever (rubella, measles, chikungunya, erythema infectiosum, infectious mononucleosis, etc.). Severe dengue with dengue shock syndrome or neurologic manifestations can mimic sepsis, meningococcal infection, or other aetiologies of encephalitis. Abdominal pain in dengue with warning signs is sometimes very severe and challenging for the differential diagnosis of an acute surgical abdomen.

1.6.2 LABORATORY DIAGNOSIS OF DENGUE

1.6.2.1 Virus isolation

DENV are found in serum/plasma and tissues for approximately 2 to 7 days, corresponding to the febrile phase. Though virus isolation by cell culture and from mosquitoes is the gold standard, it has gradually been replaced by the RT-PCR method for the detection of DENV during the febrile period (see below). Dengue serotypes can be identified by immunofluorescence assay using serotype-specific monoclonal antibodies. Virus isolation is not suitable for routine clinical management because the technique is complicated and takes 1-2 weeks to give the results [1].

1.6.2.2 Viral nucleic acid detection

The reverse transcriptase polymerase chain reaction (RT-PCR) methods have been used to identify dengue RNA in plasma or tissue since the early 1990s [85-87]. To date, various RT-PCR techniques have been developed for the diagnosis of dengue infection and serotyping based on the different amplicon sizes. More recently, real-time RT-PCR has become a quantitative method to determine the RNA viral load early in the viraemic stage, which is considered a prognostic factor of disease severity. Real-time RT-PCR can be either "singleplex", detecting one single serotype in a separate reaction, or "multiplex", identifying all four serotypes in the same reaction [88-90]. However, RT-PCR is not available at every health facilities because it requires expensive equipment and specialized training.

1.6.2.3 Viral antigen detection

NS1 is a virus protein secreted from infected cells into plasma within the first 1-3 days of fever and may persist in the peripheral blood circulation for up to 9 days from illness onset [91-93]. The availability of commercial antigen-capture assays, including ELISA and rapid test, that detect the DENV NS1 represents a major advance in early diagnosis. It has been shown that the specificity of these kits is very high, up to approximately 100%, while the sensitivity varies depending on the manufacturer. The sensitivity of NS1 assays depends on the day of illness, and is higher within the first three days of fever. Primary infection has also been documented to have higher sensitivity than secondary infection [94-98]. The hallmark of NS1 antigen detection is that this method is

simple and can give the results quickly. However, this method generally cannot differentiate between the different virus serotypes.

1.6.2.4 IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA)

MAC-ELISA is an inexpensive and simple test with a high sensitivity and relative specificity to detect DENV-reactive IgM in serum or plasma on day 5 or more after the onset of fever. It can be cross-reactive with other flaviviruses including Japanese encephalitis, St. Louis encephalitis and yellow fever probably due to the use of whole cell virus antigen [99]. However, this limitation can be reduced by using an in-house MAC ELISA with reagents derived from co-circulating flaviviruses as controls. [100]. There are also rare reports of false positive results where sera were taken from patients with malaria, leptospirosis and past dengue infection [101].

1.6.2.5 IgG antibody-capture enzyme-linked immunosorbent assay (GAC-ELISA)

GAC-ELISA follows the same procedures as MAC-ELISA and also has a broad cross-reactivity among flaviviruses [102, 103]. A fourfold or greater increase in IgG antibodies in acute and convalescent paired sera is defined as recent infections. As a consequence, IgG assays are useful for sero-epidemiological surveillance. GAC-ELISA can differentiate primary and secondary infections by comparing the IgM/IgG ratio [101, 104, 105].

1.7 EARLY DIAGNOSIS OF DENGUE AND PROGNOSIS OF SEVERE DENGUE – OBJECTIVES OF THIS THESIS

In Vietnam and many other endemic countries it is not possible to hospitalise every patient with clinically suspected dengue for observation and therefore triage is an important and necessary step [106]. An early laboratory diagnosis of dengue can assist in patient triage and management by directing clinical attention to the appearance of capillary permeability, for which supportive oral and/or parenteral fluid therapy is recommended to prevent circulatory compromise. Furthermore, an accurate dengue diagnosis helps to exclude other severe diagnoses, e.g. sepsis, and prevents unnecessary antibiotic usage [1].

A prompt diagnosis of index cases can also facilitate early vector control activities in the community so as to mitigate further transmission. Finally, given our understanding of the dynamics of viraemia, host response and clinical complications in dengue, it is likely that the success of novel interventions with anti-viral drugs or corticosteroids to modulate the host-proinflammatory response will be dependent on early (≤3 days) clinical, or better, laboratory diagnosis.

The detection of NS1 antigen of dengue virus by rapid assay will help make early diagnosis of dengue and assist clinicians in patient triage and care-management. As an example, the sensitivity and specificity of NS1 detection by ELISA and rapid diagnostic test (RDT) in Vietnamese children with dengue were recently prospectively defined [96]. In the first 3 days of illness, sensitivity and specificity were ~80% and 100% respectively in laboratory-confirmed dengue cases, suggesting a place for such a test in early diagnostic efforts [96, 98, 107, 108]. Furthermore, there are several lines of evidence to suggest that the sensitivity of early NS1 detection will be highest in children who subsequently develop severe dengue. In hospital-based studies, early NS1 concentrations [109], and viraemia levels [110, 111], were higher in the first 72hrs of fever in Thai children who developed severe dengue. However, the sensitivity of the NS1 detection assay is lower in patients with secondary infections and therefore, despite the higher NS1 levels there may be antibody antigen complexes that make the test less sensitive. Furthermore, clinical experience suggests that early clinical signs and symptoms are indistinguishable between patients with severe and uncomplicated disease evolutions.

The aims of this thesis are as follows:

1) Describe the clinical and virological characteristics of paediatric dengue infection.

2) Develop a simple diagnostic algorithm incorporating simple clinical history, examination findings and laboratory features to serve as a useful tool for making early diagnosis of dengue in the outpatient setting and compare the performance of this diagnostic tool with that of NS1 rapid test.

3) Develop a prognostic algorithm for the early identification of severe dengue in children.

By addressing these aims, physicians at the outpatient setting or primary healthcare level can get benefit of overcoming their problems related to overlooking dengue patients especially those who are at high risk of progression to severe complications. In order to fulfil these objectives, prospective studies with large sample sizes, early recruitment and well-characterized patients with different degrees of severity are important to understand better the disease pathology and to uncover early markers of dengue severity. Last but not least, by identifying cohort at risk of severe dengue, it will have implications for the targeting of future vaccination strategies and clinical trials of therapeutic interventions.

Chapter 2

MATERIALS AND METHODS

This chapter describes the general procedures including study design, patient enrolment and analysis plan used in my PhD project. A detailed presentation of the statistical methods will be included in the relevant chapters.

2.1 Human Research Ethics

The study protocol was approved by the Hospital for Tropical Diseases scientific and ethical committee and the Oxford University Tropical Research Ethical Committee (OXTREC 35-10). The accompanying parent/guardian of each child provided written informed consent.

2.2 Study design

A cohort study of dengue in children presenting to the outpatients department of collaborating hospitals in Vietnam including Hospital for Tropical Diseases (HTD), Children's Hospital 1 (CH1), Children's Hospital 2 (CH2), Tien Giang Provincial Hospital (TGH), Dong Nai Children's Hospital (DNH), Binh Duong Provincial Hospital (BDH) and Long An Provincial Hospital (LAH).

2.3 Patient enrolment

Recruitment occurred in the public sector outpatient departments of collaborating hospitals. These outpatient departments function as primary care providers to their local communities. A patient presenting to one of the study sites was eligible for enrolment if they met the following inclusion criteria - a) fever at presentation (or history of fever) and less than 72 hours of symptom history, b) in the opinion of the attending physicians dengue was the most likely diagnosis, c) 1-15 years of age inclusive, d) accompanying family member or guardian had a mobile phone and e) written informed consent for the child to participate was provided by the parent/guardian. Patients were excluded if - a) the attending physician believed they were unlikely to be able to attend follow-up or b) the attending physician believed another (non-dengue) diagnosis was more likely (Figure 2-1).

Patient enrolment occurred consecutively during normal clinical hours on weekdays without restriction. All patients were enrolled into the study before the attending physician received the results of any routine laboratory tests.

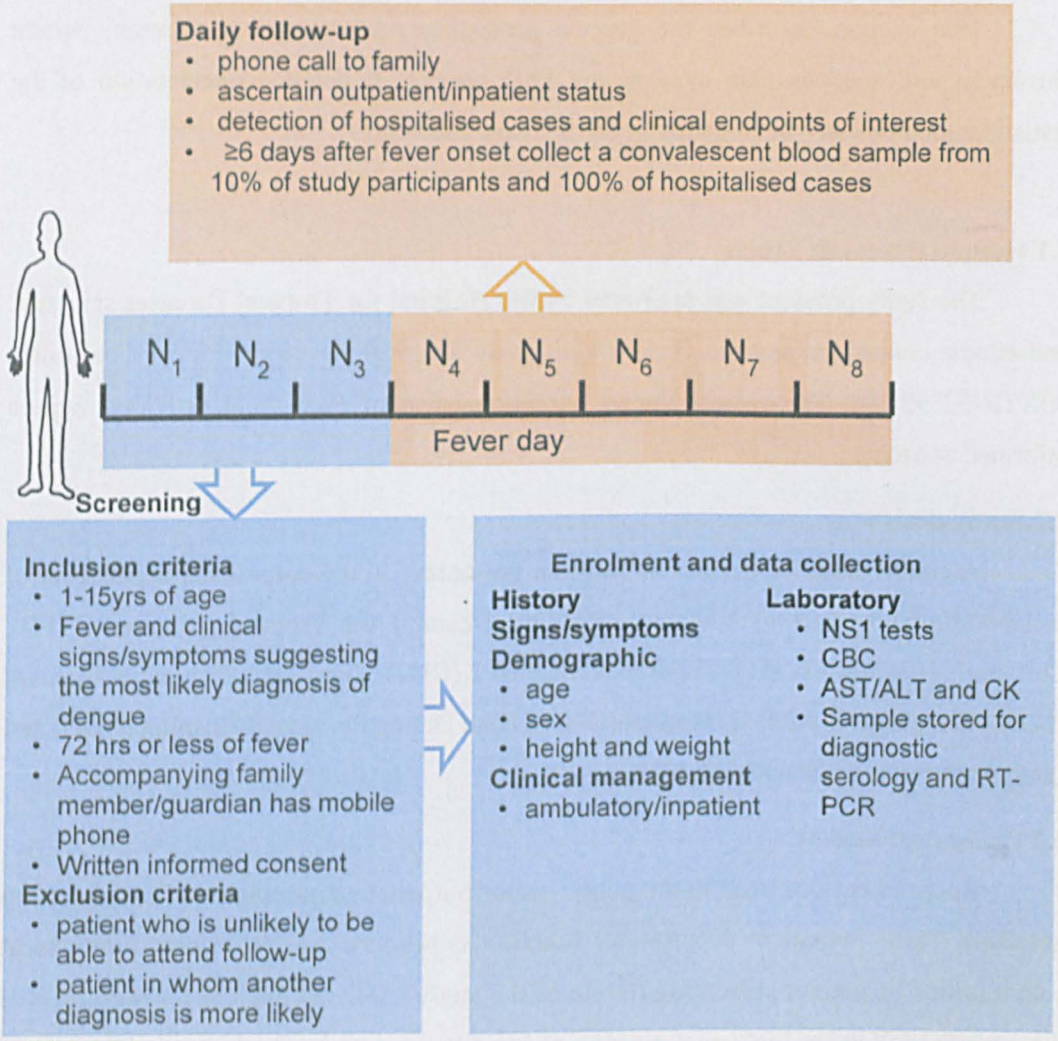


Figure 2-1. Summary of screening, enrolment, investigations and follow-up

2.4 Demographic, clinical and laboratory data collection

At the time of enrolment, information on the patient's age, sex, illness history, presenting signs and symptoms were recorded in a case report form. The definitions used to support standardized data capture are shown in Table 2-1. Blood samples were drawn

for routine haematology, biochemistry including albumin, aspartate aminotransferase, creatine kinase and NS1 rapid test. All NS1 rapid tests (NS1 Ag STRIP, BioRad) were performed on the same day of patient enrolment by one of two trained laboratory technicians at the Hospital for Tropical Diseases. Routine haematology results, but not biochemistry or NS1 rapid test results, were made available to the attending physician, who decided on the management approach, i.e. hospitalization or ambulatory follow-up.

2.5 Patient follow-up

A 2nd blood sample for the purposes of serology was collected around the time of defervescence from all patients that were hospitalized anytime during their acute illness. If the patient was managed solely on an ambulatory basis for the duration of their illness, then a 2nd early convalescence blood sample for the purposes of serology was collected only from a randomly selected 10% of this patient population. The randomization code to select ambulatory cases for follow-up was generated by a clinical data management system called "CLIRES", a software developed by the Department of Information at the Oxford University Clinical Research Unit. The rationale for collecting a convalescent blood sample from 10% of participants and 100% of hospitalized patients is explained in Section 2.6 below. This ICH-GCP compliant, clinical data management system also stores all clinical and laboratory data. Demographic and clinical data were double entered. Electronic data files containing haematological results were uploaded directly to CLIRES. Independent study monitoring was performed by the Clinical Trials Unit of the Oxford University Clinical Research Unit which examined adherence to the trial procedures, data collection and recording and compliance with ICH-GCP.

Table 2-1. Clinical and demographic features recorded at the time of study enrolment

Variable	Definition
1. Day of illness (days)	Day of illness at enrolment
1. Age (years)	Age in years
2. Sex	Male/Female
3. BMI (kg/(m)^2)	Body mass index (BMI) = weight (kg) /height (m) ^2
4. Temperature at enrolment (°C)	Axillary temperature at enrolment
5. Vomiting (Yes/No)	Any episode of vomiting in history of illness
6. Skin bleeding (Yes/No)	History or clinical examination shows that the patient has skin bleeding, e.g. petechiae or ecchymosis.
7. Mucosal bleeding (Yes/No)	Any frank bleeding from any mucosal site e.g. epistaxis, gingival bleeding, gastrointestinal bleeding, or urogenital bleeding in history of illness or medical examination
8. Abdominal pain (Yes/No)	History or clinical examination shows that the patient has a painful feeling at the abdomen by self-reporting or elicited on gentle palpation.
9. Rash (Yes/No)	Bright red-purple non-petechial dots appearing on the arms, legs, and possibly pink-red patches all over the body.
10. Flush (Yes/No)	Marked redness in the face and often other areas of the skin caused by dilatation and congestion of the capillaries that blanches with pressure.
11. Conjunctival injection (Yes/No)	Redness (bright red or pink) of the conjunctiva fading towards the limbus due to dilatation of the superficial conjunctival blood vessels.
12. WBC (10^3/mm^3)	Absolute white blood cell count at enrolment
13. HCT (%)	Haematocrit at enrolment
14. Platelet count (10^3/mm^3)	Platelet count at enrolment
15. ALB (g/L)	Blood albumin concentrations
16. AST (U/l)	Blood aspartate aminotransferase concentrations (log2-transformed)
17. CK (U/l)	Blood creatine kinase concentrations (log2-transformed)

2.6 Dengue diagnostics and case definitions

The gold standard diagnostic result was a composite derived from three tests; RT-PCR, IgM serology and NS1 detection by ELISA. First, all enrolment plasma samples were tested with a validated, quantitative RT-PCR assay to detect DENV RNA [112]. Next, any enrolment plasma samples that were negative in the RT-PCR assay were tested using the Platelia Dengue NS1 Ag ELISA assay (BioRad) and scored according to the manufacturer's instructions. Samples with equivocal results were repeated and if still equivocal they were scored as negative. Next, IgM ELISA serology (Panbio, Brisbane, Australia) was performed according to the manufacturer's instructions for patients who had paired plasma samples (enrolment and early convalescence) and who were negative in both the DENV RT-PCR assay and Platelia Dengue NS1 ELISA. Any patient who was – a) DENV RT-PCR positive, b) NS1 ELISA positive, or c) had DENV IgM seroconversion in paired plasma samples, was classified as a laboratory-confirmed dengue case. IgM seroconversion was defined as a change in the MAC ELISA test result from negative to positive in paired plasma samples with the 2nd sample collected 6 or more days after illness onset and >2 days after the 1st sample. A positive dengue IgG of either the acute or convalescent blood sample at discharge was defined as "probable secondary infection"; otherwise that both the two samples were negative for dengue IgG was considered as "probable primary infection".

Any patient who was DENV RT-PCR negative, NS1 ELISA negative and did not IgM seroconvert in paired plasma samples was classified as "not dengue". Any patient who was DENV RT-PCR negative and NS1 ELISA negative at the time of enrolment, but did not have paired samples available for serology, was classified as a "presumptive not-dengue" case. For analysis, data from "not dengue" and "presumptive not-dengue" cases were pooled.

The identification of "not dengue" cases allows the diagnostic specificity of the NS1 tests to be assessed. I did not however attempt to collect convalescent blood samples from all patients. Instead, the patients from whom I collected a 2nd sample would be determined by a randomization code and represented 10% of the overall population. In addition, I collected a convalescent blood sample from all hospitalized patients, irrespective of clinical diagnosis, at the time of discharge from hospital. The principal reason for not making the difficult effort to collect a convalescent blood sample from all study participants, and thereby identifying all of the "not dengue" cases, is that I do not believe it is necessary to have such a large population of

"not dengue" cases in which to measure specificity and negative predictive value (NPV), particularly when there is substantial existing literature, including from Vietnam, that describes the excellent specificity of these NS1 antigen detection assays [96, 113-118]. Instead, the pragmatic focus of this study is on measuring the sensitivity of the tests for an early laboratory diagnosis of cases that will develop severe complications and use this as a platform for developing a prognostic algorithm.

2.7 Laboratory methods

Qualitative NS1 ELISA

Bio-Rad Dengue NS1 Ag Platelia ELISA was carried out according to the manufacturer's instructions. Briefly, 50µl of plasma or control sera (including one calibrator, one negative and one positive control sera) were incubated directly and simultaneously with 50µl of diluent and 100µl of diluted conjugate at 37°C in microplate wells for 90 minutes. After washing, 160µl of chromogen solution was added into each well and incubated for 30 minutes in the dark at room temperature. The enzymatic reaction was stopped by adding 100µl of 1N sulphuric acid solution. The optical density (OD) was read at 450/620 nm. The presence of NS1 antigen in each sample was determined by comparing the OD of the sample to the OD of the calibrator (Sample Ratio). The test would be valid if a/ mean of calibrator OD was greater than 0.2, b/ the negative control was lower than 0.4 times the calibrator OD and c/ the positive control was greater than 1.5 times the calibrator OD. Result interpretation was as follows:

- If Sample Ratio <0.5, result was interpreted as negative
- If Sample Ratio >1.0, result was interpreted as positive
- If 0.5 ≤ Sample Ratio ≤ 1.0, result was interpreted as equivocal

Anti- DENV IgM/IgG capture ELISA assay

Briefly, 96- well plates (Maxisorp, Nunc) were coated with 100µl/well of anti-human IgM (A0425, Dako) or anti-human IgG (I2136, Sigma) at a dilution of 1:2000 in 0.05 M carbonate-bicarbonate buffer, pH9.6 (C3041, Sigma) overnight at 4°C. After plates were washed 3 times with phosphate buffer saline–TWEEN (PBST), each well was blocked with 200µl of 3% bovine serum albumin (BSA, A7906, Sigma) in phosphate buffer saline (PBS) for 2 hours at room temperature. The plate was subsequently washed again and then incubated for 2 hours at room temperature with

100µl per well of positive, negative controls or samples which were diluted 1:100 in 0.1% BSA-PBS. After washing, the test was continued by adding 100µl of pooled C6/36 cultures of DENV1-4 antigen to each well and incubating at 4◦C overnight. Next, the plate was washed again and incubated with a cocktail of mouse monoclonal anti-DENV1-4 E protein antibodies (100µl/well) for 1 hour at room temperature. The antigen-antibody complex was detected with 100µl of 1:2000 diluted anti-mouse Ig Horseradish Peroxidase (HRP) (P260, Dako). After the final washing, substrate o-phenylenediamine dihydrochloride (OPD, Sigma) was added the incubated for 30 minutes and the colour metric reaction was stopped by adding 50µl of 10% H2SO4. The optical density (OD) was read at 490nm with Microplate Reader and analysed with Microplate Manager software (Biorad).

Positive control was a mixture of plasma samples from Vietnamese acute dengue patients who had strong positive results with the IgM capture ELISA assay. Negative control was a mixture of plasma from dengue-naïve healthy adult volunteers who had just arrived from regions without known dengue transmission and who had negative results on the Panbio IgG indirect ELISA.

The cut-off value (CO) for positivity was defined as being 5 times higher than the average OD of negative control samples (ODn) after the subtraction of background OD (ODb). The sample ratio (Rs) was calculated by subtracting background OD from the test sample OD (ODs) then dividing by the assay cut-off. In summary, the formulas were calculated as below:

$CO = 5x \text{ mean } (ODn - ODb)$

$Rs = (ODs - ODb)/CO$

The interpretation of results was as follows:

- If RS < 0.8, result was interpreted as negative
- If RS > 1.2, result was interpreted as positive
- If RS from 0.8 – 1.2, result was interpreted as equivocal

One-step real-time multiplex reverse transcription-PCR

The procedure for a one-step real-time multiplex RT-PCR that can identify i) DENV1, DENV3 and Equine Arteritis Virus (EAV), a positive-sense single-stranded RNA virus as an internal control or ii) DENV2, DENV4 and EAV simultaneously was developed and validated.

The specific primers and probes for each DENV serotype and EAV are summarized in Table 2-2. A linearized plasmid containing the cloned target amplicon with a series of 10-fold dilutions was used as standard for the real-time RT-PCR.

The preparation of the PCR master mix is described in Table 2-3. Then, 6µl of RNA previously extracted from the patient's plasma, positive or negative controls, or standard DNA was added to the reaction. Amplification and detection was performed on a LightCycler II PCR machine (Roche, Germany). The DENV RNA was reverse transcribed at 61◦C for 10 minutes, followed by 1 cycle of denaturation at 95◦C for 2 minutes and amplification steps consisting of 45 cycles at 95 ◦C for 15 seconds and 60◦C for 30 seconds, then a fluorescence measurement was made.

Table 2-2. Specific primers and probes for each DENV serotype and EAV in the one-step real-time multiplex RT-PCR

Primer/Probe	Primer/Probe sequences (5'-3')	Position	Product size in base
DENV1- Forward	ATCCATGCCCAYCACCAAT	9865 - 9883	100
DENV1- Reverse	TGTGGGTTTTGTCCTCCATC	9945 - 9964	
DENV2- Forward	TCCATACACGCCAAACATGAA	9859 - 9879	125
DENV2- Reverse	GGGATTTCCTCCCATGATTCC	9963 - 9983	
DENV3- Forward	TTTCTGCTCCCACCACTTTC	9591 - 9610	118
DENV3- Reverse	CCATCCYGCTCCTTGAGA	9691 - 9708	
DENV4- Forward	GYGTGGTGAAGCCYCTRGAT	9587 - 9607	178
DENV4- Reverse	AGTGARCGGCCATCCTTCAT	9744 - 9764	
EAV- Forward	CATCTCTTGCTTTGCTCCTTAG	1847 - 1868	133
EAV- Reverse	CATCTCTTGCTTTGCTCCTTAG	1963 - 1980	
DENV1-Probe	5' (FAM) TCAGTGTGGAATAGGGTTTGGATAGAGGAA 3'(BHQ1)		
DENV2-Probe	5'(FAM) AGGGTGTGGATTCGAGAAAACCCATGG 3'(BHQ1)		
DENV3-Probe	5'(Cyan500) AAGAAAGTTGGTAGTTCCCTGCAGACCCCA 3'(BHQ1)		
DENV4-Probe	5'(Cyan500)TTCCCTCCTCTTYTTGAACGACATGGGAAAGGT G 3'(BHQ1)		
EAV-Probe	5'(Cy5)CGCGCTCGCTGTCAGAACAACATTATTGCCCACAG CGCG 3'(BHQ3)		

Y stands for C or T nucleotide, R for A or G.

Table 2-3. Components of one-step real-time multiplex RT-PCR master mix

Reagents	Working concentration	µl/reaction
Mix of DENV1 (or DENV2) Fw – Rev primers	20µM	1
DENV1 (or DENV2) probe	10µM	0.28
Mix of DENV3 (or DENV4) Fw – Rev primers	20µM	1
DENV3 (or DENV4) probe	10µM	0.28
Mix of EAV Fw - Rev primers	20µM	0.2
EAV probe	10µM	0.08
Activator		1.4
Enhancer	20X	1
RNA master mix	2.7	7.4
H2O		1.36
Total		**14**

The accompanying software of the Lightcycler II PCR machine, LightCycler® 480 SW 1.5, was used to collect and analyse the one-step real-time multiplex RT-PCR results. The units of the RT-PCR are presented as copies/ml. The limit of detection (LOD) of each serotype is presented in the Table 2-4. Viraemia levels that were lower than the LOD were considered to be negative.

Table 2-4. Limit of detection of one-step real-time multiplex RT-PCR assay

	DENV1	DENV2	DENV3	DENV4
LOD (copies/reaction)	5	1	5	10
LOD (copies/ml plasma)	300	60	300	600

2.8 Data cleaning and treatment of missing values

In addition to the quality checks that are used when the data are acquired, data discrepancy reports have been generated on an ongoing basis since the study commenced. In the first step, a team of experienced study doctors and I checked individual CRFs for the consistency and accuracy against the electronic databases. In the second step, we examined the whole database to identify outliers or implausible values, and then traced them back to the source documents for cross-checking if necessary. We also checked for duplicated records of the same patients and missing data. A complete case analysis was used because the amount of missing data was minimal.

2.9 Descriptive and univariate analyses

Continuous data will be summarized as median (interquartile range), categorical data as absolute frequency and percentage. All candidate predictors will be summarized by diagnosis (confirmed dengue vs. non-dengue).

Univariate effects of all candidate predictors on diagnosis will be estimated based on a logistic model and expressed as OR (95% CI), p- value. Predictors have a statistically significant effect in the univariate analysis if the 95 percent confidence interval (95% CI) does not include the value one.

Continuous variables will be properly scaled prior to the univariate analysis such that the OR is clinically interpretable. Continuous variables with a pronounced right-skewed distribution will be log2-transformed prior to analysis which implies that the OR now corresponds to the effect of a two-fold increase of the original variable. Natural cubic spline functions and generalized additive models will be used to assess whether there's a pronounced univariate non-linear relationship between a predictor and the outcome.

Statistical software

All statistical analyses will be performed using the latest version of the statistical software R (R foundation for statistical computing, Vienna, Austria).

Chapter 3

CLINICAL AND VIROLOGICAL FEATURES OF PAEDIATRIC DENGUE IN SOUTHERN VIETNAM, 2010-2013

Summary

We described clinical and virological features of paediatric dengue cases in southern Vietnam in a large prospective cohort study. We used the new classification of WHO 2009 to include dengue shock syndrome, severe bleeding and organ failure in the category of severe dengue in data analysis.

3.1. INTRODUCTION

Community-based cohort studies have been critical in providing insights into dengue disease burden in endemic countries [34, 119-121]. Such studies inform the natural history of dengue, and in particular the ratio of symptomatic and asymptomatic disease. However community cohort studies are not designed to provide deep detail on the clinical and virological characteristics of symptomatic cases, i.e. those who represent the greatest burden to health care services. Most prospective studies of symptomatic dengue have described inpatients alone and have not described the very large burden that is managed on an ambulatory basis. The scale of the ambulatory disease burden is important to understand for considering the economic burden of dengue and the rational assessment of individual and community level disease control interventions. In this study, we describe the characteristics of dengue cases 1 to 15 years old presenting to outpatient clinics at one of the seven hospitals in southern Vietnam. In detail, the objectives of this chapter are to describe the following points:

a) prevalence, clinical features and outcomes of dengue in a cross-sectional study of acute fever in children

b) the oscillations of DENV serotypes according to geography and time in southern Vietnam

c) the association between disease severity, plasma viral RNA concentration and NS1 rapid test results

d) the association between serological response in hospitalized dengue and plasma viral concentration, haematological and biochemical results

3.2. MATERIALS AND METHODS

Details of study design, patient enrolment and data collection, including dengue diagnostics and severity classification, were described in Chapter 2 (Materials and Methods).

For each serotype, we compared plasma viral RNA concentration between dengue categories by using multiple linear regression to calculate odds ratios adjusted for day of illness at enrolment. Logistic regression was used to evaluate the association between disease severity and NS1 rapid test status in conjunction with day of illness at enrolment and infecting serotypes. The relationship between infecting serotypes and hospitalization adjusted for day of illness was assessed by using logistic regression. Differences between primary and secondary infection in terms of plasma viral RNA concentration, haemato-biochemical results were evaluated by using multiple linear regression to calculate adjusted odds ratios for day of illness at enrolment and/or infecting serotypes.

We used the statistical software R version R v3.1.1 (R foundation for statistical computing, Vienna, Austria) for analyses. Significance was assigned at $p < 0.05$ for all parameters and was two-sided.

3.3 RESULTS

3.3.1 Study setting and population

Three hospitals, Children's Hospital 1, Children's Hospital 2 and Hospital for Tropical Diseases in HCMC started patient enrolment in Oct 2010. Children's Hospital 2 then stopped enrolment of patients since 2012 due to a lack of clinical resources in the outpatient setting. Other hospitals subsequently joined the study; Tien Giang Provincial Hospital in the third quarter of 2011, Dong Nai Children's Hospital and Long An Provincial Hospital in the fourth quarter of 2011 and Binh Duong Provincial Hospital in the first quarter of 2012. Figure 3-2 shows the geographic locations of clinical study sites in Ho Chi Minh city (Children's Hospital 1, Children's Hospital 2 and Hospital for Tropical Diseases) and Tien Giang, Dong Nai, Long An, Binh Duong provinces. At each hospital a triage system is in place to direct children with undifferentiated fevers to a selected number of outpatient rooms. These rooms are often staffed by physicians on rotation from the Dengue or Infectious Disease department of each hospital. One of these outpatient examination

rooms at each hospital participated in patient enrolment. Each examination room is staffed by a physician and nurse and is open 8hrs per day (2x4hr shifts), 6 days per week. The case burden is large, with each physician seeing 60-80 patients per 4hr shift.

The study profile of enrolment and patient outcome is shown in Figure 3-1. From Oct 2010 to December 2013 a total of 7563 patients with fever of less than 72 hours were enrolled at one of the seven clinical study sites in southern Vietnam. Of these patients, 2 cases had missing values for haematology and 17 cases had missing values for biochemistry. Of 7544 patients who had complete haematological and biochemical results available, 2060 patients (27.3%) had laboratory-confirmed diagnosis of dengue. The number of cases with severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 117 (5.7%), 156 (7.6%) and 1787 (86.7%) respectively. Among the laboratory-confirmed dengue case population, the number of cases that were RT-PCR positive was 1954 patients (94.9%) of whom 897 (45.9%) were hospitalized. Proportions of RT-PCR positivity in the groups of severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 115/117 (98.3%), 149/156 (95.5%) and 1690/1787 (94.6%) respectively.

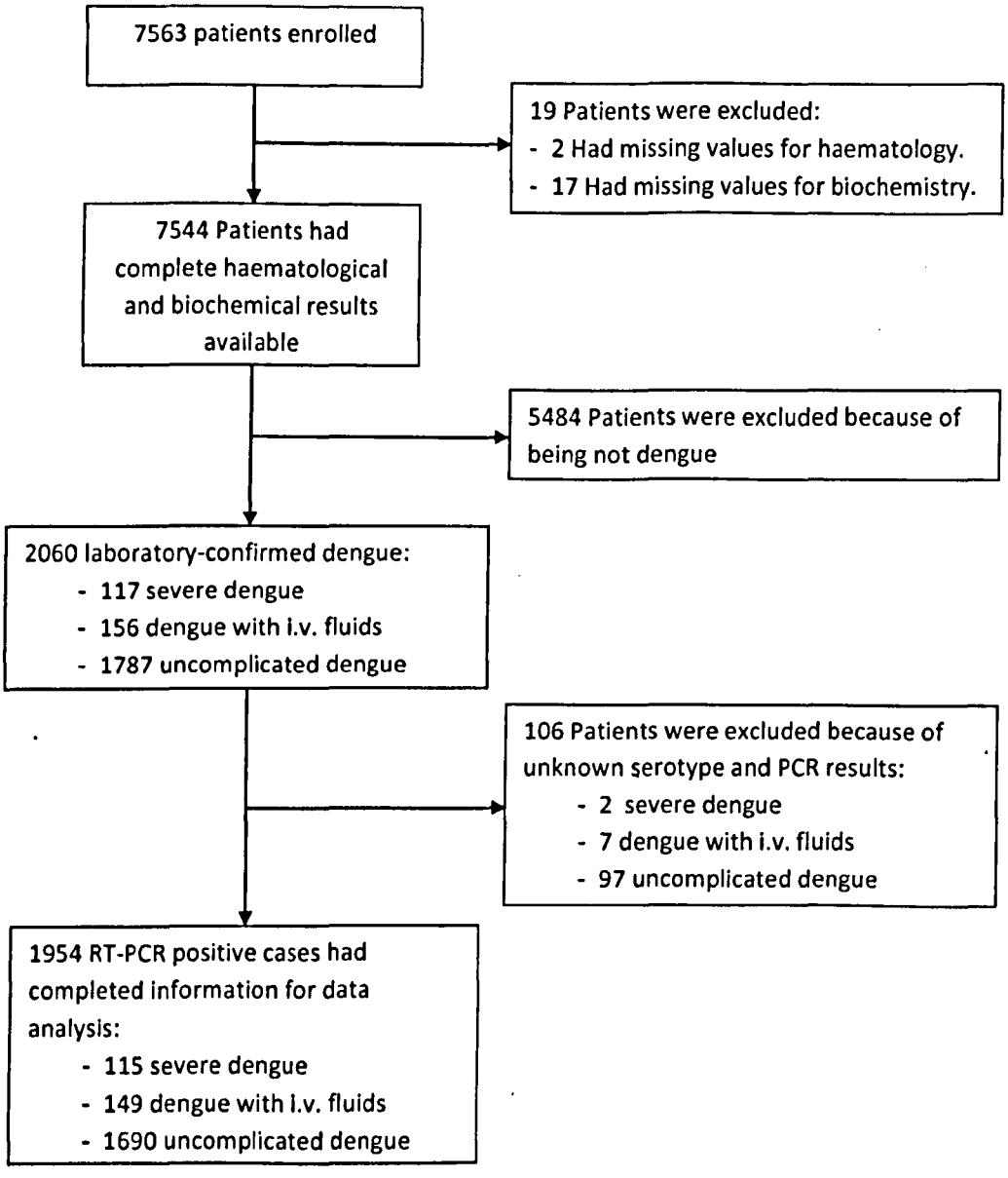


Figure 3-1. Flow chart showing patient enrolment and classification

Figure 3-2. Location of study sites

The upper right corner map shows the geographic location of Vietnam in the Southeast Asian region. The main map shows Ho Chi Minh city (blue), Tien Giang (green), Dong Nai (yellow), Long An (pink) and Binh Duong (dark pink) provinces. Provinces are separated by dark black boundaries. Locations of hospital are marked with red crosses.

3.3.2 Baseline characteristics of the study population

The baseline characteristics of all study participants, and of dengue cases only, stratified by study sites were shown in Table 3-1 and Table 3-2. The percentage of study participants with dengue was reasonably similar across each of the sites, ranging from 21% (Long An Provincial Hospital) to 42% (Children's Hospital 2). The ranges of median age were 5-9 years for all study

participants and 8-11 years for dengue patients across study sites. Interestingly, at all study sites males slightly outnumbered females in the overall study population (% male: 53.6% – 58.8% across study sites) and also in the dengue case population (% male: 53.9% – 60.1%).

The majority of dengue patients were enrolled into the study on day 2 to 3 of illness. While at enrolment symptoms of vomiting (31.3% – 59.1%) and abdominal pain (13.3% – 27.2%) remained similar in dengue patients at all sites, physical signs of skin bleeding, flush and conjunctival injection showed a remarkably difference in percentages, particularly as low as 0% to 3.6% at BDH, TGH, DNH but as high as 15.9% to 76.0% at CH1 and CH2. Quite possibly this reflects the subjective nature of these presentations, i.e. differences in clinical interpretation. Notably, symptoms of mucosal bleeding occurred in dengue patients during the first 3 days of illness at a very low rate from 0.6% to 5.9%, except for patients at CH2 (26.2%). Laboratory tests showed a low white blood cell counts (4.0 – 5.1 $x10^3$ cells/mm^3) in dengue patients at all study sites. The hospitalization rate of dengue patients was from 32.6% – 36.2% (CH1, CH2) to 57.8% – 61.5% (HTD, LAH).

Table 3-1. Baseline characteristics of all study participants by site

	CH1 (N=2158)	CH2 (N=657)	HTD (N=1311)	TGH (N=1037)	DNH (N=1015)	LAH (N=524)	BDH (N=842)
Duration of study conduction	Jan-10 to Dec-13	Jan-10 to Dec-11	Jan-10 to Dec-13	Jun-11 to Dec-13	Nov-11 to Dec-13	Feb-12 to Dec-13	Feb-12 to Dec-13
Age (years)	5 (3-8)	6 (3-9)	7 (5-11)	6 (4-9)	9 (7-11)	8 (5-11)	6 (4-8)
Sex: male	1184 (54.9)	386 (58.8)	758 (57.8)	589 (56.8)	574 (56.6)	305 (58.2)	451 (53.6)
BMI	16.3 (14.3-18.8)	16.7 (14.9-18.9)	15.9 (14.3-18.1)	15.4 (14.1-17.2)	16.0 (14.4-17.9)	15.4 (14.3-17.3)	15.3 (14.1-17.1)
Day of illness							
1	397 (18.4)	282 (42.9)	545 (41.6)	59 (5.7)	447 (44.0)	108 (20.6)	272 (32.3)
2	1136 (52.6)	210 (32.0)	447 (34.1)	542 (52.3)	372 (36.7)	149 (28.4)	359 (42.6)
3	625 (29.0)	165 (25.1)	319 (24.3)	436 (42.0)	196 (19.3)	267 (51.0)	211 (25.1)
Vomiting	580 (26.9)	361 (54.9)	584 (44.5)	436 (42.0)	397 (39.1)	167 (31.9)	275 (32.7)
Abdominal pain	268 (12.4)	148 (22.5)	222 (16.9)	289 (27.9)	250 (24.6)	89 (17.0)	89 (10.6)
Skin bleeding	258 (12.0)	134 (20.4)	11 (0.8)	7 (0.7)	11 (1.1)	18 (3.4)	0 (0)
Mucosal bleeding	79 (3.7)	112 (17.0)	13 (1.0)	12 (1.2)	20 (2.0)	10 (1.9)	12 (1.4)
Flush	598 (27.7)	277 (42.2)	265 (20.2)	8 (0.8)	20 (2.0)	9 (1.7)	7 (0.8)
Injection	171 (7.9)	451 (68.6)	124 (9.5)	3 (0.3)	5 (0.5)	6 (1.1)	6 (0.7)
WBC ($x10^3$ cells/mm^3)	8.0 (5.3-11.8)	7.4 (4.9-10.8)	7.6 (5.2-11.1)	7.1 (4.7-10.5)	7.7 (5.6-10.8)	6.9 (4.6-9.4)	9.0 (6.1-12.4)
Hct (%)	37 (35-39)	37 (36-40)	38 (36-40)	37 (35-40)	40 (38-43)	39 (37-41)	38 (36-40)
PLT ($x10^3$ cells/mm^3)	229 (177-286)	227 (175-286)	224 (181-270)	231 (185-285)	218 (179-262)	221 (179-269)	234 (194-276)
ALB (g/l)	44 (42-46)	45 (43-46)	44 (42-46)	44 (42-46)	45 (43-47)	44 (42-46)	44 (42-46)
AST (U/l)	43 (35-53)	46 (39-58)	41 (31-51)	43 (36-54)	45 (38-55)	45 (37-54)	43 (37-52)
Hospitalization	230 (10.7)	121 (18.4)	429 (32.7)	225 (21.7)	232 (22.9)	133 (25.4)	134 (15.9)
Laboratory-confirmed dengue	540 (25.0)	279 (42.5)	391 (29.8)	283 (27.3)	277 (27.3)	109 (20.8)	181 (21.5)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables. CH1: Children's Hospital 1, CH2: Children's Hospital 2, HTD: Hospital for Tropical Diseases, TGH: Tien Giang Provincial Hospital, DNH: Dong Nai Children's Hospital, LAH: Long An Provincial Hospital, BDH: Binh Duong Provincial Hospital.

Table 3-2. Baseline characteristics of laboratory-confirmed dengue patients by site

	CH1 (N=540)	CH2 (N=279)	HTD (N=391)	TGH (N=283)	DNH (N=277)	LAH (N=109)	BDH (N=181)
Age (years)	9 (5-11)	8 (5-11)	10 (7-13)	8 (6-10)	10 (8-12)	11 (8-13)	8 (6-11)
Sex: male	291 (53.9)	161 (57.7)	235 (60.1)	162 (57.2)	158 (57.0)	59 (54.1)	98 (54.1)
BMI	17.0 (14.7-19.7)	17.4 (15.2-19.8)	16.6 (14.8-18.9)	15.4 (14.1-17.7)	16.6 (15.2-18.9)	16.4 (14.8-18.5)	15.8 (14.1-18.3)
Day of illness							
1	74 (13.7)	87 (31.2)	108 (27.6)	18 (6.4)	92 (33.2)	12 (11.0)	52 (28.7)
2	246 (45.6)	94 (33.7)	147 (37.6)	124 (43.8)	118 (42.6)	27 (24.8)	82 (45.3)
3	220 (40.7)	98 (35.1)	136 (34.8)	141 (49.8)	67 (24.2)	70 (64.2)	47 26.0)
Vomiting	169 (31.3)	165 (59.1)	195 (49.9)	121 (42.8)	123 (44.4)	38 (34.9)	78 (43.1)
Abdominal pain	86 (15.9)	73 (26.2)	76 (19.4)	77 (27.2)	63 (22.7)	22 (20.2)	24 (13.3)
Skin bleeding	137 (25.4)	99 (35.3)	9 (2.3)	6 (2.1)	8 (2.9)	14 (12.8)	0 (0)
Mucosal bleeding	32 (5.9)	73 (26.2)	5 (1.3)	5 (1.8)	4 (1.4)	2 (1.8)	1 (0.6)
Flush	191 (35.4)	149 (53.4)	119 (30.4)	4 (1.4)	10 (3.6)	7 (6.4)	5 (2.8)
Injection	86 (15.9)	212 (76.0)	57 (14.6)	1 (0.4)	2 (0.7)	3 (2.8)	3 (1.7)
WBC ($x10^3$ cells/mm^3)	4.7 (3.4-6.7)	5.1 (3.6-7.6)	4.9 (3.5-7.0)	4.3 (3.4-6.1)	5.6 (4.1-7.3)	4.0 (3.2-5.5)	5.6 (4.0-7.4)
Hct (%)	38 (36-40)	38 (36-40)	39 (37-40)	39 (36-41)	41 (38-43)	40 (38-41)	39 (37-40)
PLT ($x10^3$ cells/mm^3)	169 (130-216)	192 (146-243)	180 (138-221)	182 (145-225)	180 (141-222)	170 (122-212)	192 (149-236)
ALB (g/l)	44 (41-46)	45 (42-46)	44 (42-46)	44 (43-46)	44 (42-46)	44 (41-45)	43 (41-45)
AST (U/l)	52 (40-76)	53 (41-69)	47 (38-62)	53 (43-73)	51 (42-69)	51 (42-70)	49 (41-62)
Hospitalization	176 (32.6)	101 (36.2)	226 (57.8)	143 (50.5)	152 (54.9)	67 (61.5)	78 (43.1)
Outcomes							
-Severe dengue	35 (6.5)	14 (5.0)	19 (4.9)	13 (4.6)	20 (7.2)	7 (6.4)	9 (5.0)
-Dengue with i.v. fluids	35 (6.5)	22 (7.9)	44 (11.3)	43 (15.2)	4 (1.4)	2 (1.8)	6 (3.3)
-Uncomplicated dengue	470 (87.0)	243 (87.1)	328 (83.9)	227 (80.2)	253 (91.3)	100 (91.7)	166 (91.7)
Serotype							
-DENV-1	193 (38.4)	116 (42.8)	160 (42.7)	77 (29.4)	110 (40.7)	17 (17.0)	94 (54.3)
-DENV-2	119 (23.7)	86 (31.7)	100 (26.7)	61 (23.3)	39 (14.4)	18 (18.0)	21 (12.1)
-DENV-3	56 (11.1)	39 (14.4)	36 (9.6)	8 (3.1)	16 (5.9)	2 (2.0)	31 (17.9)
-DENV-4	135 (26.8)	30 (11.1)	79 (21.1)	116 (44.3)	105 (38.9)	63 (63.0)	27 (15.6)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables. The number of unknown serotype was 37, 8, 16, 21, 7, 9 and 8 cases at CH1, CH2, HTD, TGH, DNH, LAH and BDH respectively. Dengue with i.v. fluids was hospitalized non-severe dengue with fluid infusion. Uncomplicated dengue included non-severe dengue and dengue without fluid infusion. CH1: Children's Hospital 1, CH2: Children's Hospital 2, HTD: Hospital for Tropical Diseases, TGH: Tien Giang Provincial Hospital, DNH: Dong Nai Children's Hospital, LAH: Long An Provincial Hospital, BDH: Binh Duong Provincial Hospital.

3.3.3 Characteristics and outcomes in dengue cases

The final clinical outcome for dengue cases is summarised in Figure 3-3. Most of the severe dengue was due to DSS without any other severe complication. Of 117 severe dengue cases, 101 patients were due to DSS, 10 patients had severe bleeding and 6 patients suffered from respiratory distress only. However 27 out of 101 DSS cases were accompanied by another severe manifestation such as severe bleeding (7 cases), respiratory distress (14 cases), or both (6 cases). A more detailed description of these subcategories including DSS, severe bleeding and respiratory distress in the group with severe dengue is presented in Table 3-3A, 3-3B and 3-3C.

The characteristics of patients in the "dengue with i.v. fluids" group is described in Table 3-4. The majority of intravenous fluid therapy was given on day 4 (24.8%), 5 (40.3%) and 6 (20.1%) of illness. The reasons for giving infusion to these dengue patients were one or more of the following causes including haemoconcentration (59.7%), persistent vomiting (46.3%), anorexia (20.8%) and dehydration (18.1%). Notably, of the 149 patients in the dengue with i.v. fluids group who had positive RT-PCR result, 32 (21.5%) required to switch from using crystalloid to colloid during the fluid replacement.

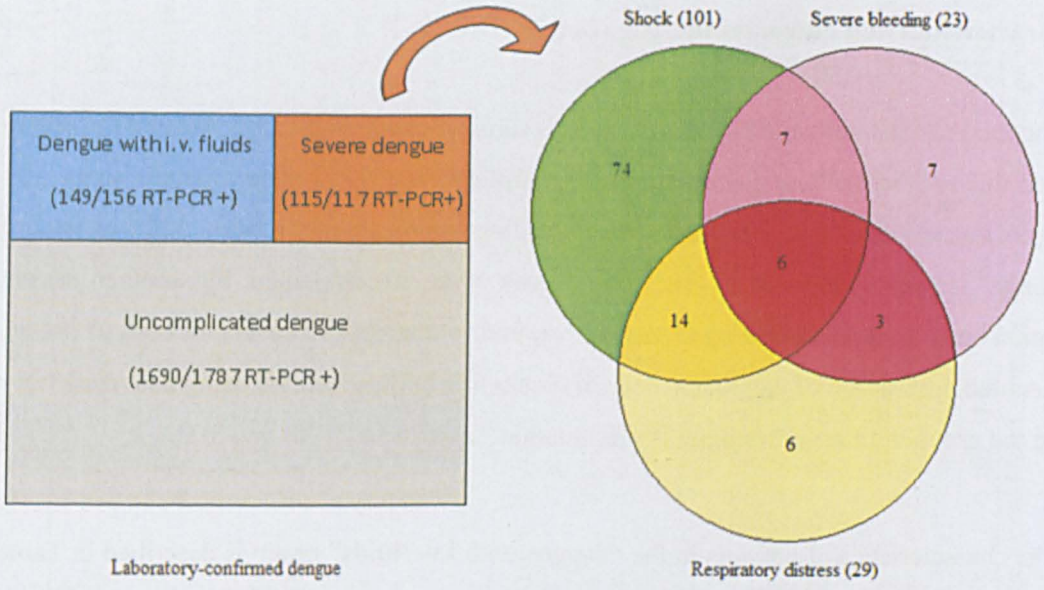


Figure 3-3. Final outcome of dengue patients

The square picture shows the outcomes of patients who were laboratory-confirmed dengue. The number of cases with severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 117 (5.7%), 156 (7.6%) and 1787 (86.7%) respectively. Proportions of RT-PCR positivity in the groups of severe dengue, dengue requiring parenteral fluids and uncomplicated dengue was 115/117 (98.3%), 149/156 (95.5%) and 1690/1787 (94.6%) respectively. The venn diagram shows 117 severe dengue cases with shock (green), severe bleeding (pink) or respiratory distress (light yellow).

Table 3-3A. Characteristics of severe dengue – Dengue shock syndrome

Shock (n=101)	Median/ n	IQR/ %
Age	9	(7-11)
Sex: Male	54	53.5
BMI	16.9	(14.3-20.1)
Day of illness at enrolment		
1	15	14.9
2	38	37.6
3	48	47.5
Day of illness at shock		
3	4	4.0
4	28	27.7
5	49	48.5
6	18	18.2
7	2	2.0
At enrolment		
- Vomiting	66	65.3
- Abdominal pain	29	28.7
- Skin bleeding	17	16.8
- Mucosal bleeding	7	6.9
- Flush	31	30.7
- Injection	19	18.8
Systolic blood pressure (mmHg)	95	(90-100)
Diastolic blood pressure (mmHg)	75	(68-80)
Pulse pressure (mmHg)	20	(20-20)
Pulse rate at shock (beat/min)	112	(100-120)
Pulse status		
- Undetectable	4	4.0
- Weak	76	75.2
- Strong	21	20.8
Poor peripheral perfusion	101	100
Re-shock	18	17.8
WBC at enrolment (x 10^3 cells/mm^3)	3.7	(2.5-5.8)
Hct at enrolment (%)	40	(38-42)
Hct highest (%)	49	(45-52)
Day of illness at highest Hct	5	(4-5)
PLT at enrolment (x10^3 cells/mm^3)	111	(89-147)
PLT nadir (x10^3 cells/mm^3)	39	(25-62)
Day of illness at lowest PLT	5	(4-6)
ALB (g/l)	43	(40-45)
AST (U/l)	100	(58-156)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables.

Table 3-3B. Characteristics of severe dengue – Severe bleeding

Severe bleeding (n=10)	Median/ n	IQR/ %
Age (years)	8	(6-11)
Sex: Male	8	80.0
BMI	16.9	(15.0-18.8)
Day of illness at enrolment		
1	0	0
2	3	30.0
3	7	70.0
Day of illness at severe bleeding		
4	5	50.0
5	3	30.0
6	2	20.0
At enrolment		
- Vomiting	8	80.0
- Abdominal pain	3	30.0
- Skin bleeding	2	20.0
- Mucosal bleeding	1	10.0
- Flush	3	30.0
- Injection	2	20.0
Sites of severe bleeding		
- GI bleeding	9	90.0
- Nose bleeding and venipuncture sites	1	10.0
Required blood product transfusion	3	30.0
Required local packing or compression	3	30.0
WBC at enrolment (x 10^3 cells/mm^3)	3.3	(2.7-5.4)
Hct at enrolment (%)	38	(37-42)
Hct highest (%)	44	(42-47)
Day of illness at highest Hct	4	(4-6)
PLT at enrolment (x10^3 cells/mm^3)	113	(89-158)
PLT nadir (x10^3 cells/mm^3)	34	(19-74)
Day of illness at lowest PLT	5	(5-6)
ALB (g/l)	44	(42-46)
AST (U/l)	113	(63-140)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables.

Table 3-3C. Characteristics of severe dengue – Respiratory distress only

Respiratory distress only (n=6)	Median/ n	IQR/ %
Age (years)	4	(3-9)
Sex: Male	4	66.7
BMI	16.9	(14.4-21.1)
Day of illness at enrolment		
1	0	0
2	2	33.3
3	4	66.7
Day of illness at respiratory distress		
4	2	33.3
5	1	16.7
6	2	33.3
7	1	16.7
At enrolment		
- Vomiting	5	83.3
- Abdominal pain	3	33.3
- Skin bleeding	1	16.7
- Mucosal bleeding	1	16.7
- Flush	2	33.3
- Injection	1	16.7
Cause of respiratory distress		
- Pleural effusions and ascites	4	66.7
- Pleural effusions only	1	16.7
- Ascites only	1	16.7
WBC at enrolment (x 10^3 cells/mm^3)	4.5	(2.7-6.9)
Hct at enrolment (%)	41	(39-43)
Hct highest (%)	48	(46-50)
Day of illness at highest Hct	4	(4-5)
PLT at enrolment (x10^3 cells/mm^3)	74	(53-109)
PLT nadir (x10^3 cells/mm^3)	47	(36-57)
Day of illness at lowest PLT	5	(3-5)
ALB (g/l)	42	(40-44)
AST (U/l)	130	(94-160)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables.

Table 3-4. Characteristics of dengue patients with i.v. fluids

Dengue with i.v. fluids (n=149)	Median/ n	IQR/ %
Age (years)	9	(7-11)
Sex: Male	75	50.3
BMI	16.1	(14.4-19.0)
Day of illness at enrolment		
1	29	19.5
2	55	36.9
3	65	43.6
Day of illness at first infusion		
2	4	2.7
3	13	8.7
4	37	24.8
5	60	40.3
6	30	20.1
7	4	2.7
8	1	0.7
At enrolment		
- Vomiting	89	59.7
- Abdominal pain	48	32.2
- Skin bleeding	22	14.8
- Mucosal bleeding	9	6.0
- Flush	47	31.5
- Injection	33	22.1
Reason for infusion		
- Haemoconcentration	89	59.7
- Persistent vomiting	69	46.3
- Anorexia	31	20.8
- Dehydration	27	18.1
Infusion by crystalloid only	117	78.5
Infusion by crystalloid and colloid	32	21.5
Total volume (ml/kg)	78	(24-113)
WBC at enrolment (x 10^3 cells/mm^3)	4.4	(3.2-6.4)
Hct at enrolment (%)	39	(37-41)
Hct highest (%)	44	(42-47)
Day of illness at highest Hct	5	(4-6)
PLT at enrolment (x10^3 cells/mm^3)	147	(111-195)
PLT nadir (x10^3 cells/mm^3)	65	(38-95)
Day of illness at lowest PLT	5	(4-6)
ALB (g/l)	43	(41-46)
AST (U/l)	58	(46-80)

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables. Dengue with i.v. fluids was hospitalized non-severe dengue with fluid infusion.

3.3.4 Natural history of time to events in dengue cases

For hospitalized cases, severe dengue patients had a longer illness history at enrolment compared to other groups with dengue and non-dengue (median day of illness (IQR): 3 (2-3) vs. 2 (2-3) and 2 (1-3) days respectively). Patients with severe dengue were also hospitalized sooner after enrolment compared to those with dengue and non-dengue (median days from enrolment to admission (IQR): 1 (0-2) vs. 1 (1-2) and 2 (0-3) days respectively). (Table 3-5). The median day of illness at severe complications was 5 (IQR: 4-5).

Table 3-5. Relationship between day of illness, day of hospitalisation and onset of severe signs/symptoms in study participants

	Severe dengue (N=117)	Dengue (N=826)	Non-dengue (N=561)	p
Day of illness at enrolment	3 (2-3)	2 (2-3)	2 (1-3)	<0.001
1	15 (12.8)	159 (19.2)	179 (31.9)	
2	43 (36.8)	327 (39.6)	207 (36.9)	
3	59 (50.4)	340 (41.2)	175 (31.2)	
Days from enrolment to hospital admission	1 (0-2)	1 (1-2)	1 (0-2)	<0.001
0	30 (25.6)	202 (24.5)	162 (28.9)	
1	34 (29.1)	286 (34.6)	255 (45.5)	
2	29 (24.8)	187 (22.6)	88 (15.7)	
3	14 (12.0)	102 (12.3)	31 (5.5)	
4	9 (7.7)	35 (4.2)	20 (3.6)	
5	1 (0.9)	12 (1.5)	4 (0.7)	
6	-	2 (0.2)	1 (0.2)	
Day of illness at first onset of severe complications	5 (4-5)	-	-	
3	4 (3.4)	-	-	
4	35 (29.9)	-	-	
5	53 (45.3)	-	-	
6	22 (18.8)	-	-	
7	3 (2.6)	-	-	

Data are expressed as median (interquartile range) for continuous variables or frequency and percentage for categorical variables. P- values were given by Chi-square tests.

3.3.5 Temporal and spatial distribution of DENV serotypes

DENV-1 and DENV-4 accounted for 39% (767/1954) and 28% (555/1954) respectively of all RT-PCR dengue positive cases. DENV-2 was identified in 444 (23%) and DENV-3 in 188 (10%) patients. The distribution of dengue serotypes by sites during the entire study period is shown in Table 3-2 and Figure 3-4. In HCMC, DENV-1 and DENV-2 were the predominant serotypes in 2010 and 2011. Interestingly, 2011 saw the emergence of DENV-4, first in Tien Giang Provincial Hospital but subsequently in all study sites during 2012/13 such that by 2013 it was the 2nd most commonly detected serotype in that year. On the contrary, a gradual but steady continuous decrease in the prevalence of DENV-2 took place at all study sites from 2011 to 2013. The number of laboratory-confirmed dengue cases in 2013 was also lowest compared to that of previous years and this was consistent with Ministry of Health surveillance data indicating 2013 was a relatively low year for dengue case burden (See Figure 1-5 in the Introduction). These data suggest that Vietnam is a dengue endemic country that experiences the co-circulation of four viral serotypes, each of which may differ in their epidemic potential.

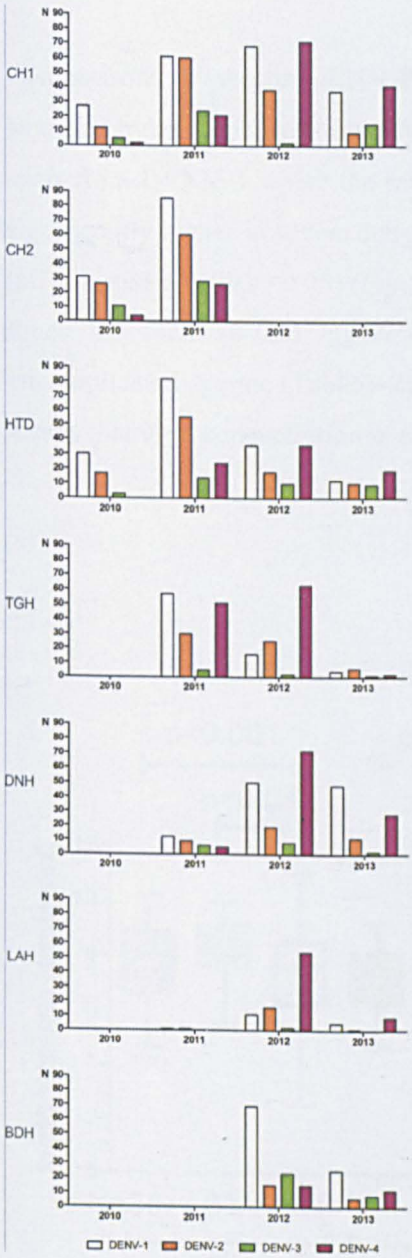


Figure 3-4. Number of dengue cases and infecting serotypes, stratified by hospital and year. *X-axis: time, Y-axis: number of dengue cases. CH1: Children's Hospital 1, CH2: Children's Hospital 2, HTD: Hospital for Tropical Diseases, TGH: Tien Giang Provincial Hospital, DNH: Dong Nai Children's Hospital, LAH: Long An Provincial Hospital, BDH: Binh Duong Provincial Hospital. CH2 stopped enrolment of patients since 2012. TGH started patient enrolment in the third quarter of 2011; DNH and LAH started patient enrolment in the fourth quarter of 2011; BDH started patient enrolment in the first quarter of 2012.*

3.3.6. Relationship between clinical outcome and DENV serotype, plasma DENV RNA concentration and NS1 rapid test results at enrolment

Table 3-6. Relationship between DENV serotype and disease outcome

	Severe dengue (N=115)			Dengue with i.v. fluids (N=149)			Hospitalized dengue (N=897)		
	n (%)	OR (95%CI)	p	n (%)	OR (95%CI)	p	n (%)	OR (95%CI)	p
DENV-1 (N=767)	38 (33.0)	0.8 (0.5, 1.3)	0.352	62 (41.6)	1.5 (0.9, 2.3)	0.184	367 (40.9)	1.0 (0.8, 1.3)	0.785
DENV-2 (N=444)	37 (32.2)	1.4 (0.9-2.3)	0.058	47 (31.5)	2.1 (1.3, 3.3)	0.028	199 (22.2)	0.9 (0.7, 1.2)	0.696
DENV-3 (N=148)	6 (5.2)	0.5 (0.2, 1.2)	0.416	9 (6.0)	0.8 (0.4, 1.8)	0.583	68 (7.6)	0.6 (0.4, 0.9)	0.034
DENV-4 (N=555)	34 (29.6)	1	-	31 (20.8)	1	-	263 (29.3)	1	-

Reference group: DENV-4

Comparisons of plasma DENV RNA concentrations between clinical outcome categories and stratified by serotype were done by using logistic regression adjusted for day of illness. Generally, apart from DENV-3 where the sample size was limited, plasma RNA viral concentrations were significantly higher in severe dengue cases than in uncomplicated dengue cases for any serotypes (all adjusted p-values <0.05) (Figure 3-5). The odds of having severe dengue increased by 1.3-1.7 times for each 10-fold higher DENV RNA concentration as compared to patients with uncomplicated dengue (Table 3-7) and this was statistically significant for DENV-1, -2 and -4. It means that viral concentration is associated with dengue severity.

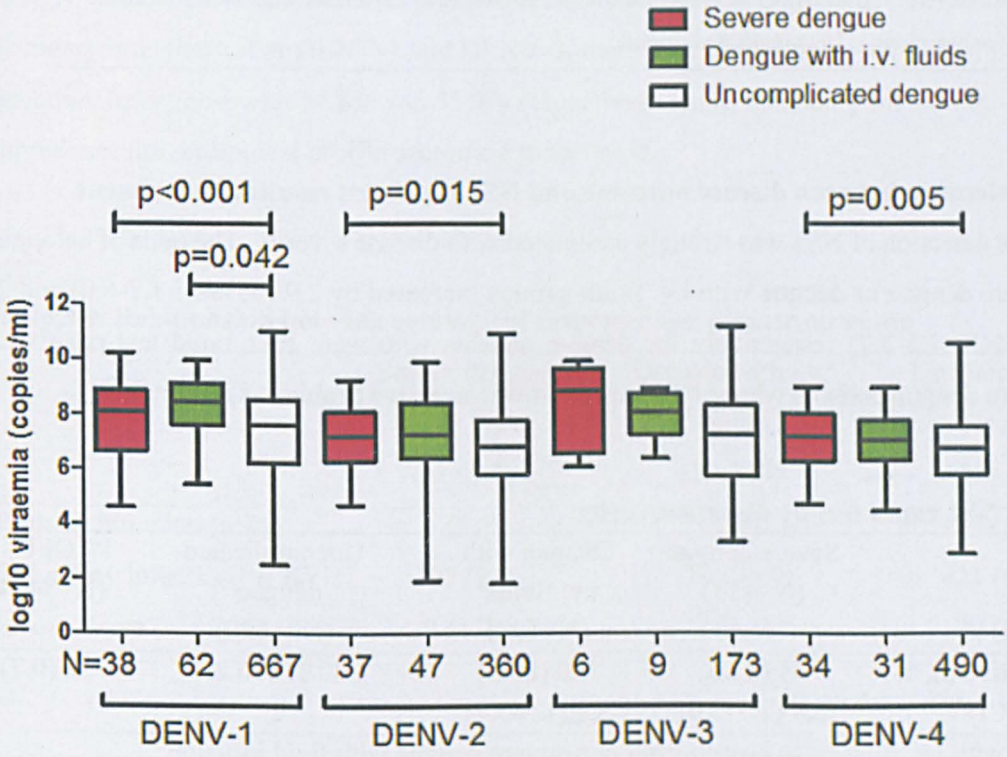


Figure 3-5. Plasma viral RNA concentration by disease severity.
Box and whisker plots of plasma viraemia levels in enrolment samples represent median, 25th and 75th percentile, minimum and maximum of the data by DENV serotypes and severity. P-values were adjusted for day of illness. The numbers of cases in each dengue category by different infecting serotypes are shown on the X-axis.

Table 3-7. Relationship between plasma viraemia and dengue severity stratified by serotypes

	Severe dengue (N=115)	Dengue with i.v. fluids[a] (N=149)	Uncomplicated dengue[b] (N=1690)
OR (95%CI)[c]			
DENV-1	1.3 (1.1-1.6)	1.7 (1.4-2.1)	1
DENV-2	1.4 (1.1-1.8)	1.2 (0.9-1.5)	1
DENV-3	1.7 (1.0-3.0)	1.4 (0.9-2.2)	1
DENV-4	1.6 (1.2-2.2)	1.3 (0.9-1.8)	1

[a] Dengue with i.v. fluids was hospitalized non-severe dengue with fluid infusion.

[b] Uncomplicated dengue included non-severe dengue and dengue without fluid infusion.

[c] OR: Adjusted odd ratios for day of illness to assess the odds of having severe dengue or dengue with i.v. fluids for each 10-fold higher DENV RNA concentrations as compared to patients with uncomplicated dengue. Reference group = Uncomplicated dengue.

3.3.7 Relationship between disease outcome and NS1 rapid test results at enrolment

The detection of NS1 was strongly associated with disease severity. The odds of belonging to the severe dengue or dengue with i.v. fluids groups increased by 2.9 (95%CI: 1.7-5.0) and 2.4 times (95%CI: 1.5-3.7) respectively for dengue patients who were NS1 rapid test positive as compared to dengue patients whose NS1 rapid test was negative (Table 3-8).

Table 3-8. NS1 rapid test by disease severity

	Severe dengue (N=115)	Dengue with i.v. fluids[a] (N=149)	Uncomplicated dengue[b] (N=1690)	OFI[c] (N=5484)
NS1 Positive (n, %)	95 (82.6)	120 (80.5)	1187 (70.2)	32 (0.7)
OR (95%CI)[d]	2.9 (1.7-5.0)	2.4 (1.5-3.7)	1	-

[a] Dengue with i.v. fluids was hospitalized non-severe dengue with fluid infusion.

[b] Uncomplicated dengue included non-severe dengue and dengue without fluid infusion.

[c] OFI: Other febrile illnesses.

[d] OR: Adjusted odd ratios for day of illness and serotype. Reference group = Uncomplicated dengue.

3.3.8 Relationship between serological response in hospitalized dengue with DENV serotype, plasma viral concentration and laboratory results.

Classification of serological responses (i.e. primary versus secondary) was only possible for hospitalized patients as only these participants had paired plasma specimens for serological investigations. Of the 897 hospitalized dengue cases with infecting serotypes identified by RT-PCR, 204 (22.7%) were primary and 647 (72.1%) were secondary infection while in the remaining 46 cases (5.1%) the results of serological diagnostics for immune status were inconclusive. Nighty-three % of the severe dengue cases and 79.9% of the "dengue with i.v. fluids" group were secondary infections respectively (Table 3-9). By serotype, secondary infections were most strongly associated with DENV-2 and DENV-4, with 87.9% and 88.6% respectively being secondary infections. For DENV-1 and DENV-3, there was a more balanced mix of primary and secondary infections, with 54.8% and 55.9% respectively being secondary infections. Figure 3-6 summarises the serological profile according to serotype.

Table 3-9. Relationship between serological response and disease outcome

	Severe dengue (N=115)	Dengue with i.v. fluids (N=149)	Uncomplicated dengue (N=633)
Primary infection (n, %)	6 (5.2)	25 (16.8)	173 (27.3)
Secondary infection (n, %)	107 (93)	119 (79.9)	421 (66.5)
Inconclusive (n, %)	2 (1.7)	5 (3.4)	39 (6.2)

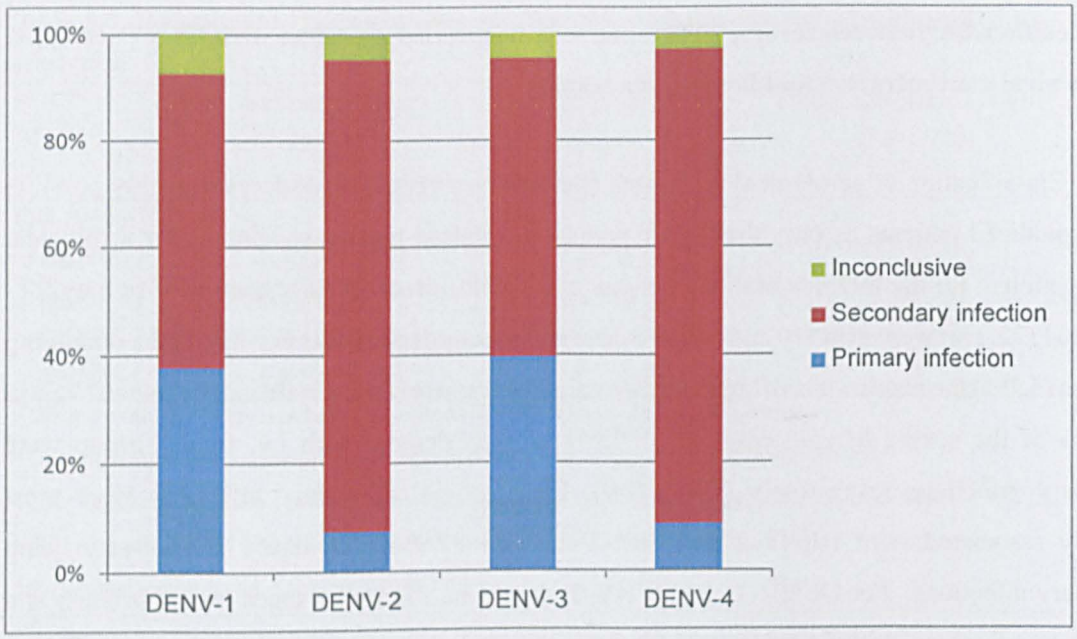


Figure 3-6. Serological response according to dengue serotype

Among hospitalized dengue patients with the same duration of illness history there was no difference in plasma viral concentration between primary and secondary infection in overall as well as in those due to DENV-1, DENV-2 and DENV-3 (all adjusted p-values >0.05). However DENV-4 infected patients had significant higher plasma viraemia levels in secondary than primary infection (median and interquartile range (IQR), 7.0 (6.1-7.8) vs. 6.7 (5.4-7.3) log10copies/ml respectively, adjusted p=0.013) (Table 3-10).

Table 3-10. Plasma viral RNA concentration vs. serological response in hospitalized dengue cases

	All hospitalized dengue [a] Median (IQR) N	Primary infection Median (IQR) N	Secondary infection Median (IQR) N	p
All serotypes [b]	7.4 (6.4-8.4) 851	7.8 (6.6-8.7) 204	7.3 (6.3-8.2) 647	0.124
DENV-1 [b]	8.0 (6.8-8.8) 340	8.1 (6.8-8.9) 139	8.0 (6.8-8.7) 201	0.748
DENV-2 [b]	7.2 (6.3-8.0) 190	7.6 (6.1-8.2) 15	7.2 (6.3-8.0) 175	0.559
DENV-3 [b]	7.8 (6.4-8.7) 65	7.4 (6.6-9.5) 27	7.8 (6.3-8.7) 38	0.935
DENV-4 [b]	6.9 (6.1-7.7) 256	6.7 (5.4-7.3) 23	7.0 (6.1-7.8) 233	0.013

[a] 46 hospitalized patients were excluded, of which the number of DENV-1, DENV-2, DENV-3 and DENV-4 serotype were 27, 9, 3, and 7 cases respectively, because of having inconclusive serological responses.

[b] log10copies/ml

P –values were adjusted for day of illness to compare viraemia between primary and secondary infection.

Compared to those with primary infections, at enrolment patients with secondary infections had lower median (IQR) albumin levels (43 (41-45) vs. 44 (42-47) g/l), white blood cell counts (4.4 (3.1-6.3) vs. 5.0 (3.5-6.8) $x10^3$ cells/mm^3), platelet counts (146 (109-184) vs. 181 (145-217) $x10^3$ cells/mm^3), higher AST (59 (45-85) vs. 49 (40-60) U/l) and higher years of age (10 (7-12) vs. (8 (6-10) years) (all adjusted p-values <0.05). Patients with secondary infections also had significantly lower median platelet nadir during hospitalization than patients with primary infections (62 (25-91) vs. 95 (46-138) $x10^3$ cells/mm^3) (Table 3-11). These data suggest that secondary infection have more severe manifestations compared to primary infection.

Table 3-11. Serological response vs. haematology, biochemistry in hospitalized dengue cases

	Primary infection (N=204)	Secondary infection (N=647)	p
At enrolment			
Sex: male (n, %)	122 (59.8)	335 (51.8)	0.045
Age (years)	8 (6-10)	10 (7-12)	0.011
ALB (g/l)	44 (42-47)	43 (41-45)	0.001
AST (U/l)	49 (40-60)	59 (45-85)	<0.001
WBC (x10^3 cells/mm^3)	5.0 (3.5-6.8)	4.4 (3.1-6.3)	0.041
Hct (%)	39 (37-41)	40 (37-42)	0.142
PLT (x10^3 cells/mm^3)	181 (145-217)	146 (109-184)	<0.001
During hospitalization			
Hct highest (x10^3 cells/mm^3)	41 (38-43)	43 (39-45)	0.280
PLT nadir (x10^3 cells/mm^3)	95 (46-138)	62 (25-91)	<0.001

Data are expressed as median (interquartile range) for continuous variables.

3.4 DISCUSSION

This large, prospective, outpatient-based study of dengue in southern Vietnam has provided a relatively unbiased clinical and virological profile of the disease burden seen by local health services. The results confirm that DSS is the commonest severe complication of dengue in children in this setting and that it occurs in ~5% of cases. The results also highlight the burden of dengue; 45.9% of all RT-PCR positive dengue cases in this study were hospitalized at some point in their illness and 13.5% required therapeutic interventions because of severe dengue or dengue with i.v. fluids at some point in their hospitalization. For the first time we have estimated, with a high level of precision, the contribution that increased (early) DENV viraemia, or the presence of a detectable NS1 antigenaemia, has on the likelihood of developing severe dengue. Furthermore, we have been able to describe serotype-specific differences in risk of clinical complications and in the profile of the disease burden. For example, primary infections with DENV-2 and DENV-4 contributed a negligible amount to the hospitalized disease burden during the study period. Our findings were in agreement with previous publications suggesting that DENV-2 appears to be significantly associated with more severe dengue compared to other serotypes [110, 122].

The great majority of dengue cases in this study were aged from 5 to 13 years. Approximately 45.9% of dengue cases were hospitalized (versus 10.2% of children with other febrile illness). Children who were hospitalized with secondary dengue were older than children hospitalized with primary infection. The age of the dengue case burden in this study (median age 9 (IQR: 6-11) years) is entirely consistent with previous hospital-based data in this setting between 2000-2009. This indicates that despite substantial socioeconomic development in southern Vietnam in the last 15 years there is no evidence that the force of infection is declining. Vietnam's dengue control program depends up the surveillance system including vector control, virological monitoring and case management. Probable explanations for why the national control program has failed to reduce the number of infected patients are the presence of unnoticed infection in the community, changes of virus transmission dynamics and the absence of effective interventions to eliminate all breeding grounds to mosquito. The difficulty in controlling dengue using existing tools underscores the need for vaccines and other novel control methods (e.g. *Wolbachia*).

Most of the early clinical manifestations of dengue are nonspecific until the disease progresses to severe complications or resolves spontaneously without complications. Of note, there was considerable variation in the incidence of physician-reported signs such as skin-bleeding, flush and conjunctival injection in dengue patients. This highlights the subjective nature of the manifestations. Eighty-five percent of dengue cases would have met the WHO 2009 case definition. The dengue case definition in Vietnam is also the same as that from WHO and is now widely used throughout the country. The next chapter will consider whether any of the early clinical or laboratory features of dengue can be employed in a diagnostic algorithm to further enhance early diagnosis.

DSS represents the commonest severe dengue complication in children followed by respiratory distress and severe bleeding. This finding is similar to previous reports which showed severe dengue in children was mainly due to DSS rather than severe haemorrhage [36, 123]. Of note, while confirmed dengue patients with DSS or severe bleeding occurred at older age (median: 8-9 years) those with respiratory distress occurred at younger age (median: 4 years). This is because the compensation of the respiratory system in young children is fragile and more susceptible to being deteriorating in case of plasma extravasation and third-space fluid loss. Most respiratory distress was due to pleural effusion alone or in combination with ascites and sometimes due to overhydration in fluid replacement. Most severe bleeding was due to gastrointestinal

bleeding. The great majority (93%) of severe cases had secondary infections and DENV-2 was proportionally more common in severe cases than other serotypes. Watts et al found that secondary DENV2 infections with the Asian genotype were more likely to cause severe dengue than the American genotype [124]. It has been hypothesized that more efficient DENV-2 replication in primed hosts confers enhanced pathogenicity [110]. The increase in susceptibility to secondary infection could be explained by the co-circulation of 4 dengue serotypes in Vietnam with sequential replacement of the dominant serotype [20, 125]. The majority of hospitalized dengue patients were due to secondary infection accounting for 76% (647/851) in our study which was similar to other reports in Southeast Asia [55, 119, 126, 127]. We found that haematological and biochemical values indicated a more severe status in patients with secondary infections, including lower albumin levels, white blood cell counts, platelet counts and higher AST at enrolment together with lower platelet nadir during hospitalization as compared to patients with primary infections. These findings agree to other studies and are consistent with the antibody dependent enhancement theory as well as its role in pathogenesis [56]. Libraty and Duyen *et al.* reported the association of secondary infection and thrombocytopenia [111, 128]. Other colleagues in the Caribbean and Pakistan found the correlation between elevated serum enzymes levels, decreased platelet count and secondary dengue infection [129, 130]. The reason for lower albumin in secondary than in primary infection is explained by a greater extent of plasma leakage in secondary than in primary infection.

Of note, no dengue cases died in this study. This is reflective of the overall low mortality rate for dengue in Vietnam, estimated at below 1/1000 hospitalised cases. This low case mortality has arisen because of the skills and experience of physicians managing dengue cases in southern Vietnam and clear hospital guidelines on fluid management. In this study the main indications for parenteral fluid infusion included haemoconcentration and persistent vomiting, both of which are considered dengue warning signs according to WHO guidelines [1]. Persistent vomiting can exacerbate haemoconcentration, a manifestation of plasma leakage which can result in hypovolemia and DSS [131]. Our study showed that colloids were also used in volume replacement in combination with crystalloids. All therapeutic modalities to dengue infection are symptomatic as regards DSS or severe bleeding by replacement of fluid loss with crystalloid/colloid solutions or blood-derived products. Consequently, it is recommended by WHO that dengue patients with vasculopathy should be given carefully-titrated crystalloid fluid to

maintain hemodynamic stability. Colloids are recommended when crystalloid fluid infusion has not elicited the desired clinical response [1, 49, 51, 132, 133].

The Ministry of Health dengue surveillance program showed that DENV-1 and DENV-2 were the predominant serotypes in southern Vietnam between 2003 and 2011 [20]. Our study described a temporal change in the distribution of dengue serotypes among infected patients presenting to outpatient clinics. DENV-4 increased remarkably from 2011 to 2013 firstly at Tien Giang Provincial Hospital then to all other hospitals to replace DENV-2 and became the second most prevalent infecting serotype. The presumed drivers of major changes in serotype prevalence in endemic areas such as southern Vietnam are the introduction of new lineages with greater epidemic potential or the interserotypic immune reactions in the population. There is no evidence about the relationship between climate change and variation in the pattern of dengue serotype. Instead, climate change might affect disease transmission, e.g. changes to the force of infection that could lead to more or less frequent oscillations in serotypes. Interestingly, this temporal change of serotypes was accompanied by a sharp decline in the number of infected cases in the following year, 2013. This observation is at odds with the general view that major serotype changes in endemic settings are often associated with increased incidence [134-136]. Perhaps DENV-4, as suggested by others [137], is less "virulent" in its epidemic potential, i.e. it has a lower R0 than other serotypes. This might be because it is less infectious for mosquitoes, as suggested by previous human-to-mosquito transmission studies by our group in Vietnam [138]. We note however that DENV-4 can cause severe dengue; the proportion of all DENV-4 cases that evolved to "severe" was similar to DENV-1, 2, 3.

We also found that early plasma viral RNA concentrations are positively associated with disease severity, as suggested by previous studies [110, 111, 139]. Plasma viraemia can be considered as a proxy for virus infection of tissues, which enhances the immune response in dengue infection leading to severe disease. Yet, plasma RNA viraemia concentrations may not be a good surrogate for the titre of infectious virus, because the assay measures nucleic acid only. With respect to describing the relationship between early viraemia and outcome, this study is unique in the sample size of the study population, the representation of all four serotypes and the breadth of clinical outcomes, from ambulatory to severe cases. Furthermore we have given precision to the

degree of association between viraemia and severe dengue, with odds ratios of 1.3-1.7 for DENV-1-4. Yet whilst significant, the strength of the association between early viraemia level and severe disease was not particularly high. This underscores the complex nature of dengue pathogenesis; the magnitude of the systemic viral infection (of which we assume viraemia to be a proxy) is only one aspect driving the complex syndrome. Other aspects, e.g. host predisposition [21, 63], flavivirus immune status [140, 141] and even clinical management [142, 143] probably all contribute to the final clinical outcome independently of viraemia level.

It is also acknowledged that viraemia is positively associated with NS1 levels in plasma [109, 111, 144]. The results of NS1 rapid tests again depends on NS1 antigenaemia [145]. Among the nonstructural proteins of dengue virus, NS1 is highly conserved and hypothesised to play a role in the severity of the disease, as increased concentration of soluble NS1 in serum correlates with viraemia levels and disease severity [109, 146]. Therefore, NS1 response is supposed to be strongly correlated with disease severity as reported in our results. However, the test has some limitations regarding the fact that the sensitivity of the test was reportedly lower in DENV-2-infected patients than DENV-1, DENV-3 or DENV-4 infected patients [63, 96, 98] and the sensitivity was also lower in those with secondary infections relative to primary infections [63, 93, 96-98, 116].

In this present study, after controlling for day of illness at enrolment, we did not find a correlation between plasma viral RNA concentration and serological response (primary vs. secondary) amongst hospitalized dengue patients. The only infecting serotype that had higher plasma viraemia in secondary infection than in primary infection was DENV-4. This finding was similar to Murgue's et al [147], but not consistent with other studies on DENV-1 or DENV-2 infections [110, 128, 148]. It might be possible because of the difference in classifying primary and secondary infections. Vaughn, Yeh and Duyen *et al.* defined secondary infection as a clear increase in IgG level by day 5-7, while in our present study we classified serological response based on IgM seroconversion and the presence of IgG. In our study, a positive dengue IgG of either the acute or convalescent blood sample at discharge was defined as "probable secondary infection"; otherwise that both the two samples were negative for dengue IgG was considered as "probable primary infection". Thus it is possible that a small number of primary cases have been misclassified as secondary cases. However we believe the number of misclassification will be small because of

the 897 hospitalized patients, convalescent samples were collected on day 6, 7, 8, 9 and 10 of illness and accounted for 92% (825 cases), 5% (45 cases), 1.5% (13 cases), 1% (9 cases) and 0.5% (5 cases) respectively. In our results, the "probable primary infection" group only accounted for 17/897 (1.9%) of hospitalized dengue patients, consistent with secondary infection being an important epidemiological risk factor.

The other explanations are DENV-2 infections were not common among hospitalized dengue patients in our study and particularly DENV-2 primary infections were rare.

In brief, this study provided a comprehensive description of DENV infection in Vietnamese children via a large, prospective cross-sectional study in outpatient departments. We documented clinical manifestations, laboratory findings and virological characteristics at enrolment and for a subset, during hospitalization. We measured with some precision the association between NS1 antigenaemia and viraemia with disease severity. Surprisingly, we found no association between early viraemia levels and secondary infection status. The results of this study will assist clinicians, epidemiologists, public health officials and scientists to implement intervention measures on treatment, control and clinical trials to achieve the aim of reducing the incidence rate, mortality rate and case fatality rate of dengue. The next two chapters will employ data from this prospective study to a)- devise algorithms to make an early diagnosis of dengue and b) devise algorithms to predict patients at risk of severe dengue.

Chapter 4

A NOVEL DIAGNOSTIC CLASSIFIER FOR THE EARLY DIAGNOSIS OF DENGUE

Summary

Dengue is a common, potentially life-threatening disease in the tropical world. We developed a clinically intuitive algorithm that can be used alone or as an adjunct to dengue NS1 rapid tests for improved early diagnosis of dengue. We demonstrate that the early diagnosis of dengue can be enhanced beyond the current standard of care using a simple evidence-based algorithm. The results should support patient management and clinical trials of specific therapies.

This chapter was submitted and published on the journal PLOS Neglected Tropical Diseases and can be found at
http://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0003638

4.1 INTRODUCTION

Dengue is an acute, systemic viral infection and a public health problem in the tropical world [1]. The aetiological agents of dengue are any of the four dengue viruses (DENV-1-4). In endemic countries it is common for all four DENV serotypes to co-circulate. Late-stage trials of a dengue vaccine with intermediate efficacy have recently been reported, offering hope of a public health intervention [6, 149].

The World Health Organisation (WHO) has a stated goal of reducing global dengue mortality by 50% by 2020 [1]. Improvements in case diagnosis and management will be central to achieving this aim. Significant loss of intravascular plasma volume leading to hypovolemic shock (dengue shock syndrome (DSS)), usually between the 4^{th}-6^{th} day of illness, is the commonest life-threatening complication of dengue [1, 150]. It's widely held that the case-incidence of DSS can be reduced via careful monitoring and the judicious use of parenteral fluids to maintain an adequate intravascular volume [1]. Ideally, this case management approach is enabled because the attending physician had made an early diagnosis and thus alerted clinicians, nurses and family caregivers to

the signs and symptoms suggestive of clinical worsening. Additional benefits of an early diagnosis include support to community level public health interventions and improvements in the sensitivity of case surveillance systems and disease burden estimates. Furthermore, it is likely that the therapeutic window of opportunity for a dengue antiviral drug lies in the first 48-72 hours of illness [151]. Thus, programmatic use of therapeutic interventions in the future will likely go hand in hand with strategies for early diagnosis.

Yet there are numerous challenges for busy primary care clinicians in making a diagnosis of dengue in the first few days of illness. Rapid lateral flow tests, based on the detection of the viral NS1 antigen, are available in some settings and can provide a confirmatory diagnosis [96, 98, 152]. The diagnostic performance of the WHO dengue case definition, which relies on non-specific signs and symptoms that overlap with other infectious diseases, is unknown in the first few days of illness [1]. Potts *et al* concluded that more prospective studies were needed to construct a valid and generalizable algorithm to guide the differential diagnosis of dengue in endemic countries [153]. To this end, several prospective studies have described the creation of classifiers for the diagnosis of dengue [32, 154, 155]. However none of these studies have exclusively focused on paediatric fever cases presenting to primary care facilities with short illness histories, a very common scenario in dengue endemic settings. Against this backdrop, the purpose of the current study was to prospectively derive a dengue diagnostic algorithm from routinely clinical and laboratory findings in paediatric patients with <72 hours of illness history and compare this approach against the diagnostic performance of a leading NS1 rapid test (BioRad NS1 Ag STRIP) in the same patients. The results provide pragmatic methods to enhance the early diagnosis of dengue in primary care settings.

4.2 MATERIALS AND METHODS

Details about study design, patient enrolment, data collection and variable definitions were presented in Chapter 2 (Materials and Methods).

Enrichment of NS1

Plasma samples were enriched for proteins with molecular weight >100kDa using Amicon filtration units (**Millipore**). Briefly, 200µl of plasma was concentrated to ~30µl and then tested

in the Platelia NS1 ELISA. All concentrated samples were tested in parallel with an aliquot of the original plasma samples and the filtrate (containing proteins with molecular weight <100kDa).

Strategy for developing the multivariable prediction models

Logistic regression was used for the development of the diagnostic algorithm [156, 157]. Continuous laboratory variables with a right-skewed distribution will be log2-transformed prior to analysis. Natural cubic spline functions and generalized additive models will be used to assess non-linearity. Potential interactions between candidate predictors will be assessed by comparing the models with and without interaction terms (e.g. appropriate likelihood ratio tests for interaction). Interaction plots will also be used to visualize whether interactions warrant inclusion into the full model.

A detailed assessment of the model assumptions of linearity and additivity was performed (supplementary statistical appendix). All pre-defined candidate predictors listed in Supplementary Table 4-1 and significant interaction terms were included in the full model. The model was then simplified using step-wise backwards selection using Akaike Information Criterion (AIC) and stability selection [158]. Alternative statistical models such as classification and regression trees (CART) and random forests (RF) were also investigated in order to find an optimal diagnostic algorithm [159, 160].

Evaluation of model accuracy and performance

The performance of the model was assessed with respect to discrimination (receiver operating characteristic curves (ROCs) and area under the ROC curve (AUC)), calibration (calibration plots and calibration intercepts and slopes), and standard accuracy criteria of binary diagnostic tests (sensitivity, specificity, negative and positive predictive values). We selected the cut-off point to classify a patient as dengue positive at a predicted risk of dengue of ≥33.3%, corresponding to assuming that the "cost" of missing a true dengue patient is twice as large as the cost of a false-positive [157].

Model validation

To avoid over-optimistic estimates of model accuracy and performance due to model derivation and evaluation on the same dataset, all accuracy measures were corrected for optimism by validation. Validation was performed for the whole model development process including variable selection. Two validation schemes were employed to mimic external validation:

a) "leave-one-site-out cross-validation", i.e. repeatedly developing the algorithm on all but one site and validation on the left-out site
b) Temporal validation with patients recruited before 15 June 2012 as the training set and patients recruited thereafter as the evaluation set [156].

Presentation of the final model

The final logistic model will be presented in a standard way as OR [95% CI] and p-values for all coefficients. It will then be simplified to a nomogram for direct clinical use.

All statistical analyses were performed using the statistical software R v3.1.1 (R foundation for statistical computing, Vienna, Austria) and its companion packages c060 version 0.2-3 (for stability selection), randomForest version 4.6-7 (for random forest) and rpart version 4.1-8 (for CART).

4.3 RESULTS

4.3.1 Study population

5729 children with fever of less than 72 hours were enrolled at one of the seven clinical study sites in southern Vietnam between October 2010 and December 2012. A summary of the patient screening, enrolment and diagnostic outcomes is shown in Supplementary Figure 4-1. A total of 5707 patients were included in the analyses. 1692 (29.6%) participants had laboratory-confirmed dengue. The baseline characteristics of the dengue and non-dengue cases are shown in Table 4-1. Notably, dengue cases were older than non-dengue cases. All four DENV serotypes were detected; DENV-1 was the commonest serotype, followed by DENV-4, -2 and -3.

Table 4-1:.Baseline characteristics of study participants

	Laboratory-confirmed dengue (N=1692)	Non-Dengue (N=4015)
Demographic characteristics		
Age (years)	9 (6-11)	5 (3-8)
Sex-Male (n, %)	945 (55.9%)	2249 (56.0%)
BMI (kg/(m)^2)	16.4 (14.6-18.9)	15.6 (14.1-17.6)
History and clinical characteristics		
Day of illness		
1	361 (21.3%)	1232 (30.7%)
2	692 (40.9%)	1732 (43.1%)
3	639 (37.8%)	1051 (26.2%)
Temperature (°C)	38.5 (38-39)	38.4 (37.8-39.0)
Vomiting (n, %)	737 (43.6%)	1442 (35.9%)
Abdominal pain (n, %)	351 (20.7%)	702 (17.5%)
Skin bleeding (n, %)	243 (14.4%)	135 (3.4%)
Mucosal bleeding (n, %)	113 (6.7%)	99 (2.5%)
Flush (n, %)	399 (23.6%)	532 (13.3%)
Hepatomegaly (n, %)	6 (0.4%)	5 (0.1%)
Rash (n, %)	62 (3.7%)	79 (2.0%)
Conjunctival injection (n, %)	343 (20.3%)	385 (9.6%)
Laboratory results*		
WBC (10^3/mm^3)	4.74 (3.50-6.80)	8.90 (6.36-12.40)
PLT (10^3/mm^3)	180 (141-227)	242 (200-292)
HCT (%)	38.6 (36.6-40.7)	37.4 (35.3-39.6)
ALB (g/L)	43.7 (41.7-45.6)	43.9 (42.0-45.7)
AST (U/l)	51 (40-67)	42 (35-49)
CK (U/l)	105 (82-140)	100 (76-131)

* All laboratory results were acquired on the day of enrolment.
Values are presented as median and interquartile range for continuous variables or frequency and percentage for categorical variables.
BMI: body mass index; WBC: white blood cell count; PLT: platelet count; HCT: haematocrit; ALB: albumin; AST: aspartate aminotransferase; CK: creatine kinase.

4.3.2 Diagnostic accuracy of the NS1 Ag Strip test and association with viraemia

Enrolment plasma samples (n=5707) were tested for the presence of NS1 by NS1 Ag Strip test in a blinded, real-time fashion. Against the composite gold-standard reference diagnostic result,

the NS1 Ag Strip test had a sensitivity of 70.4% (95%CI: 68.2-72.6%), specificity of 99.2% (95%CI: 98.9-99.5%), positive predictive value (PPV) of 97.4% (95%CI: 96.3-98.2%), and negative predictive value (NPV) of 88.9% (95%CI: 87.9-89.8%) for the diagnosis of dengue (Table 4-2). There was a striking difference in diagnostic performance by serotype, with NS1 detection being less sensitive in DENV-2 infections irrespective of the serological response (primary vs. secondary) (Supplementary Table 4-2). The detection of NS1 was strongly associated with the concentration of DENV RNA in the same plasma sample; the odds of NS1 detection increased by 1.8 (95%CI: 1.6-1.9) for each 10-fold higher DENV RNA concentration (Table 4-2). These data define the strengths and weaknesses of NS1 rapid testing; it is highly specific but is compromised by suboptimal sensitivity, especially for DENV-2 cases.

Table 4-2. Diagnostic performance of NS1 rapid test in enrolment plasma samples and odds of NS1 detection in relation to plasma viraemia

	Laboratory-confirmed dengue cases	Non-dengue Cases	Total	
NS1 rapid test positive	1192	32	1224	PPV % = 97.4% (96.3-98.2%)
NS1 rapid test negative	500	3983	4483	NPV % = 88.9% (87.9-89.8%)
Total	1692	4015	5707	
	Median (IQR) plasma viral RNA concentration (log10copies/ml)*	OR (95%CI)	Sensitivity % (95%CI)	Specificity % (95%CI)
All serotypes (n=1692)	7.3 (6.2-8.3)	1.8 (1.6-1.9)	70.4% (68.2-72.6%)	99.2% (98.9-99.5%)
DENV-1 (n=629)	7.9 (6.6-8.7)	2.0 (1.8-2.3)	80.3 (77.0-83.3%)	-
DENV-2 (n=399)	7.0 (6.0-7.9)	1.8 (1.5-2.1)	46.4 (41.4-51.4%)	-
DENV-3 (n=154)	7.5 (6.4-8.6)	1.4 (1.1-1.9)	85.1 (78.4-90.3%)	-
DENV-4 (n=433)	6.9 (6.0-7.7)	1.5 (1.3-1.8)	75.8 (71.3-79.7%)	-

* Viraemia measurement in the same plasma sample was used for NS1 testing.

PPV: positive predictive value; NPV: negative predictive value; DENV: dengue virus; OR: odds ratios for detecting NS1 for each 10-fold higher DENV RNA concentration. There were 77 dengue cases where the infecting serotype was unknown.

4.4 Manipulation of plasma specimens to improve NS1 detection

Volume enrichment of the plasma molecular weight fraction containing multimeric NS1 (>100,000kDa) was performed on plasma samples from 21 viraemic dengue cases enrolled in this study. However, despite 5-10-fold concentration of plasma, this processing failed to materially improve the diagnostic yield, with only 1 of 11 samples changing their status from negative (original sample) to positive (concentrated sample) in the Platelia NS1 ELISA (Supplementary Table 4-3).

4.5 The Early Dengue Classifier; a diagnostic rule based on clinical and simple laboratory features

Multivariate logistic regression analyses of clinical, demographic and laboratory data from 5707 patients were performed to generate a practical dengue diagnostic classifier that could replace or augment NS1-based diagnosis in the first 72 hours of illness. The most parsimonious model, derived from stability selection, used the patient's age, white cell count and platelet count at the time of enrolment to classify dengue from non-dengue cases (Table 4-3). Alternative approaches to feature selection yielded models with only slightly higher performance but relied on many more (more than ten) variables (Supplementary Table 4). The most parsimonious model, herein called the Early Dengue Classifier (EDC), had a sensitivity of 74.8% (95%CI: 73.0-76.8%), specificity of 76.3% (95%CI: 75.2-77.6%), positive predictive value of 57.1% (95%CI: 56.2-59.0%), and negative predictive value of 87.8% (95%CI: 86.8-88.5%) for correctly classifying dengue cases in the entire dataset at the pre-defined cut-off of 33.3%. Of note, this pre-defined cut-off reflecting clinical priorities was very close to the cut-off corresponding to the point on the ROC curve closest to the upper left corner (perfect model), which was 34.2% (Figure 4-1A). The area under the ROC curve (AUC) was 0.829 (Figure 4-1B) and the predicted risk of dengue agreed well with the observed risk (Figure 4-1C). The EDC had sensitivity of 72.9% (95% CI: 69.6-76.6%) for DENV1, 74.7% (95%CI: 71.0-79.7%) for DENV2, 68.4% (95%CI: 59.2-74.5%) for DENV3 and 78.2% (95%CI: 75.5-83.3%) for DENV4 infection. The overall performance characteristics of the EDC under temporal, leave-one-site-out validation or seasonality (rainy versus dry season), are summarized in Supplementary Table 4-5. These results suggest that, in settings where NS1 rapid tests are not routinely available, the EDC could assist primary care physicians in dengue diagnosis.

Table 4-3. Univariate and multivariate analysis of candidate predictors of laboratory-confirmed dengue

	Univariate analysis			Multivariate analysis					
				Full model with all candidate predictors			Final model based on stability selection		
	OR	95% CI	p	OR	95% CI	P	OR	95% CI	p
Demographic characteristics									
Age (by + 1 year)	1.24	1.22-1.26	<0.001	1.21	1.18-1.24	<0.001	1.15	1.13-1.17	<0.001
Sex: Male	0.99	0.89-1.11	0.909	0.94	0.81-1.09	0.432	-	-	-
BMI (by +1)	1.09	1.07-1.11	<0.001	1.06	1.04-1.09	<0.001	-	-	-
History and clinical characteristics									
Day of illness (+1 day)	1.45	1.34-1.56	<0.001	0.78	0.70-0.87	<0.001	-	-	-
Temperature (by +1°C)	1.23	1.14-1.32	<0.001	1.28	1.16-1.40	<0.001	-	-	-
Vomiting = Yes	1.37	1.22-1.54	<0.001	1.31	1.12-1.52	<0.001	-	-	-
Abdominal pain = Yes	1.24	1.07-1.42	0.004	0.91	0.76-1.10	0.349	-	-	-
Skin bleeding = Yes	4.82	3.87-5.99	<0.001	2.08	1.53-2.84	<0.001	-	-	-
Mucosal bleeding = Yes	2.83	2.15-3.73	<0.001	1.02	0.67-1.53	0.934	-	-	-
Flush = Yes	2.02	1.75-2.34	<0.001	1.37	1.11-1.69	0.003	-	-	-
Hepatomegaly = Yes	2.85	0.87-9.36	0.086	0.58	0.05-7.08	0.687	-	-	-
Rash = Yes	2.02	1.75-2.34	<0.001	1.23	0.78-1.93	0.381	-	-	-
Injection = Yes	2.40	2.05-2.81	<0.001	1.58	1.25-1.99	<0.001	-	-	-
Laboratory results									
WBC (+10^3/mm^3)	0.70	0.68-0.71	<0.001	0.77	0.75-0.80	<0.001	0.78	0.76-0.80	<0.001
PLT (+10^4/mm^3)	0.87	0.86-0.88	<0.001	0.97	0.95-0.98	<0.001	0.94	0.93-0.95	<0.001
HCT (+1%)	1.12	1.10-1.14	<0.001	0.97	0.95-0.99	0.013	-	-	-
ALB (+1g/l)	0.97	0.95-0.99	0.004	1.00	0.98-1.03	0.787	-	-	-
AST (per 2-fold increase)	3.65	3.23-4.14	<0.001	3.50	2.97-4.13	<0.001	-	-	-
CK (per 2-fold increase)	1.33	1.24-1.43	<0.001	0.84	0.76-0.93	<0.001	-	-	-

BMI: body mass index; WBC: white blood cell count; PLT: platelet count; HCT: haematocrit; ALB: albumin; AST: aspartate aminotransferase; CK: creatine kinase

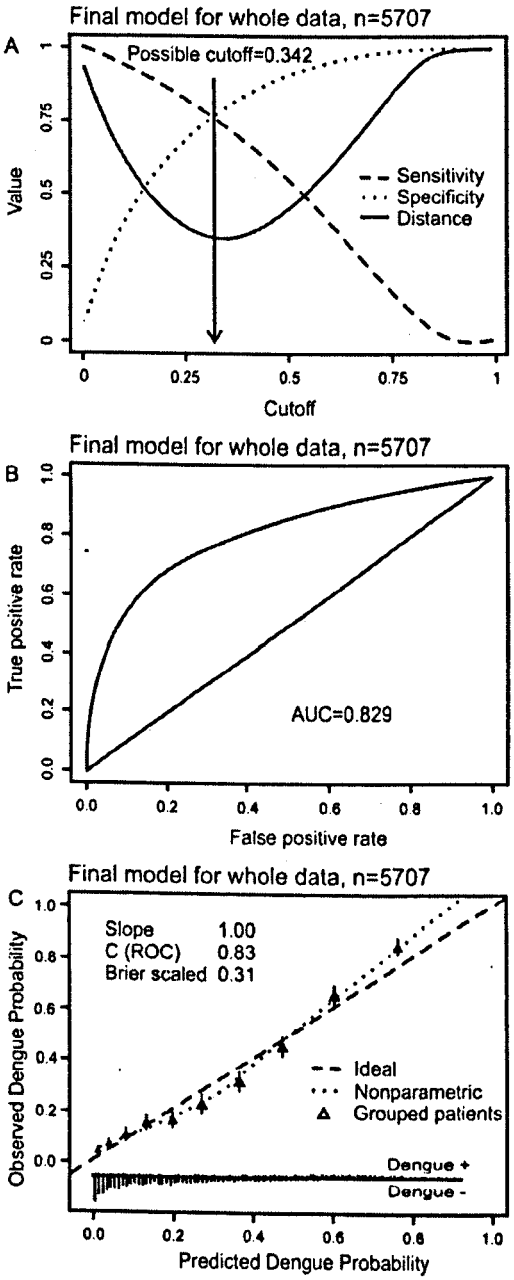


Figure 4-1. Performance of the Early Dengue Classifier (EDC) in all subjects. *Figure 4-1A displays possible sensitivity/specificity trade-offs for different cut-off values and the distance from the corresponding points on the ROC curve to the upper left corner (perfect model). Figure 4-1B displays the receiver operating characteristic (ROC) curve. Figure 4-1C is a calibration plot. It displays a scatterplot-smoother of predicted versus observed risks (dotted line), predicted versus observed risks for ten patient strata of equal size grouped according to predicted risks (triangles) and the ideal identity line (dashed line). The rugs at the bottom of the graphs characterize the distribution of predicted risks in true dengue and non-dengue cases, respectively.*

In settings where NS1 rapid tests are routinely used, the EDC can be combined with the NS1 rapid test as a composite test (classified as positive when either NS1 rapid test or EDC are positive, and classified as negative when both NS1 rapid test and EDC are negative). This composite test had sensitivity of 91.6% (95%CI: 90.4-92.9%) while the specificity was 75.7% (95%CI: 74.5-77.0%). Corresponding positive and negative predictive values were 61.7% (95%CI: 60.6-63.1%) and 95.5% (95%CI: 94.9-96.1%). If a higher specificity was desired, a higher cut-off value of the EDC could be used for the combined test instead, e.g. a cut-off of 50% would lead to a sensitivity of 86.0% (95%CI: 84.5-87.6%) and specificity of 89.6% (95%CI: 88.7-90.5%). These results imply that the EDC is useful in settings with and without NS1 rapid testing.

4.6 User-friendly applications of the Early Dengue Classifier

Figure 4-2 presents a nomogram of the EDC. The nomogram assigns points to all risk factors and translates the total point score to a predicted risk for dengue. For example, a 9-year-old patient with platelet count $100x10^3/mm^3$, and white blood cell count $5x10^3/mm^3$ has a total points score of 15+32+84=131, and the corresponding risk of dengue is about 70%. The predicted risk of dengue is larger than 33.3% so the patient would be classified as dengue positive. Alternatively, the EDC could be implemented as a smartphone app. The exact formula for the estimated risk of dengue (p) is given by the following logistic equation: logit(p)=1.236 + 0.139*age (in years) – 0.254*white blood cell (in $10^3/mm^3$) – 0.006 *platelet (in $10^3/mm^3$).

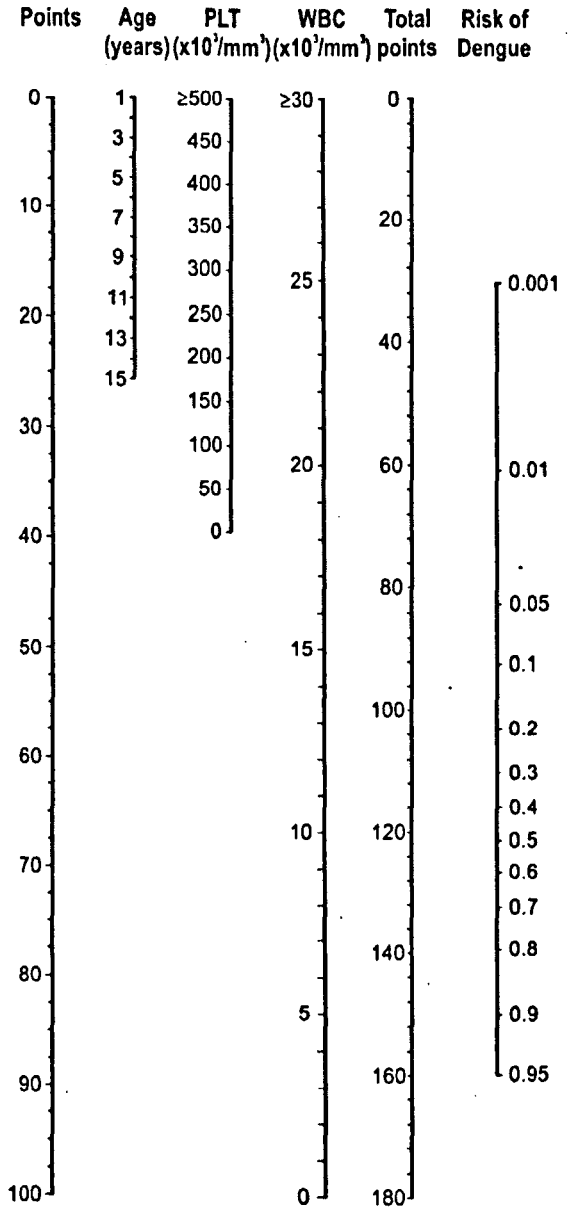


Figure 4-2. Nomogram of the Early Dengue Classifier (EDC) to predict the risk of dengue. *A horizontal line from a predictor value to the "Points" axis assigns points to the 3 required variables age, platelet count (PLT), and white blood cell count (WBC). The sum of these points (total points) can then be translated to the corresponding predicted risk of dengue. As an example, a 9-year-old patient with a PLT of 100x10³/mm³, and a WBC of 5x10³/mm³ has a score of 15+32+84=131, and the corresponding risk of dengue is about 70%. Note: As <1% of patients had platelet (PLT) count >500x10³/mm³ or white blood cell (WBC) count >30x10³/mm³, for better visualization, PLT and WBC counts were truncated at 500x10³/mm³ and 30x10³/mm³ respectively.*

4.7 DISCUSSION

The early and accurate diagnosis of dengue on the grounds of clinical signs and symptoms alone is problematic [153]. Physicians need better tools to assist in early diagnosis if the WHO ambition of a 50% reduction in global dengue mortality is to be achieved by 2020. This study characterized the performance of three diagnostic approaches; the NS1 rapid test, a stand-alone diagnostic classifier and the combination of NS1 rapid test and diagnostic classifier together. Our results highlight the utility of NS1 rapid tests for an early specific diagnosis, yet also remind that 2nd generation tests are needed with improved sensitivity. The diagnostic classifier described here could help guide diagnosis in endemic settings, or be used as an adjunct to help exclude dengue in patients returning a negative NS1 rapid test result.

There is a body of literature describing the performance of NS1 rapid tests for the diagnosis of dengue [96, 98, 108, 113, 152, 161, 162]. This current study extends that literature in several ways. First, by virtue of the large sample size we demonstrate with high precision the differential sensitivity of the NS1 Ag STRIP for different DENV serotypes. This test was sensitive (between 75-85%) for DENV-1, -3 and -4 infections, but poorly sensitive in DENV-2 infections (46.4%). Lower sensitivity was partially attributable to the great majority of DENV-2 infections being associated with secondary serological responses, although we note sensitivity was also low in primary DENV-2 infections. This suggests that there are particular virological (e.g. lower viral burdens in vivo) or intrinsic aspects of the NS1 test, that limit DENV-2 NS1 detection. [92, 128, 145, 163]. Second, we make the novel observation that 5-10 fold enrichment of proteins with molecular weight >100kDa in plasma specimens (the NS1 hexamer has predicted molecular weight of 310kDa [164]) did not lead to improved NS1 detection rates. These data suggest that dengue patients who return negative NS1 rapid test results in the first 3 days of illness have free plasma NS1 concentrations substantially below the limit of sensitivity of existing assays and that 2nd generation tests might need to be at least an order of magnitude more sensitive. Nonetheless, better NS1 rapid diagnostic tests are needed if they are going to be widely adopted by clinical services in primary care settings. In malaria, HRP2 rapid diagnostic tests for *Plasmodium falciparum* infection are an example of how improvements to assay performance can lead to recognition as a diagnostic standard of care [165]. Finally, although serum NS1 concentrations

have been proposed to have prognostic value in a small study, this is yet to be independently validated and is likely to be difficult given that blood NS1 concentrations vary widely according to the infecting DENV serotype, serological response and day of illness [98, 109, 111, 128].

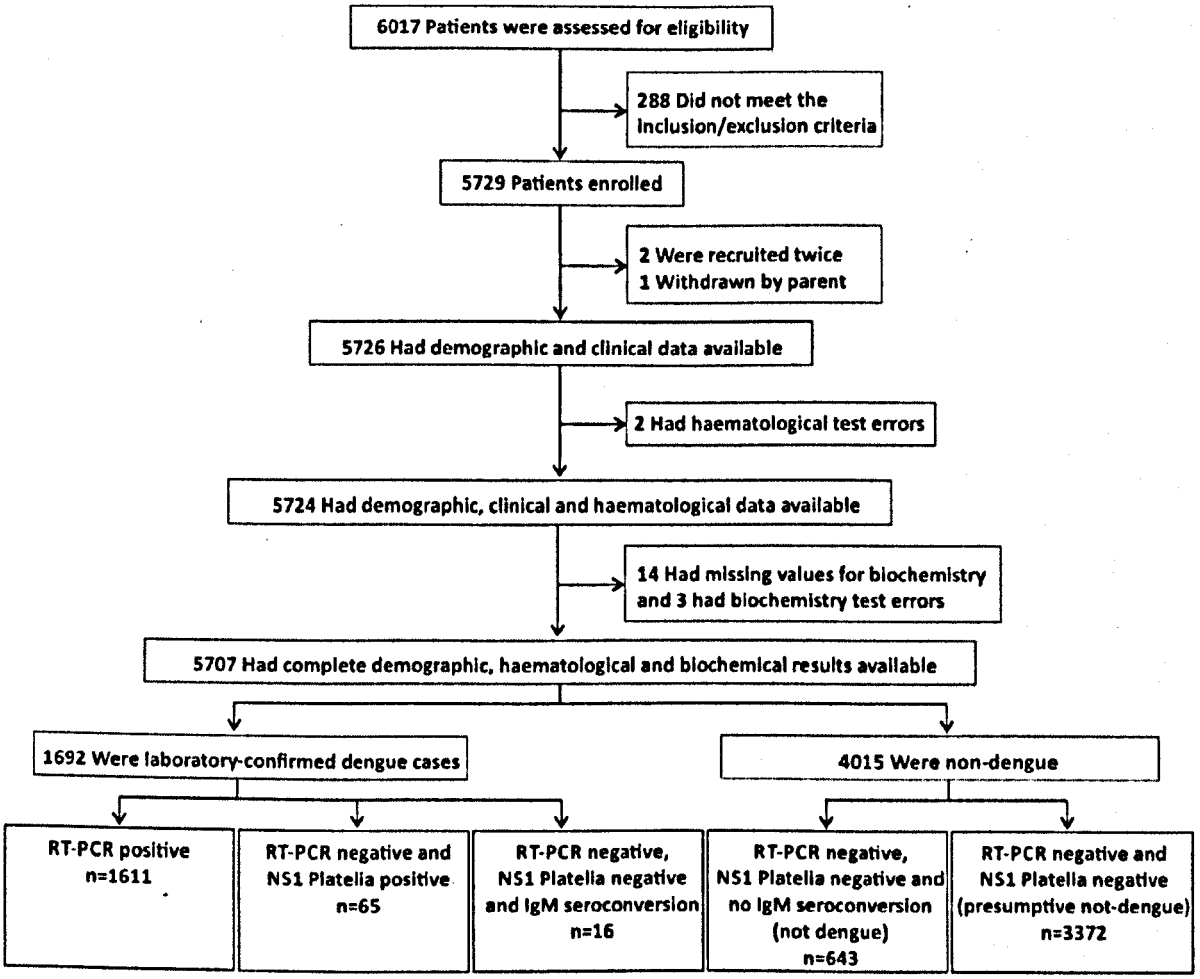
A body of literature describes clinical and/or routine laboratory findings that distinguish patients with dengue from those with other febrile illnesses [155, 166-172]. What is striking in the literature is that only three prospective studies have considered dengue diagnostic algorithms exclusively in children and of these the largest contained 1227 patients, of who 614 had dengue [32, 173, 174]. More generally, most diagnostic studies failed to report positive and negative predictive values for their diagnostic algorithms, thus making it difficult to assess their utility in routine practice. Against this backdrop, a strength of the current study is the large sample size, the presence of all four DENV serotypes, robust statistical validation techniques and transparent performance characteristics. The clinical signs and symptoms that make up the WHO case definition for dengue were not used in the final, parsimonious diagnostic EDC classifier. Instead, we found that only three variables– patient age, white blood cell count and platelet count, provided similar discriminatory information as alternative models that relied upon a much larger set of clinical data.

The purpose of this study was to explore whether it was possible to develop any kind of simple, evidence-based algorithm for early diagnosis – the results demonstrate this feasibility, albeit the performance characteristics of the end-result algorithm are not so outstanding that they will result in widespread adoption. The sensitivity (74.8%), specificity (76.3%) and positive predictive value (57.1%) of the EDC were not outstandingly high and as such will not change the practice of experienced clinicians. We concur with Potts *et al* in the belief that diagnostic rules for dengue are not a replacement for good clinical acumen and management [153]. However, the EDC described here offers an evidence-based guide that can likely improve the prevailing diagnostic accuracy of most Vietnamese physicians working in primary care who do not possess extensive experience in dengue diagnosis and management. In particular, in settings where NS1 rapid tests are not routinely available or affordable, or where DENV-2 is the most prevalent virus in circulation, the EDC could help guide clinicians in making their differential diagnosis. An early diagnosis of dengue can assist in patient triage and management by directing clinical/caregiver

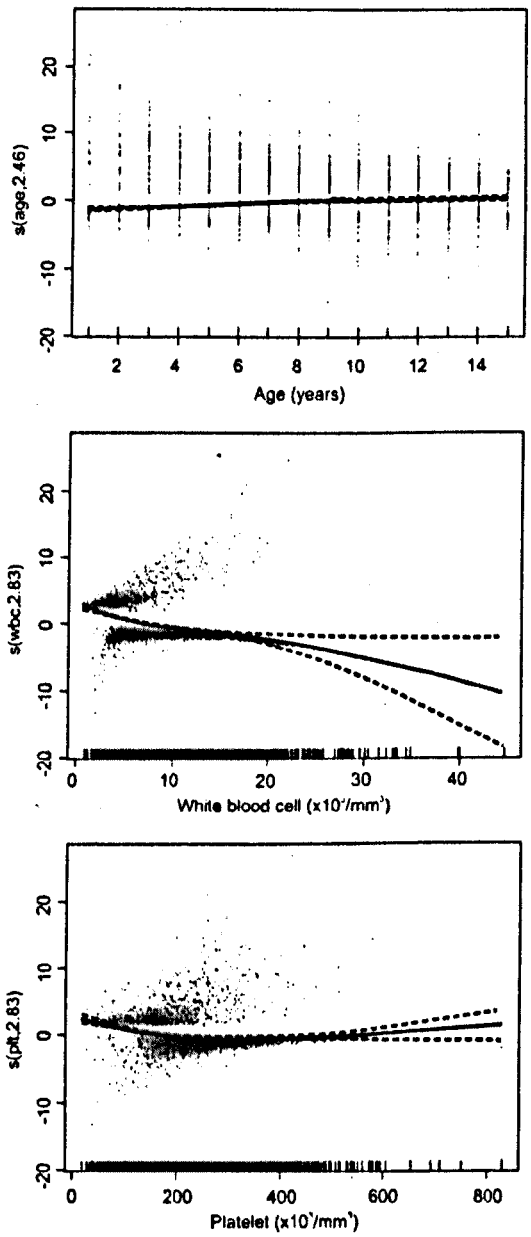
attention to clinical warning signs and/or the appearance of capillary permeability, for which supportive oral and/or parenteral fluid therapy is recommended in order to prevent circulatory compromise. Additionally, in the first days of illness many dengue cases are infectious to *Aedes aegypti* mosquitoes and hence an early diagnosis could support measures to prevent further transmission, e.g. by use of topical repellents and local mosquito control [138].

Our study has several design features and limitations that might preclude its wider generalizability. The EDC relies on routine haematology findings that are commonly accessible in primary care settings in Vietnam but might not be available everywhere. By design, our study focused on patients with <72 hours of illness and hence our results might not be applicable to patients who present to medical care at later time-points. By using the age of the patient as a component of the EDC, it's likely that the EDC would not perform well in settings where the burden of dengue falls on age-groups different from that in southern Vietnam. Nonetheless, this study has delivered the largest population-based and quantitative framework to guide early diagnosis of paediatric dengue. Further prospective validation in Vietnam and other endemic countries with similar epidemiology will be needed to establish the clinical utility of the EDC.

APPENDIX



Supplementary Figure 4-1. Flow chart showing patient enrolment and classification.



Supplementary Figure 4-2. Plots of estimated component smooth functions of a GAM for the risk of dengue which included all candidate predictors listed in Supplementary Table 4-1 and modelled continuous parameters as smooth terms. Only terms estimated to have a non-linear association with outcome are displayed. Dots correspond to individual partial residuals; solid lines correspond to smooth spline functions estimated by GAM; dashed lines correspond to the estimated smooth functions plus/minus one standard error.

Supplementary Table 4-1. Clinical and demographic features recorded at the time of study enrolment

Variable	Definition
1. Day of illness (days)	Day of illness at enrolment
2. Age (years)	Age in years
3. Sex	Male/Female
4. BMI (kg/(m)2)	Body mass index (BMI) = weight (kg) /height (m) ^2
5. Temperature at enrolment (°C)	Axillary temperature at enrolment
6. Vomiting (Yes/No)	Any episode of vomiting in history of illness
7. Skin bleeding (Yes/No)	History or clinical examination shows that the patient has skin bleeding, e.g. petechiae or ecchymosis.
8. Mucosal bleeding (Yes/No)	Any frank bleeding from any mucosal site e.g. epistaxis, gingival bleeding, gastrointestinal bleeding, or urogenital bleeding in history of illness or medical examination
9. Abdominal pain (Yes/No)	History or clinical examination shows that the patient has a painful feeling at the abdomen by self-reporting or elicited on gentle palpation.
10. Rash (Yes/No)	Clinical examination reveals generalised macular, blanching, non-petechial rash.
11. Flush (Yes/No)	Redness of the skin caused by dilatation and congestion of the capillaries that blanches with pressure.
12. Conjunctival injection (Yes/No)	Redness (bright red or pink) of the conjunctiva fading towards the limbus due to dilatation of the superficial conjunctival blood vessels.
13. WBC (103/mm3)	Absolute white blood cell count at enrolment
14. HCT (%)	Haematocrit at enrolment
15. Platelet count (103/mm3)	Platelet count at enrolment
16. ALB (g/L)	Blood albumin concentrations
17. AST (U/l)	Blood aspartate aminotransferase concentrations (log2-transformed)
18. CK (U/l)	Blood creatine kinase concentrations (log2-transformed)

Supplementary Table 4-2. Sensitivity of NS1 rapid test according to serotype and serological response in hospitalized patients

	Sensitivity % (95%CI)	
	Primary infection (NS1+ /total primary infection)[a]	Secondary infection (NS1+ /total secondary infection)[b]
DENV-1	93.6% (87.2-97.4%) (107/109)	82.2% (75.5-87.8%) (134/163)
DENV-2	42.9% (17.8-71.1%) (6/14)	57.1% (48.9-64.9%) (89/156)
DENV-3	95.2% (76.1-99.2%) (20/21)	81.1% (64.8-92.0%) (30/37)
DENV-4	70.0% (45.7-88.0%) (14/20)	81.1% (74.8-86.4%) (154/190)
Unknown	68.8% (41.4-88.9%) (11/16)	55.5% (30.8-78.4%) (10/18)
Total	**180**	**564**

Note: (a), (b) indicated the number of cases with NS1 rapid test positive compared to the total number of primary or secondary infections respectively within the same individual dengue serotypes.

Supplementary Table 4-3. Detection of NS1 in viraemic blood samples collected at the time of enrolment, before and after volume concentration

				NS1 status (Platelia ELISA)[a]			
Sample#	DENV Serotype	log10 viraemia RNA copies/ml	NS1 rapid test	Original sample	Sample concentrate	Sample filtrate	Fold concentration of sample
15-222	2	6.85	-	+	+	-	8.0
16-427	2	6.90	-	+	+	-	6.7
16-134	1	9.54	-	+	+	+	6.7
16-139	1	6.93	-	+	+	+	6.7
16-142	1	8.22	-	+	+	+	8.0
16-130	1	8.34	-	+	+	-	6.7
1-920	1	8.33	-	+	+	+	8.0
16-184	1	7.95	-	+	+	-	8.0
16-223	2	6.33	-	+	+	-	10.0
16-269	1	5.40	-	-	-	-	6.7
16-281	2	4.98	-	-	-	-	6.7
16-308	1	5.19	-	-	-	-	5.7
16-352	2	5.39	-	-	+	-	5.7
16-399	2	4.49	-	-	-	-	4.0
15-226	2	8.47	-	-	-	-	5.7
16-155	1	3.11	-	-	-	-	5.7
16-160	1	7.56	-	-	-	-	5.7
16-514	1	7.36	-	Eq	Eq	-	5.7
16-154	1	2.94	-	-	-	-	6.7
1-846	2	5.78	-	-	-	-	5.7
1-882	1	11.05	-	-	-	-	8.0

[a] NS1 Platelia test result: "+"=positive, "-"=negative, "Eq'= equivocal

Supplementary Table 4-4. Performance of models for the risk of dengue in all subjects resulting from different statistical modelling approaches

	Full model	Step-wise AIC	STAB	CART	RF
Calibration intercept	-0.414 (-0.470–0.554)	-0.432 (-0.470–0.557)	-0.200 (-0.810–0.852)	-0.211 (-0.667–0.833)	-0.256 (-0.601–0.742)
Calibration slope	0.926 (0.820–1.248)	0.920 (0.820–1.263)	1.075 (0.855–1.272)	1.076 (0.829–1.076)	1.293 (1.037–1.331)
AUC	0.855 (0.815–0.861)	0.854 (0.815–0.861)	0.835 (0.792–0.850)	0.774 (0.703–0.777)	0.872 (0.830–0.873)
Sensitivity (cutoff 0.33)	0.785 (0.651–0.868)	0.790 (0.659–0.890)	0.795 (0.659–0.912)	0.614 (0.459–0.736)	0.812 (0.579–0.846)
Specificity (cutoff 0.33)	0.760 (0.699–0.868)	0.756 (0.699–0.868)	0.726 (0.550–0.839)	0.896 (0.735–0.939)	0.809 (0.709–0.873)
PPV (cutoff 0.33)	0.535 (0.513–0.699)	0.533 (0.519–0.720)	0.505 (0.426–0.738)	0.676 (0.504–0.848)	0.600 (0.513–0.768)
NPV (cutoff 0.33)	0.909 (0.815–0.936)	0.911 (0.811–0.946)	0.909 (0.748–0.945)	0.868 (0.702–0.884)	0.924 (0.805–0.926)

The full model included all variables listed in Supplementary Table 4-1 plus interaction terms between age and WBC and between age and PLT.
Step-wise AIC selected the following variables: age, day of illness, BMI, vomiting, temperature, skin bleeding, flush, injection, haematocrit, log2(aspartate ami notransferase), log2(creatine kinase), white blood cell count, platelet count, age by white blood cell count and age by platelet count.
STAB selected the following variables from the full model: age, white blood cell count, platelet count.
CART selected the following variables: age, white blood cell count.
RF was derived from all variables listed in Supplementary Table 4-1.
AUC: area under the ROC curve, PPV: positive predictive value, NPV: negative predictive value, Step-wise AIC: step-wise backwards variable selection using Akaike information criterion, STAB: stability selection, CART: classification and regression trees, RF: random forests.
The results were from leave-one-site-out validation and were presented by mean and range of performances across left out sites.

Supplementary Table 4-5. Performance of the Early Dengue Classifier (EDC)

	All patients	Dry season	Wet season	Temporal validation	Leave-one-site-out validation
Calibration intercept	0	0	0	-0.247	-0.200 (-0.810–0.852)
Calibration slope	1	1	1	0.940	1.075 (0.855–1.272)
AUC	0.829	0.831	0.825	0.776	0.835 (0.792–0.850)
Sensitivity (cutoff 0.33)	0.748 (0.730–0.768)	0.731 (0.726–0.750)	0.783 (0.771–0.798)	0.786 (0.770–0.812)	0.795 (0.659–0.912)
Specificity (cutoff 0.33)	0.763 (0.752–0.776)	0.792 (0.781–0.803)	0.758 (0.742–0.771)	0.595 (0.583–0.612)	0.726 (0.550–0.839)
PPV (cutoff 0.33)	0.571 (0.562–0.590)	0.587 (0.579–0.602)	0.570 (0.561–0.586)	0.460 (0.451–0.482)	0.505 (0.426–0.738)
NPV (cutoff 0.33)	0.878 (0.868–0.885)	0.911 (0.889–0.925)	0.864 (0.851–0.972)	0.863 (0.850–0.877)	0.909 (0.748–0.945)

Table shows apparent performance (95%CI) in all patients and seasons; performance (95%CI) in the validation set for temporal validation; and mean (range) of performances across left out sites for leave-one-site-out validation. Dry season is January to June, and wet season is July to December. The proportion of true dengue cases in all patients, dry season and wet season was 29.6% (1692/5707), 21.1 % (405/1916) and 33.9% (1287/3791) respectively.

Statistical appendix

Assessment of linearity

A generalized additive model (GAM) with integrated smoothness estimation as implemented in the R package mgcv version 1.8-0 was used to assess potential non-linear effects of variables on the risk of dengue. The model included all candidate predictors listed in Supplementary Table 4-1 and modelled continuous parameters as smooth terms.

The GAM chose non-linear associations for several parameters including age, white blood cell count, and platelet count (Supplementary Figure 4-2). While the estimated association of outcome with age and white blood cell count was not perfectly linear, it was monotone and reasonably close to a linear function. Hence, these variables were treated as linear terms.

The estimated effect of platelet count on the risk of dengue in Supplementary Figure 4-2 indicates a decreasing risk of dengue for increasing platelet counts up to ~$300x10^3/mm^3$ but a slight increase in the risk of dengue for higher platelet counts. However, the performance of the final model in which platelet count was modelled non-linearly with a linear spline function with one knot at $300x10^3/mm^3$ was similar to the one in which platelet was included as a linear term (AUC=0.830 vs. AUC=0.829). In addition, it seemed unclear whether it could be physiopathologically plausible for dengue patients to have abnormally high platelet counts or whether this was just an artifact. Thus, we presented in the main paper the results of the models in which platelet count was treated as linear term.

Assessment of interactions

Potential interactions between age, sex, study site and haematological and biochemical results were examined by likelihood ratio tests based on the full model with all variables from Supplementary Table 4-1.

Interaction terms between age and white blood cell count ($p<0.001$) and between age and platelet count ($p=0.039$) were significant and added to the full starting model.

Chapter 5

EARLY PROGNOSIS OF SEVERE DENGUE

Summary

We developed a prognostic algorithm using both clinical and laboratory features including vomiting, platelet count, AST and NS1 rapid status to make prediction of severe dengue.

5.1 INTRODUCTION

Despite progress in management and resuscitation in intensive care, dengue with severe complications is still a cause of childhood mortality in many Asian and Latin American countries. The case fatality rate for dengue has declined over the last two decades and is now less than 1% with adequate treatment [1] but can reach as high as 26% in severe dengue if treatment is inappropriate [175]. One of the "costs" of achieving a low case fatality rate is the burden to the health care system from a large number of dengue cases being admitted for hospitalization and close observation, rather than ambulatory care.

It is a great challenge for general physicians and even experienced infectious disease physicians to predict in the first few days of the illness which dengue patients will evolve to severe dengue. Typical symptoms and signs of complications generally only occur in the critical phase, from day 4 to 6 of the disease [1, 150, 176]. Thus, in many settings, physicians working in the outpatient or emergency department setting often take a conservative approach with a suspected dengue case and admit the patient for monitoring. This has important implications for allocation of scarce healthcare resources, patient triage, reduction of unnecessary hospitalization and costs to patients. Previous studies have developed different approaches to identify early prognostic factors of severe dengue by using logistic regression or classification and regression trees [155, 174, 177, 178]. However, the results from these previous studies are generally not useful in settings with limited resources e.g. where RT-PCR techniques and serology are not routinely available. Furthermore, the majority of these studies only paid attention to dengue haemorrhagic fever (DHF), a case group who are not necessarily clinically severe [1, 154, 179, 180]. Indeed, the rationale for the refinement of the WHO case classification in 2009 was to more appropriately and inclusively

classify the clinically severe dengue cases, including those with dengue shock syndrome, severe bleeding or organ failure [1]. Additionally, most earlier prognostic studies had the notable limitation of not presenting the positive and negative predictive values of the prognostic tool, or that the predictive tools were based on data from patients who had already been hospitalized [155, 181-184].

Given the knowledge gap in the field, the aim of this study was to develop a practical, prognostic tool to enable the early identification of patients at high risk of severe dengue.

5.2 MATERIALS AND METHODS

All information about study design, patient enrolment, data collection and variable definitions were presented in Chapter 2 (Materials and Methods). To develop the prognostic models for severe dengue, we used logistic regression procedures using predefined clinical, haemobiochemical features and virus-related variables, i.e. NS1 rapid test status, viraemia and dengue serotypes. Akaike information criteria (AIC) and Bayesian information criteria (BIC) were used to downselect from the original full models to the most parsimonious model for practical clinical application.

Model validation was examined by two approaches;

a) "Leave-one-site-out cross-validation", i.e. repeatedly developing the algorithm on all but one study site and validation on the left-out study site
b) Temporal validation with patients recruited before 15 June 2012 as the training set and patients recruited thereafter as the evaluation set [156].

The final logistic regression model was converted to a nomogram for ease of use in clinical practice. All analyses were performed with the statistical software R version R v3.1.1 (R foundation for statistical computing, Vienna, Austria). Significance was assigned at $p < 0.05$ for all parameters and was two-sided.

5.3 RESULTS

5.3.1 Baseline characteristics of study participants

During the study period from Oct 2010 to Dec 2013, we enrolled 7563 patients with fever illness history of less than 72 hours at the outpatient departments of seven hospitals in southern Vietnam. A detailed description of the study profile is shown in Figure 5-1. The number of cases with complete haematological and biochemical data was 7544 patients, of whom 2060 (27.3%) had laboratory-confirmed dengue. DENV serotypes were identified by RT-PCR in 115/117 severe dengue patients (98.3%) and 1839/1943 non-severe dengue patients (94.6%). Table 5-1 describes the baseline characteristics of study patients.

A range of clinical and laboratory features at the time of enrolment were associated with the development of severe dengue. In univariate analysis within the lab-confirmed dengue case population, a history of vomiting at the time of enrolment (OR=2.91 (95%CI: 1.96, 4.33), p<0.001), abdominal pain (OR=1.65 (95%CI: 1.09, 2.49), p=0.018) and longer illness history (OR=1.53 per extra day of illness at enrolment (95%CI: 1.17, 1.99), p=0.002) were associated with development of severe dengue (Table 5-2). Furthermore, patients who developed severe dengue had significantly lower white blood cell (WBC) count (OR per 1,000 cells/mm^3 increase =0.86 (95%CI: 0.79, 0.93), p<0.001), lower platelet count (OR per 10,000 cells/mm^3 increase =0.82 (95%CI: 0.79, 0.85), p<0.001) and lower albumin level (OR per 1g/L increase =0.92 (95%CI: 0.87, 0.97), p=0.003) at the time of enrolment. Additionally, a higher haematocrit (OR per 1% higher Hct =1.14 (95%CI: 1.08, 1.20), p<0.001), elevated aspartate aminotransferase (AST) (OR per two-fold higher =3.74 (95%CI: 3.00, 4.68), p<0.001), NS1 positivity (OR=2.04, (95%CI: 1.25, 3.35), p=0.004) and higher viraemia concentrations (OR per 1 log10 RNA copies/ml higher =1.22 (95%CI: 1.07, 1.39), p=0.003) were also associated with patients who subsequently developed severe dengue (Table 5-2). These data identify objectively early clinical and laboratory signs and symptoms associated with cases that will develop severe dengue from those with an uncomplicated outcome.

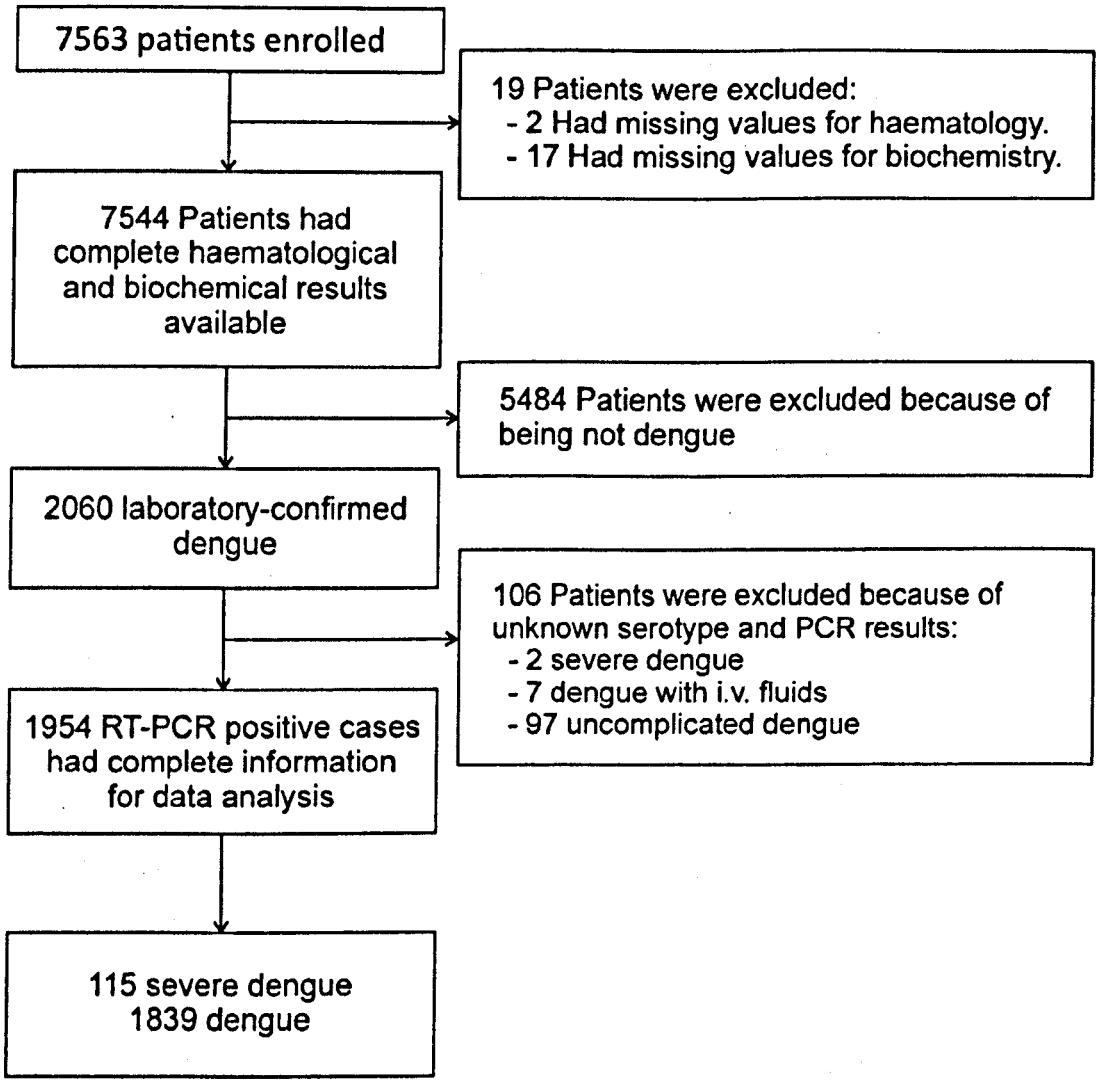


Figure 5-1. Flow diagram of the analysis for the development of severe dengue

Table 5-1. Baseline characteristics of study participants

	Severe dengue (N=117)		Non-severe dengue (N=1943)		OFI (N=5484)	
	n	Descriptive statistics	n	Descriptive statistics	n	Descriptive statistics
Age (years)	117	9 (7-11)	1943	9 (6-11)	5484	5 (3-8)
BMI (kg/(m)^2)	117	16.9 (14.6-20.1)	1943	16.5 (14.7-19.0)	5484	15.6 (14.2-17.7)
Day of illness	117		1943		5484	
1		15 (12.8)	1943	426 (22.0)	5484	1659 (30.3)
2		43 (36.8)	1943	789 (40.8)	5484	2372 (43.4)
3		59 (50.4)	1943	718 (37.1)	5484	1437 (26.3)
Vomiting (n, %)	117	79 (67.5)	1943	804 (41.6)	5484	1902 (34.8)
Abdominal pain (n, %)	117	34 (29.1)	1943	385 (19.9)	5484	930 (17.0)
Mucosal bleeding (n, %)	117	9 (7.7)	1943	112 (5.8)	5484	134 (2.5)
WBC (10^3/mm^3)	117	3.7 (2.5-5.8)	1943	4.9 (3.6-6.9)	5484	9.0 (6.4-12.5)
PLT (10^3/mm^3)	117	110 (86.5-147)	1943	182 (144-227)	5484	242 (201-291)
HCT (%)	117	40 (37.8-42.3)	1943	38.6 (36.6-40.6)	5484	37.4 (35.3-39.7)
ALB (g/L)	117	43.3 (40.5-45.1)	1943	43.8 (41.8-45.8)	5484	44.2 (42.2-46.2)
AST (U/l)	117	101 (61.5-155)	1943	50 (40-66)	5484	42 (35-49)
NS1 rapid test '+' (n, %)	117	97 (82.9)	1943	1360 (70.4)	5484	37 (0.7)
Viraemia concentration (log10copies/ml)	115	7.5 (6.4-8.3)	1839	7.2 (6.0-8.2)	-	-
Serotype	115		1839		-	
DENV-1		38 (32.5)		725 (37.5)		
DENV-2		37 (31.6)		404 (20.9)		
DENV-3		6 (5.1)		181 (9.4)		
DENV-4		34 (29.1)		519 (26.8)		
Unknown		2 (1.7)		104 (5.4)		

All laboratory results were acquired on the day of enrolment.

Values are presented as median and interquartile range for continuous variables or frequency and percentage for categorical variables.

BMI: body mass index; WBC: white blood cell count; PLT: platelet count; HCT: haematocrit; ALB: albumin; AST: aspartate aminotransferase, OFI: other febrile illness.

Table 5-2. Univariate analysis of candidate predictors of severe dengue

	Severe dengue vs. Non-severe dengue		
	OR	95%CI	p
Age (+ 1 year)	1.00	0.95, 1.06	0.920
BMI (+ 1 kg/(m)^2)	1.03	0.98, 1.09	0.223
Day of illness (+ 1 day)	1.53	1.17, 1.99	0.002
Vomiting: Yes	2.91	1.96, 4.33	<0.001
Abdominal pain: Yes	1.65	1.09. 2.49	0.018
Mucosal bleeding: Yes	1.35	0.67, 2.73	0.405
WBC (+1,000 cells/mm^3)	0.86	0.79, 0.93	<0.001
PLT (+10,000 cells/mm^3)	0.82	0.79, 0.85	<0.001
HCT (+ 1 %)	1.14	1.08, 1.20	<0.001
ALB (+ 1 g/L)	0.92	0.87, 0.97	0.003
AST (per 2-fold increase)	3.74	3.00, 4.68	<0.001
NS1 rapid test status '+': Yes	2.04	1.25, 3.35	0.004
Viraemia: (+1 log10copies/ml)	1.22	1.07, 1.39	0.003
Serotype [#]			
DENV-1	1.00	-	-
DENV-2	1.74	1.09, 2.79	0.020
DENV-3	0.63	0.26, 1.52	0.305
DENV-4	1.25	0.78, 2.02	0.355

BMI: body mass index; WBC: white blood cell count; PLT: platelet count; HCT: haematocrit; ALB: albumin; AST: aspartate aminotransferase

[#]: Univariate effect of serotype on severe dengue estimated from univariate logistic regression, using DENV-1 as reference group.

5.3.2 Prognostic models for early identification of severe dengue cases from amongst all true dengue cases

We sought to determine whether a simple, clinically useful prognostic algorithm for severe dengue could be derived from the informative clinical and laboratory signs identified above. We used logistic regression analysis to examine the impact of predefined clinical, haemobiochemical features, NS1 rapid test status, viraemia and dengue serotypes on severe dengue. For simplicity, all continuous variables were treated as linear terms in the multivariable model development of the prognostic algorithm (Supplementary Table 5-1, Supplementary Figure 5-1 and Statistical appendix). There was no interaction between age or day of illness and all other predictors. The most parsimonious prognostic model, herein called the Early Severe Dengue Identifier (ESDI), included vomiting, platelet count, AST (per two-fold increase) and NS1 rapid test status at the time of enrolment (Table 5-3). Alternative approaches to feature selection yielded models with only slightly higher performance but relied on many more variables and/or viraemia concentration by RT-PCR which is usually not feasible in clinical practice (Supplementary Table 5-2). Figure 5-2 and Table 5-4 illustrate the performance characteristics of the ESDI at various cut-offs in the population of confirmed dengue cases. At the cut-off of 0.05 the ESDI had a sensitivity of 77% (95%CI: 69-84%), specificity of 71% (95%CI: 69-73%), positive predictive value of 14% (95%CI: 12-17%), and negative predictive value of 98% (95%CI: 97-99%) for correctly discriminating severe dengue cases in the dengue population. The rationale for the electing a risk threshold of 0.05 was that the "cost" of missing a true severe dengue case as 20 times higher than the cost of a false-positive [157]. Note, this predefined cut-off, based on clinical priorities, was very close to the point on the ROC curve closest to the upper left corner (perfect model), which was 0.048 (Figure 5-2A). The area under the ROC curve (AUC) of the ESDI amongst the population of true dengue cases was 0.83 using the cut-off of 0.05 (Figure 5-2B).

The calibration plot shows that the ESDI overestimates the risk of severe dengue in the highest decile of predicted probability (Figure 5-2C), i.e. patients scored as having the highest decile of risk actually have a lower true risk than the model suggests (Figure 5-3 and Table 5-4). A summary of the performance characteristics of the ESDI by temporal and leave-one-site out validation is presented in Table 5-5. The ESDI clearly showed good discriminative performance for both temporal and leave-one-site out validation with an AUC of at least 0.89.

Table 5-3. Multivariate analysis of candidate predictors for severe dengue amongst RT-PCR positive dengue cases

	Full model with predefined candidate predictors			Reduced model by step-wise BIC			Alternative model (ESDI)		
	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
Age (+ 1 year)	0.94	0.87, 1.01	0.109	-	-	-			
BMI (+ 1 kg/(m)^2)	1.02	0.96, 1.09	0.547	-	-	-			
Day of illness (+ 1 day)	1.69	0.58, 1.18	0.295	-	-	-			
Vomiting: Yes	1.82	1.15, 2.88	0.011	1.86	1.21, 2.87	0.005	2.00	1.30, 3.07	0.002
Abdominal pain: Yes	0.95	0.57, 1.58	0.847	-	-	-			
Mucosal bleeding: Yes	1.37	0.61, 3.09	0.451	-	-	-			
WBC (+1,000 cells/mm^3)	1.07	0.96, 1.19	0.227	-	-	-			
PLT (+10,000 cells/mm^3)	0.85	0.80, 0.89	<0.001	0.86	0.83, 0.90	<0.001	0.87	0.84, 0.91	<0.001
HCT (+ 1 %)	1.07	1.00, 1.14	0.061	-	-	-			
ALB (+ 1 g/L)	0.98	0.92, 1.06	0.655	-	-	-			
AST (per 2-fold increase)	2.70	2.06, 3.53	<0.001	2.70	2.09, 3.48	<0.001	2.64	2.04, 3.40	<0.001
NS1 rapid test '+': Yes	2.46	1.30, 4.66	0.006	-	-	-	2.63	1.52, 4.57	<0.001
Viraemia: (+1 log10copies/ml)	1.42	1.18, 1.70	<0.001	1.44	1.23, 1.69	<0.001			
Serotype									
DENV-1	1.00	-	-	-	-	-			
DENV-2	2.18	1.25, 3.82	0.006	-	-	-			
DENV-3	0.69	0.27, 1.76	0.434	-	-	-			
DENV-4	1.61	0.92, 2.81	0.096	-	-	-			

Full model with predefined candidate predictors: clinical, haemobiochemical features, NS1 rapid test status, viraemia concentration, serotype.

Alternative model included only 4 variables: vomiting, platelet count, AST (per 2-fold increase), NS1 rapid test status.

WBC: white blood cell count; PLT: platelet count; HCT: haematocrit; ALB: albumin; AST: aspartate aminotransferase; ESDI: Early Severe Dengue Identifier

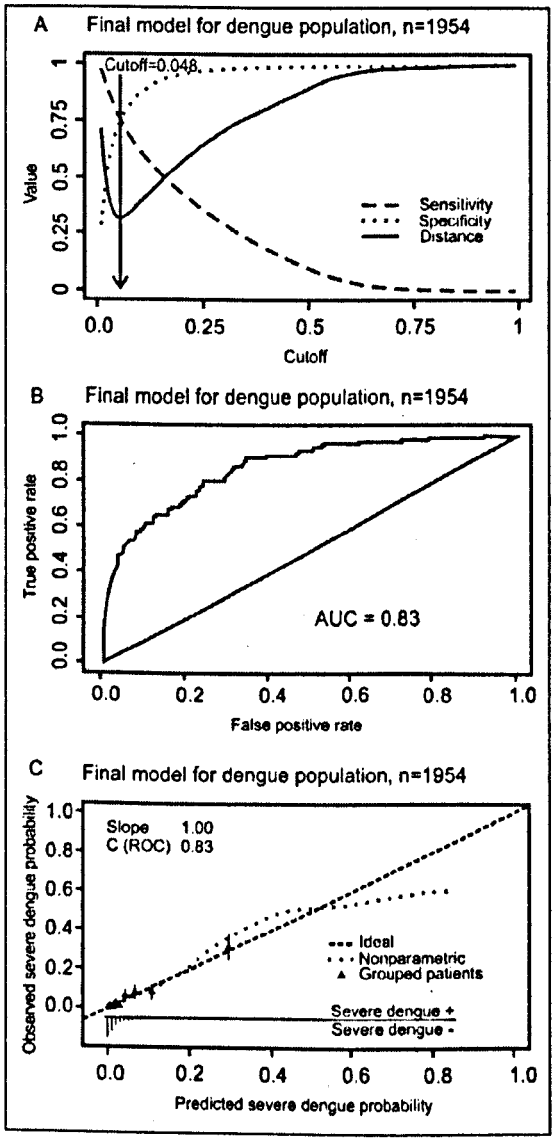


Figure 5-2. Performance of the Early Severe Dengue Identifier in RT-PCR positive dengue patients. *Figure 5-2A displays possible sensitivity/specificity trade-offs for different cut-off values and the distance from the corresponding points on the ROC curve to the upper left corner (perfect model). Figure 5-2B displays the receiver operating characteristic (ROC) curve. Figure 5-2C is a calibration plot. It displays a scatterplot-smoother of predicted versus observed risks (dotted line), predicted versus observed risks for ten patient strata of equal size grouped according to predicted risks (triangles) and the ideal identity line (dashed line). The rugs at the bottom of the graphs characterize the distribution of predicted risks in severe dengue and non-severe dengue cases, respectively.*

Table 5-4. Number of true positive and false positive severe dengue cases according to different cut-offs for classification

Risk thresholds	Number of true positives	Number of false positives
0.000	115	1839
0.005	114	1603
0.010	111	1370
0.050	89	526
0.100	71	223
0.200	48	72
0.300	37	32
0.400	28	16
0.500	11	8

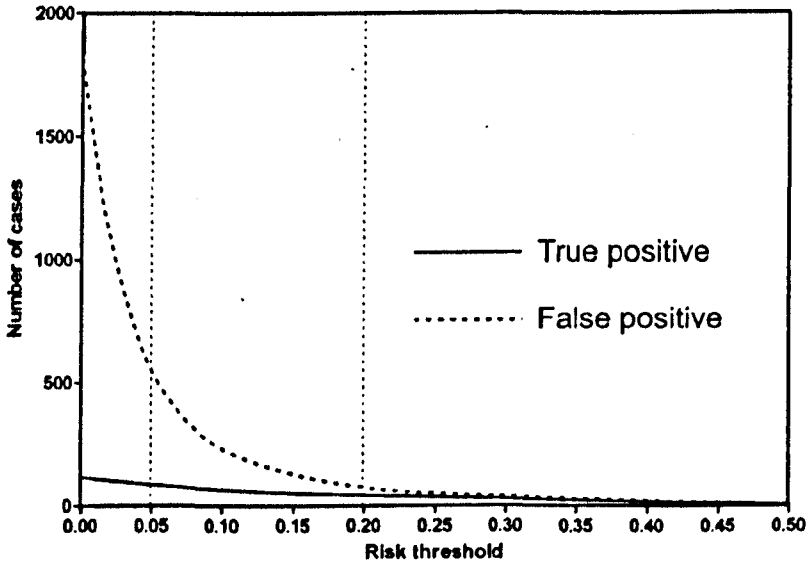


Figure 5-3. Changes in number of true and false positive severe dengue cases according to different cut-offs for classification when the ESDI is applied on the whole data.

Table 5-5. Performance of the Early Severe Dengue Identifier (ESDI)

	All RT-PCR positive patients	All study participants	Temporal validation	Leave-one-site-out validation
Calibration intercept	0	0.06	0.18	0.51 (-0.09 – 0.69)
Calibration slope	1	1.12	1.63	1.35 (0.81 – 1.39)
AUC	0.83	0.95	0.90	0.89
(95%CI)	(0.79, 0.87)	(0.92, 0.98)	(0.76, 0.87)	(0.78 – 0.92)
Sensitivity (cutoff 0.05)	0.77	0.87	0.91	0.88
(95%CI)	(0.69, 0.84)	(0.80, 0.92)	(0.85–0.96)	(0.68 – 0.93)
Specificity (cutoff 0.05)	0.71	0.88	0.73	0.76
(95%CI)	(0.69, 0.73)	(0.87, 0.89)	(0.70–0.75)	(0.73–0.82)
PPV (cutoff 0.05)	0.14	0.10	0.18	0.14
(95%CI)	(0.12, 0.17)	(0.09, 0.12)	(0.16–0.20)	(0.13–0.23)
NPV (cutoff 0.05)	0.98	0.998	0.99	0.98
(95%CI)	(0.97, 0.99)	(0.996, 0.999)	(0.98–0.99)	(0.97–0.99)

The prognostic model for severe dengue amongst RT-PCR positive dengue patients was the reduced model by step-wise BIC, derived from the original full model which included all pre-defined clinical, haemobiochemical features, NS1 rapid test status, viraemia concentration and serotype. The table shows apparent performance (95%CI) in all dengue patients; performance (95%CI) in the validation set for temporal validation; and mean (range) of performances across left out sites for leave-one-site-out validation.

5.3.3 Performance of the ESDI amongst all enrolled cases (dengue and non-dengue)

The ESDI had better performance when applied to the whole study population, (using the cut-off of ≥ 0.05) with an AUC of 0.95, sensitivity of 87% (95%CI: 80-92%), specificity of 88% (95%CI: 87-89%), positive predictive value of 10% (95%CI: 9-12%), and negative predictive value of 99.8% (95%CI: 99.6-99.9%) for the prognosis of severe dengue (Table 5-5).

5.3.4 A practical tool for physicians to predict severe dengue

A nomogram was developed to predict severe dengue using the 4 independent parameters in the ESDI (Figure 5-4). The nomogram is used by totaling the points assigned on the scales for each independent parameters. This total point is subsequently identified on the total points scale to achieve the risk of severe dengue. For example, a patient with vomiting, a PLT of 50,000/mm^3, positive NS1 rapid test and an AST of 240U/L (6-fold increase compared to the upper normal value of 40U/L) has a score of 8+92+12+30=142, and the corresponding risk of severe dengue is ~30%.

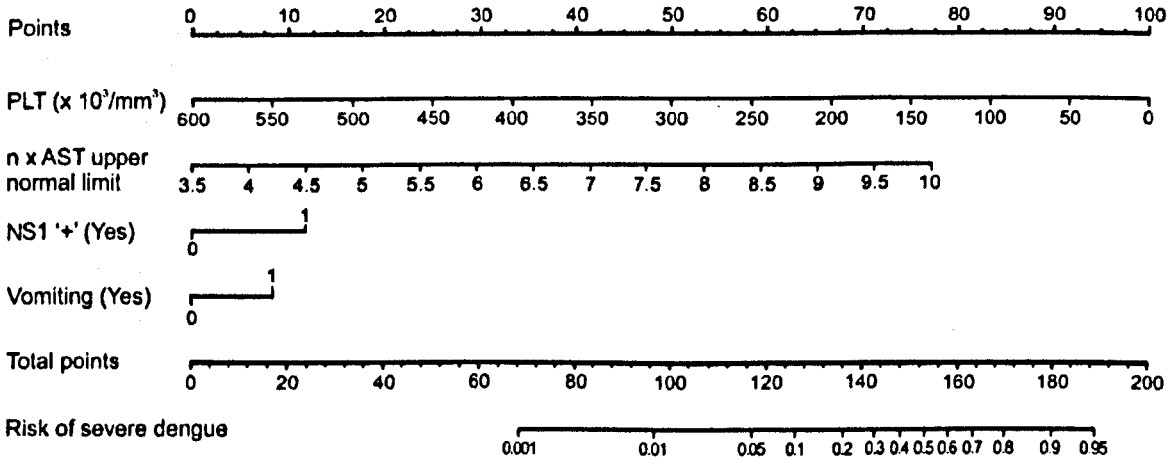


Figure 5-4. Nomogram of the prognostic model to predict the risk of severe dengue. *A vertical line from a predictor value to the "Points" axis assigns points to the 4 required variables: vomiting, platelet count (PLT), NS1 rapid test status and AST. The sum of these points (total points) can then be translated to the corresponding predicted risk of severe dengue. As an example, a patient with vomiting, a PLT of 50x10^3/mm^3, positive NS1 rapid test and an AST of 6 times higher compared to the upper normal limit has a score of 8+92+12+30=142, and the corresponding risk of severe dengue is about 30%.*

5.4 DISCUSSION

The great majority of clinically severe complications in paediatric dengue patients occur between the 4th and 6th day of illness [1, 150, 176]. Thus there is a window of opportunity in the first few days of illness to both make a diagnosis and try to predict which patients are at greatest risk of severe complications well before their onset. Yet the current standard of care in relation to prognosis relies on random clinical judgment.

Here we demonstrate the feasibility of early diagnosis and prognosis (<72 hours after illness onset) of severe dengue using simple clinical and laboratory investigations that are available in many endemic settings. A parsimonious scoring system, the ESDI, provides a simple tool for prognostic classification. The results will be particularly helpful in two scenarios. First, the ESDI might enable physicians to improve triage and clinical management (e.g. through more careful and frequent observations) of dengue cases in the outpatient setting- potentially resulting in fewer cases developing life-threatening severe outcomes. Second, the ESDI can assist clinical research studies aiming to enrol cohorts of patients who will develop severe disease at a higher frequency than would occur if simple consecutive enrolment of cases occurred, as is currently the standard approach in therapeutic trials [185-187]. Thus the ESDI could deliver efficiencies to clinical trials, i.e. cohorts enriched for severe clinical events that are not possible with the current standard of care.

The ability to make an early, evidence-based prognosis of severe dengue could have practical rewards to clinical care, health systems and clinical research. First, the ESDI could assist clinical services in identifying at risk patients for triage to more regular observations than would occur under the current standard of care; this could mean hospitalization or more regular visits in an outpatient setting. Additionally, for outpatient care, communication to the patient's caregivers could be appropriately calibrated and with attention to WHO-nominated clinical warning signs [1]. The benefits of early prognosis, with accompanying closer clinical management, could potentially include a reduction in the frequency with which cases progress to severe disease and hence cost to the health care system and to families. However, the generally low positive predictive value of the ESDI (10% at a cut-off of 0.05) inevitably means that a large number of cases identified as being at risk of severe disease will in fact have uneventful disease evolutions. Nonetheless we believe

the ESDI delivers a useful tool to clinicians because they currently work in a vacuum of evidence with respect to predicting outcome in paediatric dengue cases with <72 hours of fever.

NS1 rapid tests are used as screening tools in trials of candidate dengue therapeutics [185-187]. By themselves these tests are helpful for early and rapid diagnosis, but it does not greatly assist the task of enriching clinical intervention studies with patients at greater risk of severe dengue. As an example, a clinical trial of early prednisolone therapy in 225 Vietnamese children observed that only 6.7% developed DSS in the placebo arm. Treatment trials in adult dengue cases observe an even lower incidence of severe dengue [185, 187, 188]. Thus, early phase treatment studies endeavouring to use severe dengue as a primary endpoint require large sample sizes to meet their objectives. The ESDI could enable efficiencies in trial performance by enriching the population of participants with cases at higher risk of developing severe dengue.

Most, but not all, of the parameters used in the ESDI are routinely collectable in primary care settings in Vietnam and other endemic countries. For example, the NS1 antigen rapid test is available in most hospital emergency department/outpatient settings in Vietnam. NS1 rapid tests are recommended by WHO as a routine screening test for patients with clinically suspected dengue in the acute febrile phase [189] and are becoming more widely used in Vietnam. The diagnostic performance, and limitations, of this test has been extensively described in this patient cohort [190] and elsewhere [96, 98, 107, 113, 114, 116, 145, 163]. Previous studies also demonstrated that viraemia levels were strongly correlated with NS1 rapid test status [63, 98, 109, 128, 191]. Haematology (platelet counts) and biochemistry (AST) were also important to the operation of the ESDI. However biochemistry is not routinely performed in outpatient settings and thus this represents an unmet need with respect to the operation of the ESDI. Potts *et al* previously observed that elevated AST in the first three days of illness was a predictor for severe dengue [178]. Presumably, early elevations in blood AST concentrations are a signal of the severity of disseminated virus infection and tissue damage. Some of the WHO-nominated warning signs of severe dengue, e.g. mucosal haemorrhage, clinical fluid accumulation and hepatomegaly were not prominent at the time of enrolment. This is not surprising since clinical experience indicates these are more likely to be present nearer to the time of severe complications occurring, i.e. day 4-6.

Not all DENV serotypes were equally associated with severe outcomes. DENV-2 in particular was over-represented amongst severe cases, consistent with previous studies [110, 122]. For reasons of study design, we could not dissociate whether DENV-2 was simply a proxy for secondary infection, itself a well-recognised risk factor for severe dengue, or was independently associated with severe dengue. We believe the former is more likely, since previous studies in this setting indicate that primary DENV-2 infections are rarely detected amongst hospitalized patients.

Only one previous prospective study, of 1384 febrile Thai children (including 37 with DSS), by Potts *et al*, is similar in study design to that reported here. Potts *et al* used classification and regression tree analysis to derive an algorithm from laboratory variables collected at the time of enrolment (platelet count, white blood cell count, monocyte percentage and haematocrit). The best algorithm had 97% sensitivity and 48% specificity for the identification of patients who progressed to DSS [178], however positive and negative predictive values were not reported. The study described here includes several important points of difference including-a) a much larger sample size and inclusion of clinically severe cases who did not have DSS, b) acquisition of clinical and laboratory data and c) validation of model performance.

Our study, and the resulting ESDI, has some inherent limitations. First, the ESDI will not be suitable in all outpatient settings because NS1 rapid tests and biochemistry are not always available. The ESDI is only applicable to observations made in the first 72 hours of illness; it's uncertain what the test performance will be outside this window of presentation. The evolution of dengue is probably impacted by clinical management; hence the incidence rate of severe dengue described in this study is likely to be context dependent; for the same patient population other settings might observe a lower or higher incidence of severe dengue and this could impact the prognostic classifier. Finally, though the ESDI has a good discriminative ability (AUC=0.83), the low PPV (common to many algorithms seeking to classify relatively rare events) means that the number of true severe dengue cases will be overestimated. Application of the ESDI may result in excessive hospitalizations, unnecessary follow-up procedures and the associated economic burden than would occur under the current standard of care. Further "pilot phase" research is needed to understand the benefits and disadvantages of the ESDI in routine practice and clinical research.

APPENDIX

Supplementary Table 5-1. Linearity tests and interaction tests in the multivariable logistic regression model for the development of severe dengue (based on the original full model including all pre-defined clinical, haemobiochemical features, NS1 rapid test status, viraemia and serotype)

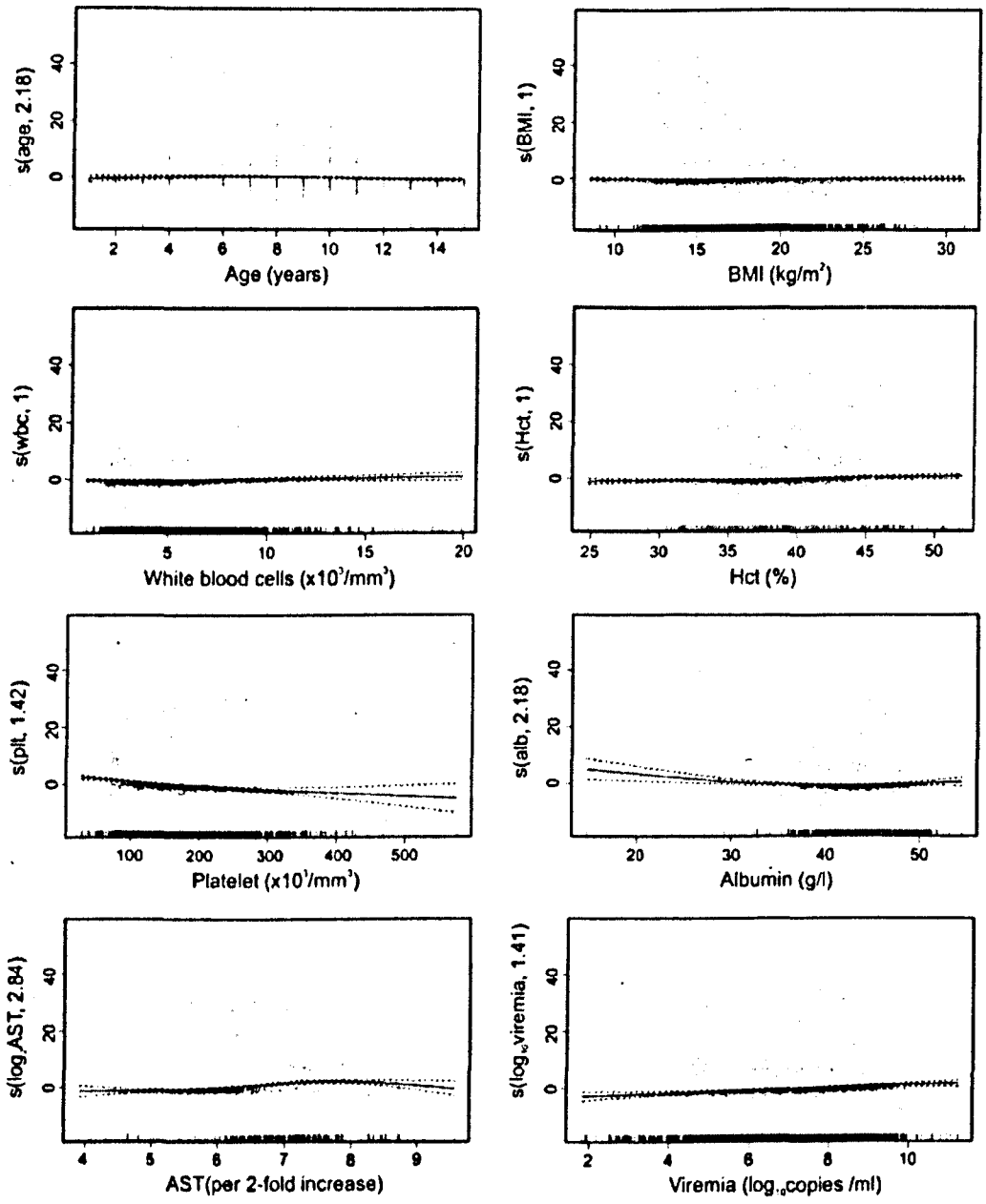
	Non-linear transformation	Deviance	df	p value
Linearity tests				
Age	Restricted cubic spline with 4 knots	7.97	3	0.05
BMI	Restricted cubic spline with 4 knots	0.44	3	0.93
WBC	Restricted cubic spline with 4 knots	5.92	3	0.12
PLT/10000	Restricted cubic spline with 4 knots	13.04	3	0.00
Hct	Restricted cubic spline with 4 knots	0.94	3	0.82
Albumin	Restricted cubic spline with 4 knots	10.12	3	0.02
log2AST	Restricted cubic spline with 4 knots	14.08	3	0.00
log10viraemia	Restricted cubic spline with 4 knots	1.03	3	0.79
Interaction tests				
Age vs. all others		15.32	10	0.12
Day of illness vs. all others		18.29	10	0.06

BMI: body mass index; WBC: white blood cell count; PLT: platelet count; AST: aspartate aminotransferase

Supplementary Table 5-2. Performance of prognostic models for severe dengue amongst laboratory-confirmed dengue

	Full model with predefined candidate predictors		Alternative model
	Reduced model by step-wise AIC	Reduced model by step-wise BIC	(ESDI)
AUC	0.87	0.85	0.83
Sensitivity	0.81	0.80	0.77
(95%CI)	(0.73, 0.87)	(0.72, 0.86)	(0.69, 0.84)
Specificity	0.74	0.72	0.71
(95%CI)	(0.72, 0.76)	(0.70, 0.74)	(0.69, 0.73)
PPV	0.16	0.15	0.14
(95%CI)	(0.14, 0.20)	(0.13, 0.18)	(0.12, 0.17)
NPV	0.98	0.98	0.98
(95%CI)	(0.98, 0.99)	(0.97, 0.99)	(0.97, 0.99)

The starting full model with predefined candidate predictors included all variables listed in Table 5-1.
Reduced model by step-wise AIC: age, vomiting, white blood cell count, hct, platelet count, log2(aspartate aminotransferase), NS1 rapid test status, log10(viraemia), serotype.
Reduced model by step-wise BIC: vomiting, platelet count, log2(aspartate aminotransferase), log10(viraemia).
Alternative full model: vomiting, platelet count, log2(aspartate aminotransferase), NS1 rapid test status.
AUC: area under the ROC curve, PPV: positive predictive value, NPV: negative predictive value, AIC: Akaike information criteria, BIC: Bayesian information criteria, ESDI: Early Severe Dengue Classifier



Supplementary Figure 5-1. Plots of estimated component smooth functions of a GAM for the risk of severe dengue which included all candidate predictors listed in Supplementary Table 5-1 and modelled continuous parameters as smooth terms. Only terms estimated to have a non-linear association with outcome are displayed. Dots correspond to individual partial residuals; solid lines correspond to smooth spline functions estimated by GAM; dashed lines correspond to the estimated smooth functions plus/minus one standard error.

Statistical appendix

Assessment of linearity

A generalized additive model (GAM) with integrated smoothness estimation as implemented in the R package mgcv version 1.8-0 was used to assess potential non-linear effects of variables on the risk of severe dengue. The model included all candidate predictors listed in Table 5-1 and modelled continuous parameters as smooth terms.

The GAM chose non-linear associations for several parameters including age, BMI, white blood cell count, platelet count, Hct, AST, albumin and viraemia (Supplementary Figure 5-1). The estimated association of outcome with all of these variables was reasonably close to a linear function. Hence, these variables were treated as linear terms.

Assessment of interactions

Potential interactions between age, day of illness and haematological and biochemical results were examined by likelihood ratio tests based on the full model with all variables from Table 5-1.

No interaction between age, day of illness and haematological and biochemical results were statistically significant (all p-values > 0.05).

Chapter 6

GENERAL DISCUSSION AND FUTURE RESEARCH

The incidence of dengue has grown dramatically around the world in recent decades. The actual numbers of dengue cases are underreported and many cases are misclassified. Dengue is one of the diseases of most public health concern in Vietnam. The annual incidence of dengue per 100,000 persons is tens to hundreds of patients, significantly affecting the country's socio-economy. Vietnam is also an endemic country where four serotypes circulate throughout the year posing a huge disease burden to every family in the country. Classic preventive interventions have not brought expected outcomes to achieve a reduction in virus transmission. The majority of vector control programs such as aerial spraying with insecticides, killing mosquito larva also have limited effectiveness. These lead to a high pressure on the hospital sector to follow up and provide medical treatment to patients during the outbreaks. The aims of this thesis were to document the range of clinical outcomes in children with dengue and to develop a simple tool to help physicians not only make early diagnosis of dengue but also prediction of severe dengue.

Although the case mortality rate has reduced significantly in Vietnam over the past 20 years, to below 0.1% for 2014, in many other endemic regions of the world the disease has a greater mortality rate. Dengue was ranked as the fastest-spreading, vector-borne viral disease in 2012. Dengue cases have been reported in several nonendemic countries and territories. Main drivers for dengue transmission are urbanization, population growth with poor sanitary condition and long distant transportation. The actual numbers of dengue cases are underreported because the reports are based on hospitalized dengue patients in public hospitals. With the blooming growth of private hospitals and clinics in most major cities, it is essential for these healthcare providers to make reports to the surveillance system so as we can take intervention measures to prevent the outbreak from occurring. Furthermore, many dengue cases are misclassified

because just a small proportion receive specific diagnostic tests and the final classification is taken from the discharge diagnosis, i.e. there will be reported dengue cases that are actually not dengue and vice versa. Therefore, a need for active surveillance system in the endemic regions must be implemented to document the index case on a weekly or even daily basis to help local authorities have preparedness and plan for disease control. Health education for community to change attitude and behaviours with regard to mosquito as well as larva elimination, and above all active surveillance system are the principle measures to tackle epidemic dengue.

The majority of dengue cases reported from the healthcare services are based on clinical judgment, using clinical findings and the complete blood counts alone. It is important to have a more specific and affordable laboratory test as a screening platform in parallel with clinical manifestations and complete blood count to achieve a better sensitivity and specificity in dengue diagnosis. Such a test is the rapid NS1 antigen detection assay now available with the sensitivity and specificity of approximate 70% and 99% respectively. Of note, the NS1 rapid test status is associated with dengue severity because it has close relationship with plasma viral RNA concentration. Although the sensitivity of the NS1 rapid test is not excellent, it is highly specific and does not require expertise or complicated facilities compared to RT-PCR and serological techniques. It is also difficult to interpret a single anti-dengue IgM alone because a positive result can be the persistence of antibodies up to 3 months after initial infection. Therefore, this may lead to misclassification of acute febrile illnesses as dengue due to persistence of anti-dengue IgM occurring after dengue infection. On the contrary, a single negative result of anti-dengue IgM cannot absolutely exclude dengue infection from an acute febrile patient with clinically suspected dengue because anti-dengue IgM antibody generally occurs 3-5 days after illness onset and is usually less sensitive in secondary infection. Therefore, the NS1 rapid test should be incorporated to the surveillance system

and healthcare facilities at all level should use it routinely in early diagnosis and implementation of effective intervention. It is, however, necessary to have more studies in evaluating new generation of NS1 rapid test to combine NS1 antigen and antibody results to exploit the temporal diagnostic characteristics of each analyte to achieve higher performance of the test [192].

Our project was the largest prospective cohort study in Vietnam to comprehensively investigate clinical and virological characteristics of children with dengue in the hottest endemic regions in southern Vietnam. The results from the study provide evidence-based insights of dengue and are especially useful in terms of early diagnosis and prognosis of severe dengue. In endemic countries like Vietnam, dengue occurs throughout the year and mainly in the wet season. It is impossible to perform hospitalization for every dengue patient in a setting with limited resources. It is essential for physicians to recognize patients with dengue early and for those at risk of developing severe complications to direct appropriate management such as hospitalization or oral fluid intake. The effort of WHO to design a new reclassification of dengue disease and incorporate this scheme to the Integrated Management Childhood Illness algorithm helps health workers assess a sick child's condition, classify the illness, treat the child, and counsel the parent. The algorithm consists of case management guidelines developed based on evidence from clinical research. The guidelines are syndromic meaning they rely on systematic assessment of signs and symptoms with a focus on sick children less than 5 years old. Our diagnostic tool has an acceptable sensitivity and specificity as well as applicable usage for children from 1 to 15 years of age. Different age setting and region or country-specific validation would be useful to increase confidence in the diagnostic algorithm.

Early prediction of severe dengue is a challenge depending mainly on close monitoring every day. The issue is to identify a small proportion of dengue cases at risk of developing to severe complications or requiring intravenous fluid

infusion to guide attending doctors in the outpatient settings to have appropriate management by indication for hospitalization without making more pressure on the overload of inpatient wards. The ESDI serves as an adjunct tool to help physicians especially general practitioners make clinical judgment about the probability of severe dengue of a child with acute onset of fever. The ESDI has a low positive predictive value because it predicts a rare outcome; yet it can define a population at high risk of severe dengue so that early intervention measures can be taken to reduce the actual complications or clinical trials with new medications can be conducted on this target group. However, due to a low PPV for severe dengue as commonly seen in prediction of a rare outcome, the ESDI can induce overestimation of this severe population as well as hospitalization of false positive severe cases. There has been robust evidence to establish the association between plasma viral RNA concentration and dengue severity. The RT-PCR technique is also highly sensitive and specific in making early diagnosis of dengue as well as quantitation of dengue viraemia. It is time to consider dengue viraemia by RT-PCR as a necessary laboratory test in endemic season where it can help in patient diagnosis, virus surveillance and perhaps in prognosis. It is also valuable to use dengue viraemia as an important endpoint in clinical trials in therapeutic perspective or preventive measures such as vaccine or the usage of biological agent, i.e. *Wolbachia*, to eliminate dengue. These studies are in progress and have initial encouraging results to set up further investigation about the effectiveness of these interventions.

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