Dengue virus evolution in Southern Vietnam over the last ten years and utility of DENV-NSI as a diagnostic marker

Thesis

Dengue virus evolution in Southern Vietnam over the last ten years and utility of DENV-NSI as a diagnostic marker

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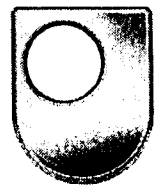

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ABSTRACT

Dengue is a major public health problem in many parts of the tropical developing world. Dengue is caused by infection with one of four serotypes of dengue virus (DENV1-4), which are arboviruses belonging to the Flaviviridae family. Although most DENV infections are asymptomatic, mild, or self-limited, a proportion of dengue fever cases result in clinically apparent disease that varies in severity from mild undifferentiated fever through to more severe syndromes, primarily dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).

The main work described in this thesis was to examine molecular epidemiology of DENV over the last 10 years in southern Vietnam (1999-2008) and to document DENV molecular epidemiology in the 2007 dengue outbreak in Dong Thap province, a Mekong Delta province, southern Vietnam. A further purpose was to assess the utility of two commercial NS1 antigen test kits, Platelia NS1-ELISA and NS1-Lateral Flow Rapid (NS1-LFRT), for early diagnosis of acute dengue infection in relation to immune status, DENV-reactive measurable IgM/IgG, viraemia level, and duration of illness prior to patient specimen collection.

Sequence analyses of Vietnamese DENV-1 and DENV-2 revealed that these viruses comprised significant genetic diversity. DENV-2 exhibits 3 distinct genotypes, American/Asian, Asian 1 and Cosmopolitan. DENV-1 viruses have become predominant in Vietnam since 2006, and circulate as 6 distinct lineages within genotype I. The most striking finding of this work was the replacement of DENV-2 genotype in the context of serotype shift occurring in southern Vietnam; both of which were temporally linked to increased disease incidence. The American/Asian genotype which has circulated in HCMC and surrounding provinces since at least 1987 was displaced by the DENV-2 Asian 1 genotype preceding the change in serotype from DENV-2 to DENV-1. However, negative selection appears to be the major evolutionary factor impacting DENV population dynamics.

Evaluation of the diagnostic accuracy for the two commercial NS1 test kits consistently showed that viraemia level in acute phase NS1-positive patients is higher than in NS1-negative patients. NS1 sensitivity was also greater in patients with primary infection.

Proportion of NS1 positivity was generally greater in DF cases. Duration of fever time prior to patient blood samples collected also contributes to NS1 sensitivity. Both DENV-reactive IgG and IgM were negatively correlated with NS1 sensitivity, but IgG showed stronger influence on NS1 positivity rate. NS1 sensitivity is also different in individual infecting serotypes; DENV-2 consistently shows lower positive NS1 results for both test kits. Overall, NS1-LFRT was modestly less sensitive than Platelia NS1-ELISA but importantly, both Platelia NS1-ELISA and NS1-LFRT retain extremely high specificity of 100%.

With a large number of whole DENV genome sequences generated in this study, it is a massive contribution to the DENV genome data in GenBank. In addition, results presented in this study contribute to current knowledge of the DENV genetic diversity which is of a fundamental importance in vaccine or drug designs. Results from the evaluation of the utility of NS1 antigen as a diagnostic marker provide further information of the strength and the weakness of the current NS1 antigen detection assays.

LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ADE	Antibody-Dependent Enhancement
Ag	Antigen
ALT	Alanine aminotransaminase
Am/As	American/ Asian
Asn	Asparagine
Asp	Aspartic acid
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BHQ 1	Black hole quencher 1
Bp	Base pair
BSA	Bovine serum albumin
^0C	Celsius degree
cDNA	Complementary DNA
CDS	Coding sequence
CFR	Case fatality rate
CH#1	Children's Hospital #1
CH#2	Children's Hospital #2
CIs	Confidence intervals
CO	Cut-off
DALYs	Disability adjusted life years
DENV	Dengue virus
DENV-NS1	Dengue nonstructural glycoprotein1
DHF	Dengue haemorrhagic fever
DF	Dengue fever
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dN	Nonsynonymous substitution
DOI	Day of illness
dS	Synonymous substitution
dsDNA	Double stranded DNA
DSS	Dengue shock syndrome
DTH	Dong Thap Hospital
DTT	Dithiothreitol
E protein	Envelope protein
EAV	*Equine arteritis* virus
E. coli	*Escherichia coli*

EIP	Extrinsic incubation period
ELISA	Enzyme linked Immuno Sorbent Assay
FEL	Fixed Effects Likelihood
F primer	Forward primer
FAM	5-carboxyfluorescesein
FN	False negative
FP	False positive
GAC ELISA	IgG antibody-capture ELISA
GPI	Glycosyl-phosphatidylinositol anchor
$GTR+I+G_4$	General time reversible, Invariant sites, gamma distribution
HCMC	Ho Chi Minh City
HGT	High gelling temperature
HRP	Horseradish peroxidase
HTD	Hospital for Tropical Diseases
IPTG	Isopropyl-b-D-thiogalactopyranoside
JEV	Japanese encephalitis virus
kb	Kilo base
kDa	Kilo Dalton
LFRT	Lateral Flow Rapid Test
LNA probe	Locked Nucleic Acid probe
MAb	monoclonal antibody
MAC ELISA	IgM antibody-capture ELISA
MEGA	Molecular Evolutionary Genetic Analysis
ml	Millilitre
Ml	Maximum Likelihood
mM	Millimoles
mNS1	Membrane-associated NS1
NC	Negative control
ng	Nanogram
NJ	Neighbour Joining
nm	Nanometer
NPV	Negative predictive value
nt	Nucleotide
NTPase	Nucleoside triphosphate hydrolase
OD	Optical density
OD_B	Mean OD of background sample
OD_N	Mean OD of negative samples
OD_S	Optical density of samples
ODP	o-Phenylenediamine dihydrochloride
OFI	Other febrile illness
OR	Odds ratio

PAUP*	Phylogenetic analysis using parsimony *and other methods
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PPV	Positive predictive value
PrM	Pre-membrane protein
PRNT	Plaque Reduction Neutralization Test
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase - Polymerase Chain Reaction
R Primer	Reverse Primer
RT	Room temperature
SC5b-9	Terminal complement complex (*SC5b-9*)
SD	Standard deviation
SE-Asian	Southeast Asian
SILs	Small Insert Libraries
sNS1	Secreted-hexamer form
TAMRA	N'tetramethyl-6-carboxyrhodamine
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA Buffer
TMB	Tetramethylbenzidine
TN	True negative
TP	True positive
UTR	Un-Translated Regions
VN	Vietnam
WGA	Whole genome amplification
WGS	Whole genome sequencing
WHO	World Health Organization
X- gal	5-bromo-4-chloro-3-indolyl-b- D galactopyranoside

Chapter 1

1. Introduction

1.1. History

1.1.1. The term "Dengue"

Epidemics of dengue fever (DF) have occurred in Africa, Asia, Europe and the Americas, since the first was described in 1780 in Philadelphia [1-3]. Patients with DF showed some or all of the following symptoms: fever, headache, severe muscle pain, joint pain, nausea, vomiting, rash, thrombocytopenia, haemorrhagic manifestations and restlessness. According to the Oxford English Dictionary, the word "dengue" originated in West Indian Spanish as *dinga* (from *kidingapopo* – dislike of movement by affected patients). In Spain, "dengue' was initially used in Madrid in 1801 to describe a fatigued appearance in patients with syndromes such as bone and joint pains, haemorrhage, and jaundice [1, 3].

1.1.2. Discovery of vector and virus

In 1903, Graham described experiments in which he claimed to show that dengue was transmitted by mosquitoes and was caused by protozoa inhabiting red blood cells and closely resembling the plasmodia of Malaria [4]. Many subsequent studies conducted have attempted to confirm that the protozoa described by Graham were the agent of dengue, but all were unsuccessful. Although Graham's interpretation of the agent causing dengue erred, his results regarding the mechanism of transmission in dengue were valuable and well-grounded. In 1906, Ashburn and Craig experimentally infected nine volunteers with mosquitoes and two volunteers with an intravenous injection of filtered and unfiltered dengue blood [4]. This research confirmed the role of mosquito vectors in dengue transmission and conclusively proved that a causative agent was ultramicroscopic in size. However, the real agent causing dengue was not identified until 1943-1944. During World War II, dengue assumed considerable military importance because of its tendency to occur in massive outbreaks affecting large numbers of men. Japanese scientists first identified the virus in 1943 [5] and were quickly followed by the U.S researchers – Sabin and his co-workers. Using active cross

immunity tests in human volunteers (he inoculated one strain of DENV into volunteers, re-infected these volunteers with the same strain/or a different strain within intervals of several to 18 months, then recorded the level of protection or developed disease result) or a dermal neutralization test with viruses diluted in convalescent/healthy sera in human volunteers, Sabin showed that of infected sera sent from Hawaii (n=1), New Guinea (n=4) and India (n=2); the Hawaiian strain, one of the four New Guinea strains, and the two Indian strains belonged to one group, while the other three New Guinea strains belonged to another different antigenic group. Thus, two antigenic types of dengue virus were identified in 1944-1945 [6]. By 1956-1958 two more serotypes of the virus were identified through the outbreak in Manila in 1953 by Hammon [5] and every outbreak of the disease since then has been related to a virus belonging to one of the four serotypes. Herein, DENV-1, -2, -3 and -4 refer to dengue serotype 1, 2, 3, and 4.

1.1.3. Global expansion of the dengue as a disease of public importance

The first known epidemic of dengue hemorrhagic fever (DHF) occurred in Manila, Philippines, in 1953 – 1954 [7]. The disease subsequently spread throughout Southeast Asia and became one of the ten leading diseases causing hospitalization and death in children in at least eight tropical Asian countries [8]. In the early 1970s, dengue was reintroduced to the Pacific islands after an absence of more than 25 years [2]. The large dengue epidemic in Cuba in 1981 caused by DENV-2 led to ~10,000 DHF/DSS cases and 158 deaths [9]; since then, dengue epidemic transmission has intensified in the Americas [8]. Interestingly, there are no records of DHF outbreaks in Africa, though surveillance is weak in most of this continent [2]. Within four decades, dengue has become a major international public health problem, particularly in tropical and sub-tropical areas. It has been estimated about 50 million DENV infections occur annually around the world producing at least 500,000 cases of DHF [10]. The case fatality rate is as high as 5% in places with less clinical experience of the disease or

where resources are limited [10]. In addition, dengue is not only problematic in endemic/epidemic areas but is also an important problem amongst international travelers. Dengue was the second most common vector-born infection, after malaria, in travelers [11].

1.1.4. Major Dengue outbreaks in Vietnam

Dengue is hyper-endemic in Vietnam and occasionally epidemic; at times, a certain serotype is more prevalent than the other serotypes. Dengue was first recorded in northern Vietnam (Ha Noi and Hai Phong) in 1959 and in the South in 1960. The first epidemic of DHF appeared in southern Vietnam in 1963 resulting in 331 DHF children being hospitalized, of whom 116 died. From 1963 - 1994 the Ministry of Health recorded 1,577,452 cases and 12,942 deaths [12]. During the last 30 years, epidemic peaks of dengue have occurred regularly at 3-4 year intervals [1975, 1978/79, 1983, 1987, high endemic between 1988 and 1997, then outbreak in 1998, 2001, and 2005; according to Pasteur Institute HCMC, http://www.pasteur-hcm.org.vn/english/]. The first nationwide outbreak occurring in 1983 resulted in ~150,000 DHF cases of which 1,795 died. After an interval of three years, the second nationwide outbreak occurred in 1987 which was the most severe with ~ 354,517 DHF cases and 1,566 deaths with DENV-2 being the predominant serotype [13]. A widespread DHF epidemic in 1998 affected 19 provinces in southern Vietnam and resulted in ~120,000 DHF cases and 342 deaths; most of which were caused by DENV-3 [14]. Since 1999 the dengue control program for southern Vietnam has been based at the Pasteur Institute, HCMC. Dengue is a notifiable disease in Vietnam and the Pasteur Institute collates this data on an annual basis. Virus surveillance is also conducted by isolation of viruses from blood samples submitted by city, district and provincial hospitals. **Fig.1.1** depicts dengue epidemics and death toll in southern Vietnam from 1963 to 1998 and other major outbreaks that occurred in 1983, 1987 and 1998. Serotype prevalence in southern Vietnam from 1987 to 2007 and disease incidence between and 1996 and 2007 is described in **Fig.1.2**.

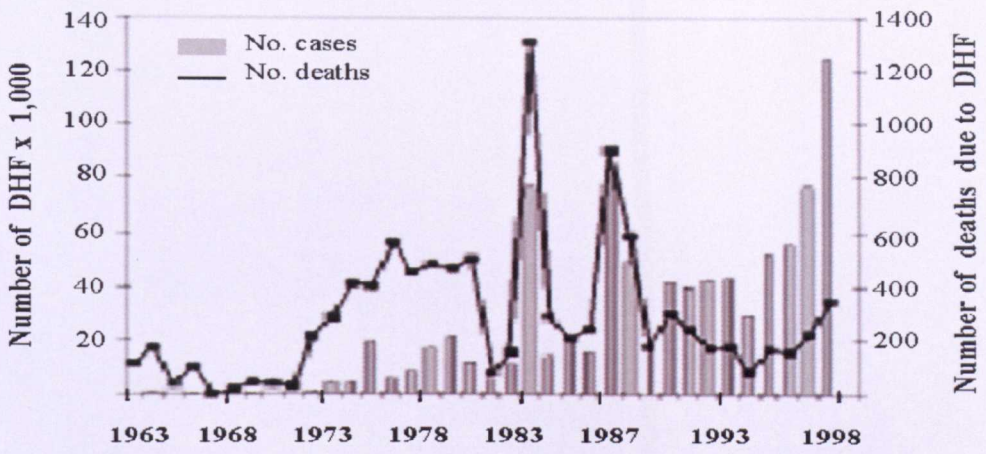


Fig.1.1. Dengue epidemics and number of deaths in southern Vietnam from 1963 to 1998. [Data used with authors' permission. (Ha, DQ et al. 2000)]

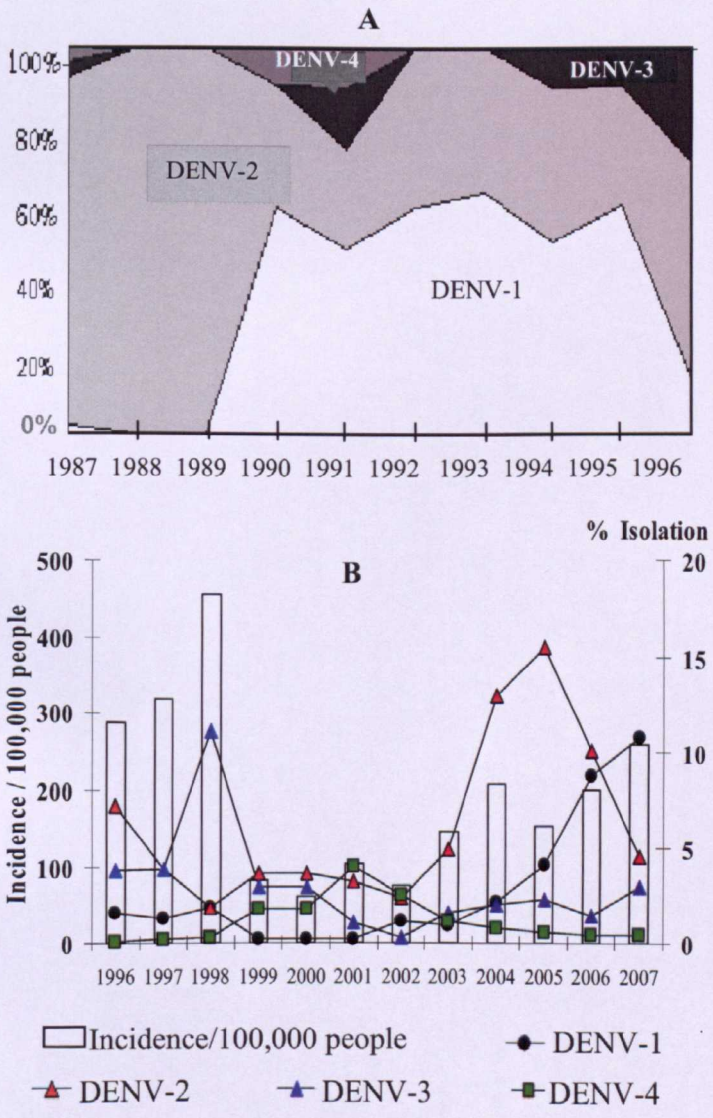


Fig.1.2. Disease incidence and dengue serotype prevalence in southern VN between 1996-2007. (**A**) shows DENV serotype detected in southern Vietnam between 1987 and 1996 (Pasteur Institute based, Do Quang Ha, http://www.ykhoanet.com/tapchiyhoc/9802/YH8CD-Ha(30-35).htm). (**B**) shows disease incidence and dengue serotype prevalence in southern Vietnam from 1996 to 2007. Data are shown as incidence per 100,000 people in the left Y-axis corresponding to the bars. The percentage with which each serotype was detected in surveillance samples is shown on the right Y-axis and expressed by the lines. The black dots, red triangles, blue triangles, and green squares represent DENV-1, 2, 3, and 4, respectively. Data courtesy of Pasteur Institute - HCMC.

1.2. Burden of dengue

1.2.1. In other countries

The relative burden of dengue can be estimated by use of DALYs (disability-adjusted life years - the number of healthy years of life lost due to premature death or disability, measured by DALY/year/million people). The average loss to dengue in Puerto Rico (1984-1994) is similar to the losses to meningitis, hepatitis, or malaria in the Latin American and Caribbean region (log10 DALY, 2.8 versus 3.1, 2.5 and 2.9, respectively [15]. Estimates of DALYs with respect to dengue in Thailand suggest it is of the same order of importance as hepatitis (DENV DALY: $4.27x10^3$/million people, Hepatitis DALY: $5.56x10^3$/ million people, 2000) [16]. However, this result may not reflect the true burden of dengue. Dengue surveillance programs usually give an account of notified or hospitalised dengue cases and do not capture data on non-hospitalised cases or cases that do not present to health care facilities. A prospective study in Thai children suggested that non-hospitalised dengue patients represented about 44 – 73% of the total DALYs lost to dengue each year [17].

1.2.2. In Vietnam

Few systematic studies of the economic impact of dengue in Vietnam have been conducted. Aside from the incidence data reported by the Pasteur Institute-HCMC for the southern provinces, the only other information on disease burden information is from hospital records. In recent years, the number of dengue patients admitted to the Hospital for Tropical Diseases (HTD) in HCMC has been increasing and all four serotypes of dengue virus have been detected (**Fig.1.3**). Recently, the number of adults hospitalised with DHF has increased to ~60% of all dengue patients at the HTD (**Fig.1.4**). However, the overall burden of DSS has remained much higher in children (**Fig.1.5**). The basis for this trend towards adults with dengue is unknown. One possible explanation is migration of dengue naïve adults from the North to the South creating a large pool of susceptible adults.

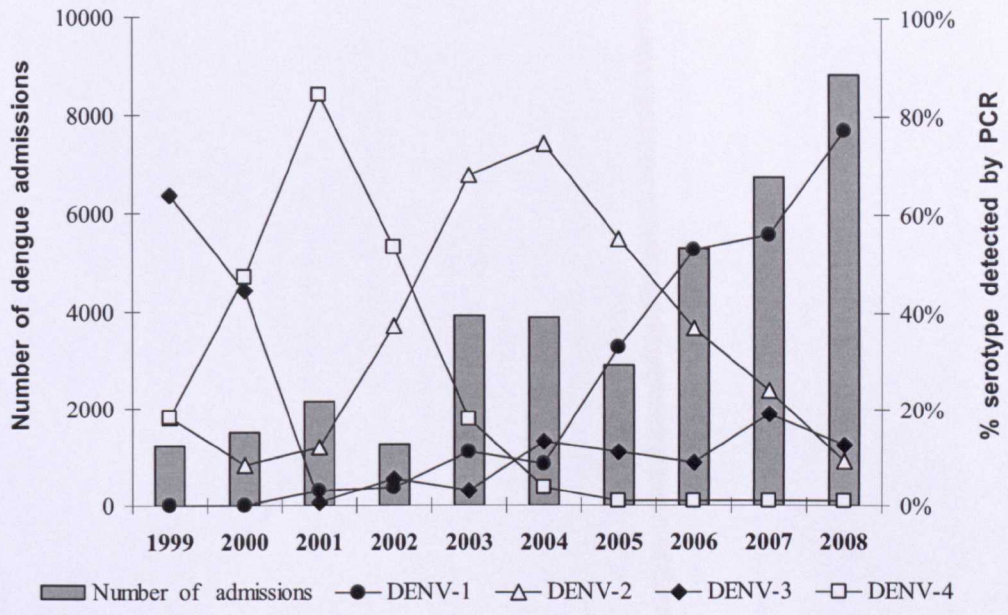


Fig.1.3. Number of dengue admissions at HTD and the relative prevalence of each DENV serotype detected by PCR from 1999 to 2008. Bars represent the number of dengue cases admitted to HTD and corresponds to the left Y-axis. The percentage of each serotype detected by PCR is indicated in the right Y-axis and expressed by the lines. The black dots, white triangles, black diamonds, and white squares represent DENV-1, 2, 3, and 4 respectively.

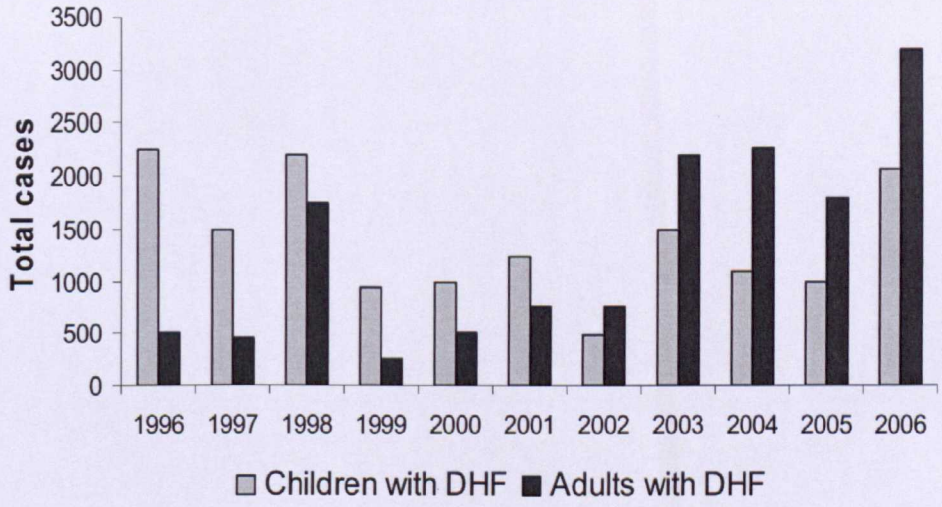


Fig.1.4. DHF in adults and children admitted to HTD between 1996 and 2006. Chart shows number of adults (>15 years, black bar) and children (grey bar) with DHF admitted to HTD between 1996 and 2006.

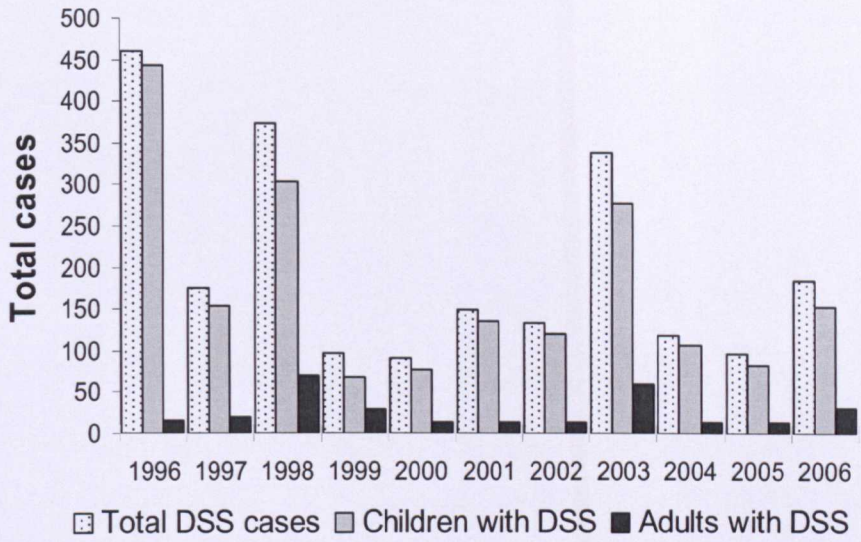


Fig.1.5. DSS in adults and children at HTD from 1996-2006. The graph shows the number of children and adult inpatients with DSS by year since 1996. (Source: HTD).

1.3. Dengue diagnosis

Accurate and timely diagnosis of dengue can play an important role not only in clinical management, but also in surveillance activities and outbreak control. Laboratory approaches to the diagnosis of dengue consist of virus detection, virus antigen detection, and identification of virus-specific antibodies. In the early acute phase of illness from day one to day five, direct virus detection via viral isolation and genomic nucleic acid amplification (PCR) can be useful tools. At the end of the acute phase, serological assays for the detection of virus-specific IgM antibodies are the method of choice.

1.3.1. Virus detection

1.3.1.1. Virus isolation

The intrathoracic inoculation of mosquitoes or intracranial inoculation in suckling mice are systems for the isolation of the dengue virus, but because of the particular skill and special facilities required these methods are rarely used. Virus isolation in cell cultures is the alternative method. The mosquito cell line *C6/36* (clone obtained from *Ae. albopictus*) is the

host cell of choice for virus isolation. Virus isolation generally requires from one to two weeks and is usually carried out in research or reference laboratories rather than hospital settings.

1.3.1.2. RNA genomic amplification

The high sensitivity, specificity, and rapid detection of minute quantities of viral RNA are advantages of the RT-PCR technique. Many real-time RT-PCR assays utilizing TaqMan or SYBR Green have been developed [18, 19]. SYBR Green is a double stranded DNA binding dye. It has an undetectable fluorescence when it is in free form, but once bound to dsDNA, it emits fluorescence, and therefore it is not a specific signal to target DNA sequence. TaqMan real-time PCR offers greater specificity via the sequence-specific hybridization of probes. During a TaqMan assay, a specific probe labeled with two different dyes hybridizes within the region of specific amplification. When the two dyes are close together (intact oligonucleotide probe), one of the two dyes (for example TAMRA or BHQ1) acts as a quencher for the second dye. The 5' exo-nuclease activity of Taq polymerase degrades the hybridizing probe leading to the separation of these two dyes in the reaction mixture resulting in a fluorescent signal. The amount of fluorescence measured in a PCR mixture is proportional to the amount of specific PCR product. The advantages of real-time TaqMan RT-PCR over conventional RT-PCR are speed, quantitative measurement, high sensitivity and specificity, and lower contamination rates relative to nested PCR assays. The relative high price of probes and real-time thermal cyclers is a disadvantage of this technique.

1.3.2. Detection of viral antigen NS1 (non-structural protein 1)

In vivo, the existence of viral NS1 was first reported in the sera of primary dengue cases [20]. Studies conducted afterwards have shown that this viral protein appears in the bloodstream from day one of the acute phase of illness to day nine with no significant difference in NS1 concentration between primary and secondary patient specimens (0.04 - 2.0 μg/ml in primary sera and 0.01 - 2.0 μg/ml in secondary sera) [21]. The NS1 protein can also be detected in

patient sera until day 18 after the onset of symptoms [22]. This protein could be detected even when viral RNA was not detected by RT-PCR or in the presence of IgM antibodies [21, 22]. In the bloodstream, NS1 levels varied amongst individuals (ranging from 0.07μg /ml to several μg/ml, up to 15μg/ml) [20]. Mean plasma levels of NS1 were highest in the first few days of illness and reached a peak at day 3 [23].

Recently, commercial assays for the detection of DENV NS1 have been developed. NS1 assay kits from different manufactures have been evaluated in several settings for the diagnosis of acute dengue [24-30]. Detection of dengue NS1 antigen in acute-phase serum has been shown to be more sensitive in patients with primary infection than patients with secondary infection [25, 27].

1.3.3. Detection of virus-specific antibodies

A variety of serological assays have been used to detect anti-DENV antibody. These tests include the haemagglutination-inhibition test, complement fixation test, plaque reduction neutralization test (PRNT), and enzyme-linked immunosorbent assay (ELISA). Of these tests, only the ELISA assay is widely used in diagnostic laboratories in endemic countries.

1.3.3.1. IgM antibody-capture ELISA (MAC ELISA)

The IgM antibody-capture ELISA has been widely used for routine dengue diagnosis. Briefly, patients' total IgM are captured in a microplate coated with anti-μ chain antibodies (specific to human IgM). Dengue antigens (usually pooled culture supernatant from DENV1-4) are overlaid on the captured IgM. Antigens bound by DENV-specific IgM are detected by monoclonal or polyclonal DENV antibodies labeled with an enzyme (e.g. horseradish peroxidase). In the presence of a substrate, an enzyme-dependent color-change reaction occurs. The optical density (OD) is measured by a spectrophotometer. All steps of this assay are illustrated in **Fig.1.6**. A diagnosis of acute dengue can be made if IgM sero-conversion occurs between acute and convalescent sera, or there is evidence of a rising level of IgM

between paired specimens. Generally, in primary infection, IgM level rises rapidly then peaks two to three weeks after the onset of fever [31]. Anti-DENV IgM levels remain positive longer in patients with primary infections compared to patients with secondary infections [31]. IgM in anamnestic (secondary) responses appears at a lower level [31, 32]. In both primary and secondary infections, anti-DENV IgM declined after days 15-30 and became undetectable in the majority of patients by day 60 [31].

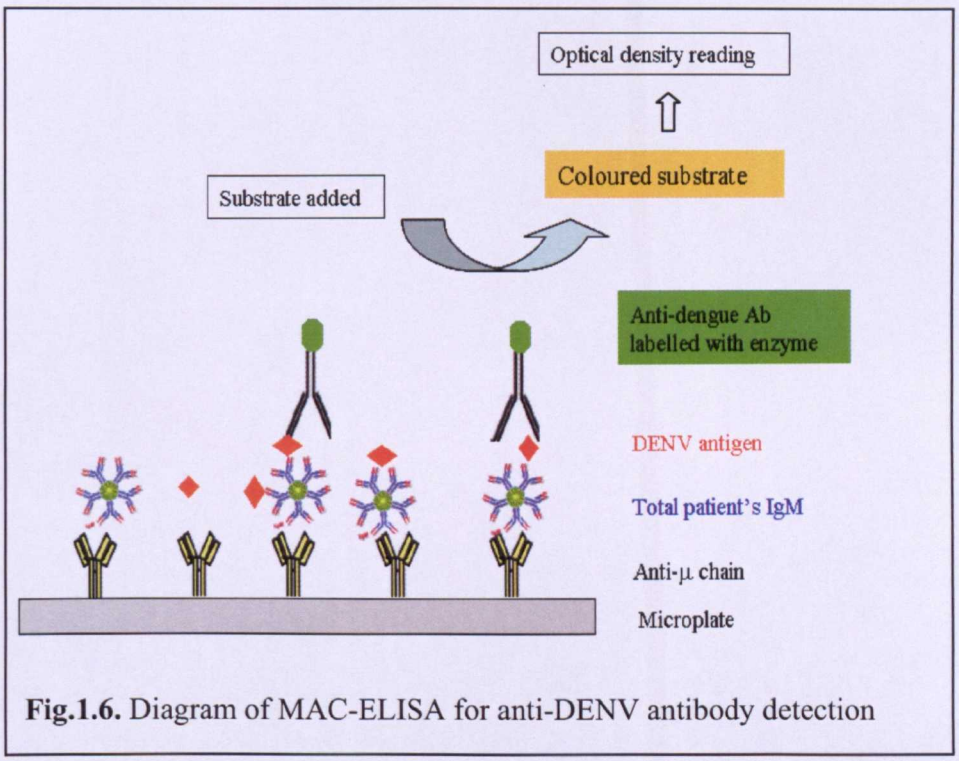


Fig.1.6. Diagram of MAC-ELISA for anti-DENV antibody detection

1.3.3.2. ELISA assays for detection of IgG

Two approaches are used to detect DENV-reactive IgG. The DENV IgG capture ELISA is used to detect DENV-reactive IgG in human plasma/serum based on the similar principle described above but the micro-plate is coated with human anti-γ chain antibodies instead of anti μ-chain. A positive IgG antibody-capture ELISA result indicates either current or recent Flavivirus infection, which in countries such as Vietnam usually means dengue. A second approach for detecting DENV-reactive IgG is to use an indirect ELISA assays (**Fig.1.7**) in

which the micro-plate is coated with recombinant E proteins or purified virions. Dilutions of plasma/serum are overlaid and any bound IgG is detected with a conjugated detector antibody. The four-fold increase in titre of IgG antibody level in convalescent samples over acute samples is indicative of acute dengue [33]. Indirect ELISA assays are useful for seroprevalence studies. In secondary infection, IgG rises quickly and appears at high levels for two to three weeks then slowly declines over 3-6 months [8].

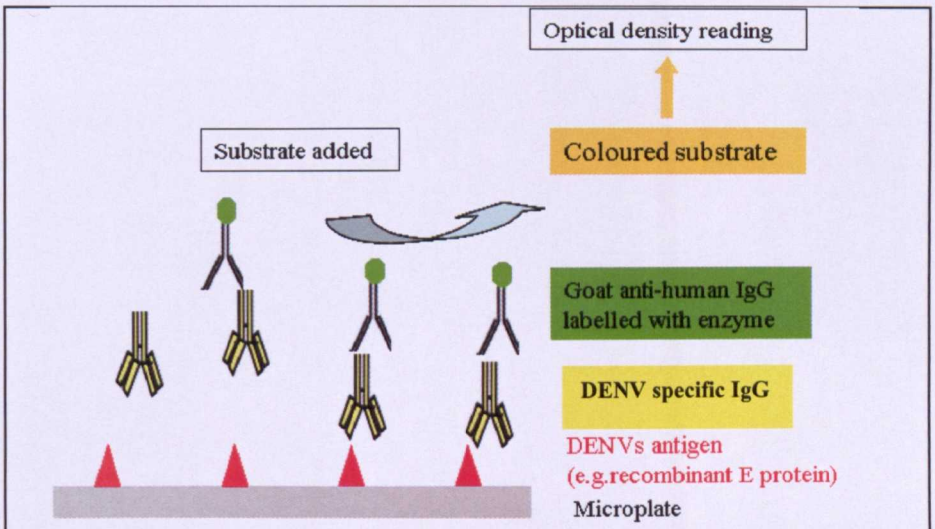


Fig.1.7. Diagram of indirect IgG ELISA for DENV antibody detection

1.4. Clinical features and management of Dengue

1.4.1. General characteristics

Dengue is an acute systemic viral infection. After an incubation period of about 3-7 days, DENV infections can result in a variety of clinical outcomes. Most DENV infections are clinically inapparent while a number of patients may present with classic Dengue fever (DF) or develop severe/potentially life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [10]. Hepatomegaly and increased liver enzymes are commonly observed in severe dengue [34, 35]. Increased disease severity is also associated with high viraemia level and secondary infection [36, 37]. Symptoms caused by DENV infection may last from 3 to 10 days, with an average of 5 days [8]. Viraemia levels was also correlated

with duration of symptoms [38]. Symptoms of dengue include, but are not limited to, fever, retro-orbital pain, muscle pain (myalgia) and joint pain (arthralgia), sometimes abdominal pain, severe headache and hemorrhage. Hemorrhagic manifestations may present in a variety of forms such as petechiae (pinpoint round red spots that are the results of blood leaked from capillaries into the skin, **Fig.1.8**), purpura (larger red spots of bleeding into the skin, **Fig.1.9**) or less frequently, gingival or gastrointestinal hemorrhage.

The time for virus clearance from the bloodstream is 5 to 7 days after the onset of fever, and this coincides with defervescence [36, 37]. Laboratory findings include increased aspartate transaminase (AST) and alanine aminotransaminase (ALT), leucopenia and thrombocytopenia. Platelet count at admission in those who later developed DHF was significantly lower than in those who did not develop DHF. It is also shown that aspartate transaminase (AST) and alanin aminotransaminase (ALT) were greatly raised, sometimes at 10 times greater of the upper normal limit [39] and associated with poor prognosis [40, 41] and AST in DHF patients was higher than DF [42]. Vascular leakage of plasma in DHF/DSS patients is a critical point of differentiation from patients with milder disease.

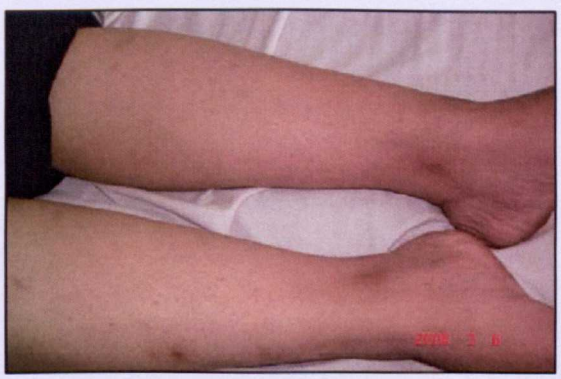


Fig.1.8. Petechiae in DHF patient in acute phase

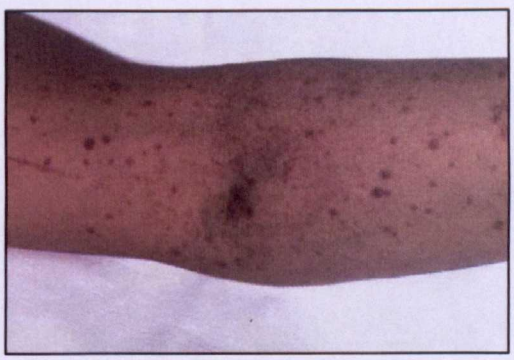


Fig.1.9. Purpura in DHF patient in acute phase

1.4.2. World Health Organization (WHO) Classification

The WHO Dengue classification scheme (**Fig.1.10**), although under revision [3, 43, 44], is used to categorize patients with dengue.

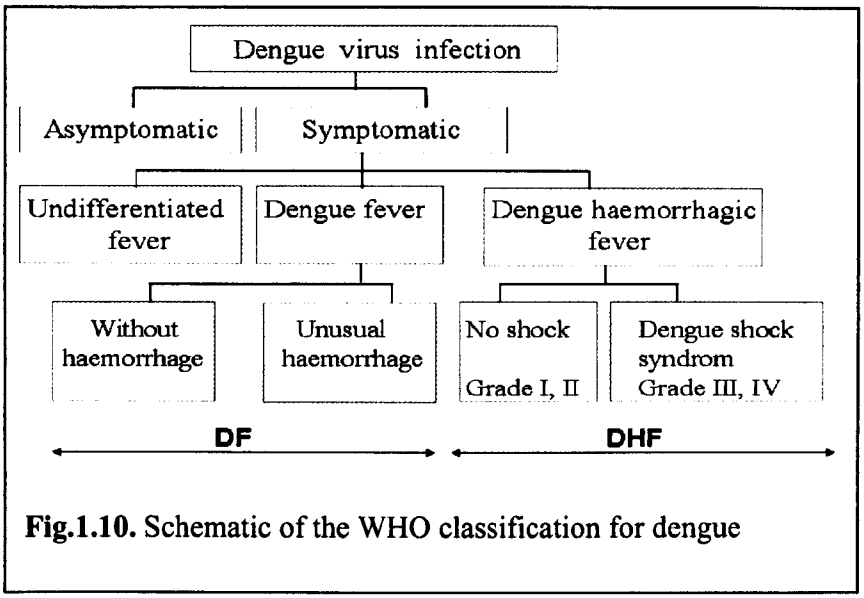


Fig.1.10. Schematic of the WHO classification for dengue

Asymptomatic fever may be the most common manifestation of dengue. A prospective study of dengue infections in 4 to 16-year-old students in Bangkok, Thailand, found that 87% of students were either asymptomatic or minimally symptomatic and were absent from school only one day [45]. Symptomatic dengue can vary from undifferentiated fever, dengue fever (DF), to dengue haemorrhagic fever (DHF). Criteria for DHF diagnoses include all symptoms of DF - fever and hemorrhagic manifestation, plus thrombocytopenia (lower than 100,000/mm^3) and evidence for plasma leakage. Plasma leakage can be detectable by either elevated haematocrit (packed-cells volume/whole blood volume) which is defined as $\geq$ 20% increasing over the baseline (or a similar drop after volume replacement treatment), low plasma protein level (normal level: 7g/dl), or clinical signs such as pleural effusion (an abnormal accumulation of fluid in the pleural space) or ascites (excess fluid in the peritoneal cavity) or thickening of the gallbladder.

The severity of DHF is divided into four grades. In grade I and II, plasma leakage is mild to moderate, and patients usually recover with or without intravenous fluids. The difference between grade I and grade II is that in grade I patients manifest haemorrhage after a tourniquet test (positive result for tourniquet test) but we can see spontaneous bleeding in grade II. Grades III and IV are dengue shock syndrome (DSS). Intense and sustained abdominal pain, persistent vomiting; rapid change from fever to hypothermia, with sweating and prostration (absolute exhaustion), restlessness and accompanied with markedly decreased platelet count are the first warning signs of shock. Patients with DHF grade III have incipient shock with signs of circulatory failure manifested by rapid, weak pulse with a narrowing blood pressure of ≤ 20 mm Hg or profound hypotension (systolic pressure is less than 90 mm Hg in five year olds or old patients) and cold extremities/clammy skin and restlessness. Grade IV is the most severe status in DHF. Plasma loss is critical, resulting in profound shock with undetectable blood pressure and pulse.

1.4.3. Clinical management

There is no specific anti-viral drug to treat DENV infection, although these are in development [46, 47]. The mainstay of care is supportive treatment with oral and when necessary intravenously administered fluids. Patients with cardiovascular shock require urgent fluid replenishment, sometimes with large amounts of intravenously administered fluids. Without fluid transfusion, cardiovascular shock is life-threatening. Mortality in DHF/DSS patients can be as high as 10-20 % without appropriate treatment [48, 49]. Hospitals with a good triage system, well-trained staff and enough essential equipment and intravenous fluids can reduce mortality from DSS to as low as ≤ 0.2 % [50-52]. Patients recover rapidly after appropriate volume-replacement therapy. The absence of fever for 48 hours, stable haematocrit, platelet count greater than 50,000/mm^3, and return of appetite are the first signs of recovery. Around the defervescence phase, macular rash (normally described as small white bumps on an area of

red skin, **Fig.1.11**) appears and lasts for several days and may be accompanied by itching or scaling.

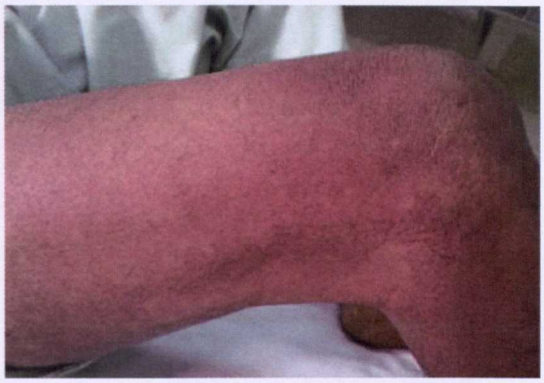


Fig.1.11. Macular (recovery) rash in an adult patient in the defervescence phase

1.5. Current hypotheses for dengue pathogenesis

It is believed that many factors contribute to DENV infection outcome. Chief amongst these are host immune status, inherent virus virulence, age, host genotype and nutritional status.

1.5.1. Inherent virus virulence

1.5.1.1. Epidemiology observations

Several studies have suggested that viral virulence has an influence on infection outcome. For example, epidemiological studies have suggested the DENV-2 American genotype viruses are not associated with DHF even in secondary infection [53], whereas DENV-2 Asian viruses are more potentially associated with DHF [54-56]. Evidence for capacity of DENV-2 Asian genotype to cause DHF has been revealed in four countries, there, the "native" American genotype that has been associated with mild disease has gradually been displaced [54]. The two major DHF epidemics in Cuba also associated with DENV-2 Asian genotypes; Asian 2 in the 1981 [55] epidemic and American/Asian (Am/As) in the 1997 epidemic [56]. A phylogenetic study of DENV-3 in Sri Lanka revealed that the introduction/emergence of the new clade of subtype III, which originated in the Indian subcontinent, coincided with the DHF outbreak in 1989 [57].

1.5.1.2. In vitro studies of viral traits

Full genome sequencing of DENV-2 American and Asian genotypes revealed distinct nucleotide changes in E (encoding residue 390), 5', and 3' un-translated regions (UTR) [58]. The $E_{390\text{-}Asp}$ reduced virus output in monocyte-derived macrophage [59] and $E_{390\text{-}His}$ effected neurovirulence in suckling mice [60]. In vitro experiments have shown that the Southeast Asian genotype can out-replicate the American genotype. The Southeast Asian genotype (American/Asian and Asian 1) generates higher virus output than the American genotype in human dendritic cells [61] and disseminated more efficiently than its American counterpart in mosquitoes [62, 63]. These observations have led to the hypothesis that SE-Asian genotype viruses are more virulent than the American genotype.

1.5.2. Infection status in dengue pathogenesis

The 1981 Cuban epidemic provided a "natural experiment", in which the introduction of a dengue serotype 2 mainly related to DENV-2 New Guinea 44 virus [55] - after dengue serotype 1 epidemic in 1977 - was associated with the dramatic increase of cases of DHF/DSS [64]. Secondary infections were demonstrated in almost 98-99% of individuals experiencing DHF/DSS [64]. The 1997 DENV-2 epidemic in Santiago de Cuba, mainly related to Am/As genotype [56], provided other epidemiologic support for the association between secondary infection and DHF, and its effect can last for as long as 20 years [9, 64]. A leading hypothesis to explain DHF in secondary infection is that the pre-existing antibodies augment heterotypic virus uptake into mononuclear cell lineages through Fcγ receptors. This hypothesis is based on the premise that heterologous antibody-virus complexes can enter cells bearing Fcγ receptors more efficiently. Animal models and in vitro experimental results suggested that enhancing antibody leads to a greater proportion of infected mononuclear cells. For example, after six weeks to six months of initial infection with DENV-1, 3, and DENV-4, primates challenged with DENV-2 had DENV-2 viraemia at significantly higher titres compared to monkey's with

primary infection [65]. In vitro, dengue replicates at higher titres in cultured human monocytes in the presence of heterotypic antibody or homotypic antibody at subneutralising concentrations than in cultures treated similarly but without flavivirus antibody [65]. This phenomenon is called antibody-dependent enhancement (ADE).

Since dengue virus exists as four distinct serotypes; people living in dengue endemic areas can be exposed to more than one serotype during their lifetime. Individuals who have experienced dengue develop serum antibodies that can neutralize the infecting serotype but weakly or not at all heterologous serotypes. The presence of non-neutralising Abs elicited by the previous infection is believed to increase virus replication and account for DHF in secondary infection. Non neutralising Ab may also account for DHF in first infection of infants born to Dengue immune mothers [66, 67]. However, not all DHF/DSS cases are related to secondary infection; in one study, about 20% of DHF/DSS cases occurred in primary infection [68]. ADE can not wholly explain this phenomenon; primary DHF/DSS cases have been reported sporadically in Thai children [69] as well as in travelers [70].

1.5.3. Role of DENV-NS1 in pathogenesis

1.5.3.1. NS1 biology

NS1, a 46-50 kDa glycoprotein, was originally described as a soluble complement-fixing antigen in infected cell cultures [71]. When newly synthesized, NS1 is trans-located in endoplasmic reticulum (ER) and exists as a water-soluble monomer then followed by a rapid dimerization before it passes through the Golgi apparatus [72, 73]. In infected cells, the NS1 protein is expressed as 2 discrete forms: an intracellular-dimer membrane-associated form (mNS1) [72, 73] and a secreted-hexamer form (sNS1) in mammalian cells [74]. Dengue NS1 is expressed on the infected cell surface via a glycosyl-phosphatidylinositol (GPI) anchor [75]. The co-localization of NS1 with ds-RNA replicative form within infected-cell is suggestive of the role of NS1 as a cofactor for viral replication [76]. Avirutnan and others have shown that

NS1 binds to many uninfected-cell types including sets of mammalian and human peripheral blood cell lines through glycosaminoglycans heparan sulfate and chondroitin sulfate E [77].

1.5.3.2. Role of DENV-NS1 in pathogenesis via in vivo and in vitro observations

Several studies have suggested NS1 may play a role in the pathogenesis of dengue. sNS1 levels in dengue patient plasma correlated with viraemia levels and were higher in patients with DHF than in those with DF [23, 78]. High NS1 level (of 600ng/ml) on presentation predicted the subsequent development of DHF with a sensitivity of 72% and a specificity of 79% [23]. sNS1 is believed to contribute to dengue pathology by activating complement which is enhanced by polyclonal and monoclonal antibodies against NS1 [78]. Plasma SC5b-9 levels in DHF patients were higher than in DF patients; and large amounts of NS1 and complement activation products (C5a, SC5b-9) were present in pleural fluids from DSS patients [78]. Moreover, secreted DENV NS1 differentially attaches to some types of endothelial cells such as lung and liver but not intestine or brain endothelium of mouse tissues suggesting that the discriminatory vascular leakage that occurs in severe dengue infection may be related to the relative ability of endothelial cells in different tissues to bind soluble NS1 [77]. These observations suggest a possible role for NS1 in the pathogenesis of vascular leakage in DHF/DSS patients.

1.5.4. Role of host genetic background

Previous studies have shown that several host genetic factors can contribute to clinical outcome. For example, variations in the HLA-A locus were associated with susceptibility to DHF while HLA-DRB1 and TNF genes were shown to be negatively associated to DHF [79]. However, genetic mechanisms of underling the DENV-host interaction remain largely unknown; this could be due to the limitations in sample size and study design in previous studies. Recent developments in biology such as high-throughput sequencing, microarray, genome-wide association studies, and bioinformatics advance the understanding of host-

pathogen interactions [80-82]. In dengue particularly, microarray analyses of gene expression have revealed that some aspects of the host response can be used as potential biomarkers of disease severity (e.g. IP-10 and I-TAC) and some can be used to control dengue viral replication (for example, over-expression of viperin reduced virus replication) with implications for drug therapy [83].

1.6. Vectors and Transmission

1.6.1. Mosquito biting behaviors and breeding habits

Aedes mosquitoes *Aedes aegypti, Aedes albopitus*, and to a much lesser extent, *Aedes polynesians* are the main vectors of DENV transmission. In Vietnam *Aedes aegypti* plays a major role in dengue transmission; it is highly anthropophilic (human-preferring) lives around human habitations or indoors. *Aedes aegypti* is primarily a daytime feeder and mainly bites in the morning and late in the afternoon in covered areas. Female mosquitoes prefer to lay eggs in stagnant clean water such as artificial water containers, e.g. discarded tyres, water jars. The mainstay of dengue prevention programs is vector control. The fight against dengue in terms of mosquito eradication is complicated firstly due to mosquito resistance to commercial aerosol sprays [8] and secondly, *A. aegypti* eggs can withstand months of desiccation [8]. Furthermore, mosquito eradication programs work best with community involvement and this is not always sustainable.

1.6.2. Transmission

Mosquito dispersal and survival rate have an important role in transmission. Mosquito flight depends on environmental conditions such as climate and geography; it can be 50 – 160 meter in distance [84], its dispersal sometimes can be as far as ~360 meters in natural open environment [85]. Importantly, human movements such as travel or transportation results in considerable contribution to mosquito global spread [7]. The transmission cycle of DENV by mosquitoes begins when an uninfected female *Aedes aegypti* bites an infected individual(s)

and ingests blood containing DENV. DENV replicates in the mosquito midgut then disseminates to the rest of the body and infects other organs including the salivary glands – this period is called the extrinsic incubation period (EIP). Infected mosquitoes transmit DENV to susceptible individual through their saliva. The extrinsic incubation period is influenced by the ambient temperature, humidity, virus strains and mosquito strains [63, 86]. Female mosquitoes can pass viruses to the next generation of mosquitoes through trans-ovarian transmission (TOT) in the laboratory setting [87] and in nature also [88-93]. However, the role of this transmission pattern in maintenance of dengue in nature is still controversial [94-96].

1.7. Dengue virus

1.7.1. Virus structure

Dengue virus exists as four serotypes [DENV-1 (10735nt), DENV-2 (10723nt), DENV-3 (10696nt), and DENV-4 (10653nt)] and belongs to the genus Flavivirus, family Flaviviridae. DENV-1 and DENV-2 were first isolated in 1944 [5, 6]. DENV-3 and DENV-4 were isolated in Manila, the Philippines in 1956 [5]. The mature dengue virion is icosahedral (having twenty equal sides or faces), and is approximately 50 nm in diameter [97]. The RNA genome is of approximately 10.7 kb in length, surrounded by a nucleocapsid (or core protein-C) and covered by a lipid envelope containing the envelope (E) and membrane proteins (M) (**Fig.1.12**).

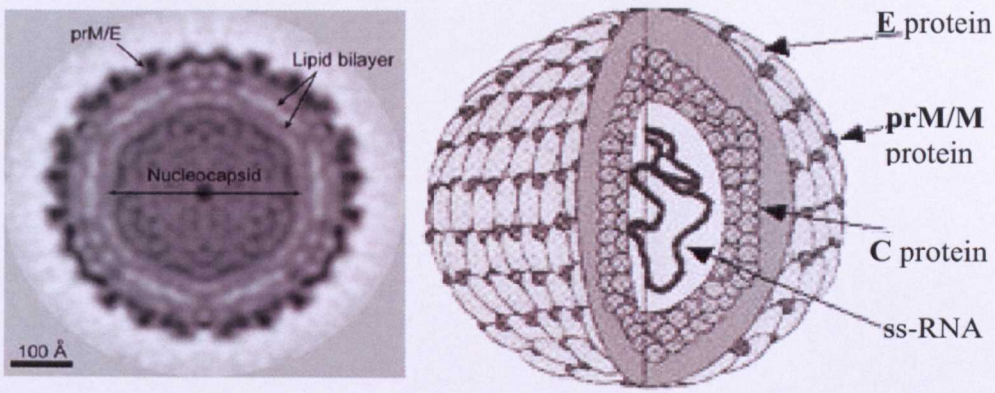


Fig.1.12. Dengue virus structure. (**A**): Sectional structure. (0Hhttp://www.sciencecentric.com/images/news/dengue_virus_1440_1440.jpg) **(B)**: Outer and inner dengue virus tructure (Source: Marlade Institute)

1.7.2 Genome organization

The viral genome is a positive single-stranded RNA, has a 5'cap but it lacks a 3' poly - A tail. The 5' (~97nt) and 3' UTRs (~450nt) play an important role in viral replication [98]. Colona et al [99] generated a panel of chimeric infectious clones in which they introduced the $E\text{-}_{390}$ substitution and the 5' and 3' UTRs of DENV-2 American genotype (Iquito strain) into the backbone of the SE Asian genotype (16681-strain, Asian 1 genotype) singly and in various combinations. The 5' and 3'-terminal and $E\text{-}_{390(Asn \rightarrow Asp)}$ mutations give a larger reduction of virus output compared to the $E\text{-}_{390(Asn \rightarrow Asp)}$ mutation only. The genome contains an open reading frame of 10,176; 10,173; 10,170 and 10,161 nucleotides in length in DEN-1.-2,-3, and 4, respectively, which encodes a precursor polyprotein of 3,392; 3,391; 3,390 and 3,387 amino acids. The gene order of the 3 structural and 7 nonstructural proteins from the 5'terminus is C - PrM/M - E and NS1- NS2A - NS2B- NS3 - NS4A - NS4B - NS5 (**Fig.1.13**). The ten proteins are formed by cleavage of the precursor polyprotein by host and viral proteases during co- and post translational phase.

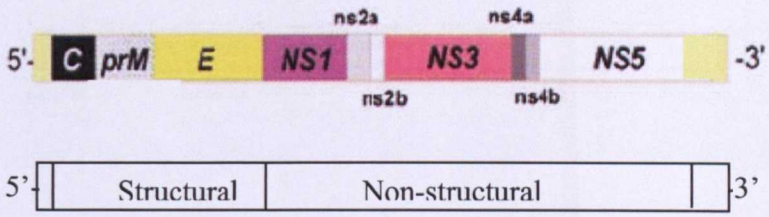


Fig.1.13. Genome organization of Dengue virus. C, prM and E encode structural protein. NS1 to NS5 encode nonstructural proteins.

1.7.3. Dengue structural proteins

The capsid (C) protein is ~ 12-14 kDa, positively charged and rich in lysine and arginine residues (27%), contains 114 amino acids. During infection, the C protein localizes to the nuclei of infected cells [100]. There are two forms of M protein; prM in the immature DENV particles is a glycoprotein precursor (18.1-19 kDa, 166 amino acids) that is cleaved to M protein (7-9 kDa, 75 amino acids). prM protein forms a prM-E heterodimer to prevent the E protein from undergoing low-pH induced conformational changes prior to virion maturation [101]. The 55-60 kDa E protein (493-495 amino acids), which is exposed on the surface of the virion is associated with a number of biologic activities including haemagglutination [102], receptor binding [103], and low-pH mediated endosomal fusion [104]. The E protein is also the major antigenic target of the humoral immune response and neutralising antibodies map to this protein [105, 106]..

1.7.4. Dengue non-structural proteins

Of the 7 nonstructural proteins, the100 kDa NS5 protein (899-900 amino acids) is the RNA-dependent RNA polymerase required for viral replication [107]. The 67 to 70 kDa NS3 protein (618 to 619 amino acids) possesses multiple enzymatic activities and in complex with NS2B it catalyzes the cleavage of NS2A-NS2B-NS3-NS4A and NS4B-NS5 sites in the polyprotein [108]. NS3 also contains NTPase-helicase on that is essential for virus replication [109].

Functions of the four small non-structural proteins – NS2A (18-22 kDa and 218 amino acids), NS2B (13-15 kDa and 130 amino acids) and NS4A (16.0-16.4 kDa and 150 amino acids), Ns4B (27- 28 kDa and 248-249 amino acids) - remain poorly defined. The 46-50 kDa NS1 protein consists of 352 amino acid, it is expressed in infected cells in both membrane - associated (mNS1) [72] and secreted (sNS1) forms [74], and possesses both group specific and type specific determinants [110]. NS1 is also attributed to play a role in pathogenesis [23, 77, 78].

1.8. Molecular evolution in dengue viruses

1.8.1. Genetic diversity

Genetic variation in DENV may result from multiple factors [111]. First, DENV replication relies on an error-prone RNA-dependent RNA polymerase resulting in roughly 1 error per genome per round of replication [112, 113]. Second, recombination facilitated by a co-infection of a single host (mosquito or human) could also drive this genetic diversity [114]. Recombination has been observed within a DENV serotype. Worobey et al [115] performed a diversity analysis of 71 published dengue virus sequences and found 7 recombination sequences which were comprised of hybrid gene regions with conflicting evolutionary histories.

Genetic diversity is an inherent characteristic of DENV illustrated by the existence of 4 distinct serotypes. In each serotype, there are multiple genotypes (with up to 8% difference in the nucleotide sequence) that can be distinguished via sequence analysis of all or part of the genome. Phylogenetic studies revealed 4 distinct genotypes in DENV-1 (1 sylvatic and 3 human lineages, [116, 117]); 6 genotypes in DENV-2 (5 human lineages and 1 sylvatic lineage, [118-120]); 4 human-associated genotypes in DENV-3 [118, 121]; and 4 genotypes in DENV-4 (3 human lineages and 1 sylvatic lineage, [122]). Additionally, virus populations within infected hosts are not homogeneous but consist of different variants described as

forming a quasispecies population [123, 124]. The extent of sequence variation differed amongst patients [123] and varied between mosquito and human hosts [125]. It was revealed that intra-host genetic variation was significantly lower in severe dengue cases than classical DF [126].

1.8.2. Serotype/Genotype/Clade turnover in DENV evolution

A common viral feature in endemic settings is the phenomenon of serotype turnover [118, 127]. Thus, in areas with four serotypes co-circulating, a certain serotype is more prevalent than the others for a period of time and then is gradually replaced by another. The mechanism underlying this shift is unknown. It could be due to stochastic factors or it is probably related to the level of herd immunity that is reached in the affected population [128].

In addition to serotype turnover, genotype/clade shift is also a feature of DENV evolution in endemic settings. This phenomenon has been observed in all 4 serotypes [118, 129-131]. For example, lineage replacements have been observed in DENV-1 genotype I [118] and DENV-3 genotype II in Thailand during a long sampling study of 30 years [132].

Many factors contribute to phylogenetic shifts. They may be due to stochastic factors (such as the oscillation of mosquito population vectors, changes in weather/climate [130, 132] or associated with selection pressure [129] or it was related to herd immunity [118, 128]. For example, the observed lineage replacement in DENV-4 in Puerto-Rico during the sampling time of ~18 years (1981-1998) was associated with adaptive selection in NS2A gene [129]. However, the replacement of DENV-1 genotype I, lineage A by DENV-1 genotype III lineages (B and C) in Myanmar (between 1998 and 2000) was attributed to seasonal variations (dry season and monsoon) [130, 132]. Additionally, introduction (gene flow) of a "foreign" genotype/clade can alter genetic structures. For example, the DENV-2 Southeast Asian viruses that are fitter than the native lineage [61-63] have spread over the Americas and displaced the American genotype in at least four countries [54]. Also, the Cosmopolitan

genotype effectively replaced Asian 2 in the Philippines in 1998 [133], and a lineage of DENV-3 genotype III replaced the resident in Sri Lanka in 1989 [57]; both of which were associated with the emergence of DHF cases.

1.8.3. Selection pressure on DENV viruses

Analyses of selection pressure on DENV population have shown that the overall pressure impacting on DENV is of purifying selection [118, 122, 125, 132], which is the process that decreases the prevalence of deleterious mutations diminishing individual's capacity to fitness, practically expressed as ratio of non-synonymous substitution rate (*dN*, amino acid changed) and synonymous substitution rate (*dS*, amino acid unchanged), with $dN/dS < 1$. However, there was some evidence for positive selection pressures (the process that increases the prevalence of adaptive traits, $dN/dS > 1$) which differed among serotypes, genotypes and viral proteins [129, 134-136].

1.9. Purposes of this thesis

Understanding the evolution of DENV and whether there is an association between viral genotype and epidemic potential or disease severity is of fundamental importance for vaccine and drug development. Although many studies of the molecular epidemiology of DENV have been conducted, there has been limited research at the level of the entire virus genome. Conventional approaches focusing on DENV genome segments to study epidemiology and genetic relationships may not have the sensitivity to accurately reflect the epidemiological and evolutionary dynamics of DENV. This shortcoming can be addressed using a robust process of whole genome sequencing, which in general has become a genomic cornerstone for understanding pathogen biology [137]. Potentially important questions in dengue epidemiology and pathogenesis could be answered with the sensitivity given by a whole genome sequence, e.g. can the molecular fingerprint given by the genome sequence be used to understand virus transmission patterns better?

High-throughput sequencing of DENV genomes has been used to provide a higher resolution for studying recombination, phylogenetic dynamics, comparative sequence divergence and the association between viral genetics and pathogenesis [58, 136-139]. However, a number of important questions about the role of viral virulence in pathogenesis have not been answered such as, (1) is there a particular gene or variant of a gene associated with disease severity? and (2) is clinical outcome associated with a particular DENV genotype or lineage? Furthermore, although the occurrence of lineage replacement is one of the most intriguing aspects of DENV molecular epidemiology, its mechanistic basis is unclear. In particular, it is uncertain whether such lineage replacement events reflect large-scale epidemiological processes that are independent of viral genotype, such as random population bottlenecks, caused by large-scale declines in mosquito numbers during the annual dry season [130, 132], or differences of viral lineages in fitness such that one is able out-compete another [54, 61-63], including as mediated by cross-immunity. Differentiating between these two mechanisms is of central importance because it enables predictions to be made on the future course of DENV evolution.

Dengue is endemic in Vietnam, with local outbreaks consecutively occurring in different areas, and a continual increase in disease incidence over the last decade, yet little is known of what drives epidemic dynamics, and how the DENV viral population is evolving. We hypothesise that (1) the recent increased in dengue incidence is associated with change in the phylogenetic structure of the DENV population and (2) the genetic diversity revealed by the genome sequence of DENV can be used to explore aspects of transmission dynamics in time and space. These questions are addressed in Chapter 3 and Chapter 4.

Further, while awaiting specific drugs or vaccines for dengue, an early, sensitive, and accurate diagnosis for dengue is helpful for clinical management, research activities and disease surveillance. The NS1 protein has the potential to be a valuable diagnostic marker. However, further evaluations of commercial assays in different ethnic groups and virus serotypes are

needed. There also remains a practical question about the utility of NS1 as a prognostic marker for dengue shock syndrome. In addition, the impact of the IgM response on the sensitivity of NS1 detection is still controversial [21, 22, 24, 27, 29, 140]. Previous studies have also shown that NS1 sensitivity differed in infecting serotypes [25, 29]. We hypothesise that different sensitivity in relation to infecting serotypes may be caused by the differential binding of mAbs used in capture and detection to the target epitope(s) on NS1 in different serotypes. Therefore, the aim of Chapter 5 and 6 of this thesis is to address the following questions; 1) how does the sensitivity of NS1 detection differ amongst DENV serotypes circulating in Vietnam; 2) is this difference in sensitivity influenced by host infection status and/or viral factors, and 3) what is the specificity of NS1 assays for the diagnosis of acute DENV infection?

Chapter 2

2. Subjects and Methods

2.1. Study subjects

2.1.1. Patients with acute dengue

Acute dengue patients (adults and children) were recruited from 3 referral hospitals in HCMC and 1 provincial hospital in Dong Thap (Dong Thap Hospital, herein abbreviated as DTH). Acute dengue virus infection was confirmed by serology and/or RT-PCR and/or NS1-ELISA and/or virus isolation. There were 3 main study groups from which patient samples were collected: ***a)*** DENCO study in HCMC (Children's Hospital #1, Children's Hospital #2 and Hospital for Tropical Diseases - abbreviated as HTD), samples were collected between 2006-2007 ; ***b)*** FG study (DTH), samples were collected between 2006-2007; and ***c)*** MD, DF, FL, FR studies in HCMC (HTD), samples were collected between 1999-2008. Severity of dengue was defined according to WHO classification (refer to section 1.4.2. in Chapter 1). All study procedures were approved by the Hospital for Tropical Diseases Ethics Committee (DENCO, MD, DF, FR and FL), the Children's Hospital #1 Ethics Committee (DENCO), the Children's Hospital #2 Ethics Committee (DENCO), Dong Thap Hospital Ethics Committee (FG) and the Oxford Tropical Research Ethics Committee UK (OXTREC) (All studies).

2.1.2. Control group

Patients with other acute febrile illness (OFI) including other viral infections (from DENCO study), malaria, enteric fever, Japanese encephalitis or leptospirosis from other studies in HTD were used as a control group for assessment of the specificity of the NS1 antigen test kits.

2.1.3. Inclusion and exclusion criteria for patient recruitment

The study inclusion criteria were: clinical suspicion of dengue with a history of fever less than 72 hrs (FG, FR studies) or 7 days (DENCO, MD, FL studies), or dengue shock syndrome (DF study); and written informed consent obtained. Patients were excluded who had dual acute infections (e.g. meningitis plus dengue), or no laboratory evidence of acute dengue (except for the control group defined above), or a lack of informed consent.

2.1.4. Sample collection

A 2-3ml venous blood sample from dengue patients collected in EDTA anticoagulant-containing tubes at the enrollment day (admission samples) and hospital discharge day (convalescent samples) were sent to the Oxford University Clinical Research Unit at the Hospital for Tropical Diseases for serologic, virologic, NS1 diagnostic investigations and genome sequencing. These samples were centrifuged to separate the cells and the plasma. Each cell pellet or plasma was aliquoted into 3 aliquots and stored at -20°C for further experiments.

2.2. Methods

2.2.1. Methods for viral amplification and sequencing

2.2.1.1. RNA extraction

RNA extraction from plasma or plasma-derived culture was carried out with Qiagen viral RNA extraction (Qiagen, Cat. No 52906). The procedure was performed according to the manufacturer's instructions. For TaqMan RT-PCR, as an internal control, an amount of $10\text{-}10^2$ copies/reaction of single-stranded positive sense RNA virus [*Equine arteritis* virus (EAV)] was added to each patient's plasma sample prior to extraction. Viral RNA was eluted in 60μl of elution buffer. RNA was stored at minus 80°C until use. For whole genome amplification, no internal control was used.

2.2.1.2. cDNA synthesis

Viral RNA was subjected to cDNA synthesis by using either random hexamers or specific reverse primers, or a combination of random hexamers and specific reverse primers.

a. cDNA generation using random hexamers

Random hexamers (Cat #1034731, Roche, Lewes, UK) of $50A_{260}$ unit equal to 2 mg in dry form were reconstituted with 1000μl of distilled water to give a concentration of 2mg/ml, and then diluted to a working solution of 1ng/μl. A mixture of 14μl of 1x First-strand buffer

(Invitrogen) containing a standard amount of viral RNA, 2μl random hexamers and a mixture of 0.5 mM deoxyribonucleotide triphosphates (dNTPs) (Cat #03622614001, Roche) was incubated at 65°C for 5 min and immediately chilled on ice for at least 1 min. A mixture of 6 μl of 1x First-strand buffer containing 5mM dithiothreitol (DTT, 0.1 M), 20 units of RNase inhibitor (40U/μl, Cat #10777-019, Invitrogen) and 100 units of SuperScript III Reverse Transcriptase (200U/μl, Cat #18080-094, Invitrogen) was added to make up a reverse transcription volume of 20 μl. The content was pretreated at 25°C for 5 minutes. cDNA was synthesized at 50°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min. cDNA would then be used for TaqMan RT-PCR.

b. cDNA generation using specific reverse primers

cDNA was generated similarly to the above procedure but using a working concentration of 2μM of each specific reverse primers (DENV-2 Reverse 1: AGAACTGGCYAAAATGCTCACC, position: 4170-4191; DENV-2 Reverse 2: TGGTCGTAGTGCCAYGATGT, 8438-8457 and DENV-2 Reverse 3: CACCATTCCATTTTCTGGCG, 10684-10704; in which Y stands for C/T nucleotide) instead of random hexamers without pre-treatment at 25°C. cDNA would be used for whole genome amplification in 3 fragments

c. cDNA generation using combination of random hexamers and specific reverse primers

For genome sequencing conducted with the Broad Institute, cDNA was generated using 1ng/μl random hexamers and 2μM specific reverse primer of fragment 14 of each dengue serotype (DENV-1 R14: AGAACCTGTTGATTCAACAGCAC, DENV-2 R14: AGAACCTGTTGATTCAACAGCAC, DENV-3 R14: CATTTTCTGGCGTTCTGTGC). The procedure for cDNA generation is shown in **Table 2.1**.

Mix 1 (working concentration)	Volume (μl) (For 1 reaction)	Final concentration
5x First-strand buffer	2	1x
Random hexamers (1ng/μl)	2	0.1ng/μl
(and/or) R specific primer (2μM)	2	0.2μM
dNTPs (10mM)	1	0.5mM
Viral RNA (template)	5	
dH_2O	up to 14	
Mix well and briefly centrifuge. Heat mixture at 65°C for 5 min and quickly chill on ice for at least 1 min. Add mix 2 into mix 1		
Mix 2	Volume (μl)	Final concentration
5x First-strand buffer	2	1x
DTT (0.1M)	1	5mM
RNase inhibitor (40U/μl)	0.5	20 U
Superscript III reverse transcriptase (200U/μl)	0.5	100 U
dH_2O	up to 6	
Run at 25°C for 5 minutes followed by 50°C for 1 hour, then inactivate the reaction at 70°C for 15 minutes		

Table 2.1 cDNA synthesis procedure. For 14 fragment amplification strategy, a combination of random hexamers and specific reverse primer (R14) of each dengue serotype was used. For 3 fragment amplification strategy, only specific reverse primers were used. For TaqMan RT-PCR, only random hexamers were used.

2.2.1.3. Dengue serotyping by TaqMan RT-PCR amplification

a. Standard curve establishment

The standard curve was generated using serial dilutions (10^{6}-10^{-1}) of a linearized plasmid containing the cloned target amplicon (177 nt in NS5 gene). Results were expressed as cDNA equivalents per milliliter of plasma. Viraemia level was estimated from a cros-over threshold (Ct value) calculated using quantitative RT-PCR

b. Primers and probes

Oligonucleotide primers and fluorescence-labeled probes used for DENVs serotyping were adapted from Laue [18]. DENV specific primers and probes are shown in **Table.2.2**.

Oligo name	Oligonucleotide sequence	Position
Denv-1 F Primer	ATCCATGCCCACCAYCAATG	9960 - 9980
Denv-1 R Primer	CAGGGATCCACACCAYTGATC	10100 - 10121
Denv-2 F Primer	ACAAGTCGAACAACCTGGTCCAT	9937 - 9941
Denv-2 R Primer	GCCGCACCATTGGTCTTCTC	10095 - 10113
Denv-3 F Primer	TTTCTGCTCCCACCACTTTCAT	9719 - 9741
Denv-3 R Primer	TGGCGTTGGATGCYAGTCT	9915 - 9934
Denv-4 F Primer	GYGTGGTGAAGCCYCTRGAT	9587 - 9607
Denv-4 R Primer	AGTGARCGGCCATCCTTCAT	9744 - 9764
Den-1-probe-ver2	5'-FAM TCAGTGTGGAATAGGGTTTGGATAGAGGAA 3'TAMRA	
DEN-2 Vers4LNA.	5'-FAM GTTTTgTCTTCCATCCA 3' BHQ-1	
Den-3-probe-ver2	5'-FAM AAGAAAGTTGGTAGTTCCCTGCAGACCCCA 3'TAMRA	
Den-4-probe-ver2	5'-FAM ACTTCCCTCCTCTTYTTGAACGACATGGGA 3'TAMRA	
EAV F Primer	CATCTCTTGCTTTGCTCCTTAG	1847-1869
EAV R Primer	AGCCGCACCTTCACATTG	1962-1980
EAV Probe	5'-Cy5CGCGCTCGCTGTCAGAACAACATTATTGCCCAC AGCGCG3'BHQ-3	

Table 2.2 DENVs Oligonucleotide primers, fluorescence-labeled probes used for serotyping and EAV primers and probes used as internal control. Y stands for C/T nucleotide, R for A/G. F: forward; R: reverse; EAV: Equine Arteritis virus (causing systemic infection in equids)

c. PCR Amplification procedures

Four μl cDNA generated with random hexamers as described above were added to 21 μl of amplification reaction containing a final concentration of 1x HotstarTaq buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.6 μM of each sense and anti-sense primer and 0.2 μM probe corresponding to each serotype, and one unit HotstarTaq (Qiagen). The amplification reaction was pretreated at 95°C for 15 min then run for 45 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s. The internal probes for three dengue serotypes 1, 3, and 4 were labeled with two fluorescence dyes: FAM reporter dye (5-carboxyfluorescence) and TAMRA quencher dye (N, N, N', N'tetramethyl-6-carboxyrhodamine). The DENV-2 probe contained LNA (locked nucleic acid) residues and was labeled with FAM reporter dye and BHQ1 (Black Hole Quencher 1). The PCR procedure and conditions are described in **Table 2.3**.

TaqMan RT-PCR procedure		
Reagents (working concentration)	µl/reaction	Final concentration
DENV FW Primer (10 µM)	1.5	0.6 µM
DENV RV Primer (10 µM)	1.5	0.6 µM
EAV FW Primer (10 µM)	1.5	0.6 µM
EAV RV Primer (10 µM)	1.5	0.6 µM
$MgCl_2$ (25mM)	2.5	2.5 mM
10x HotstarTaq Buffer	2.5	1x
dNTPs (10mM)	1	0.4mM
DENV Probe (1 µM)	5	0.2 µM
EAV Probe (5 µM)	1.5	0.3 µM
HotstarTaq (5U/µl)	0.2	1 Unit
cDNA	4	
H_2O	up to 25 µl	
TaqMan RT-PCR conditions		
Number of cycles	T°	Time
1	95°C	15 min
45	95°C	30 sec
	60°C	30 sec
	72°C	30 sec

Table 2.3 TaqMan RT-PCR procedure and conditions

2.2.1.4. DENV-2 whole genome amplification in 3 fragments and shotgun sequencing at the Sanger Institute

a. Amplification strategy

DENV-2 genome was amplified in 3 overlapping fragments. The first amplicon was 4,179 base pairs (bp) covering 5'UTR/C/M/E/NS1/NS2A and one-third of NS2B. The second amplicon (4,448 bp) had 180 nucleotides overlapping the first amplicon that spanned from NS2A/NS2B to the first one-third of NS5. The last amplicon (2,349 bp) covered the last two-thirds of NS5 and 3'UTR with 100 nucleotides overlapping the second amplicon. The amplification strategy is shown in **Fig.2.1**.

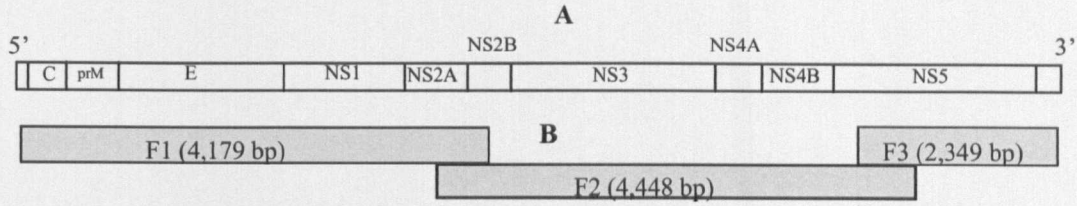


Fig.2.1 Schematic of the DENV RNA genome amplification strategy. **(A)** shows the genome organization and **(B)** is RT-PCR strategy for 3 overlapping fragments with length indicated in each block. F=fragment

b. Oligonucleotide primer design

To design primers for 3 overlapping fragments, 35 DENV-2 whole genomic sequences available from GenBank were aligned using AlignX (Vector NTI suit 7.1). Primer design software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design 3 pairs of oligonucleotide primers. These primers would generate 3 overlapping DNA fragments (namely amplicon 1, 2 and 3) covering 99.7 % of the DENV-2 genome. Oligonucleotide primers for whole genome amplification in 3 fragments are shown in **Table 2.4**.

Oligo name	Oligo sequence	Position	Melting T°	Product size in base pairs
F1	ACGTGGACCGACAAAGACA	13-31	50.8	
R1	AGAACTGGC**Y**AAAATGCTCACC	4170 - 4191	54.5	4179
F2	CCCCACTG**H**T**Y**TT**R**ACATCC	4010 - 4029	50.9	
R2	TGGTCGTAGTGCCA**Y**GATGT	8438 - 8457	52.3	4448
F3	CG**Y**AACATTGGAATTGAAAG	8356 - 8375	49	
R3	CACCATTCCATTTTCTGGCG	10684 - 10704	55	2349

Table 2.4 Oligonucleotide primer sequences used for amplification of DENV-2 in 3 overlapping fragments. (Y= C/T, R= A/G and H= A/C/T nucleotide)

c. DENV2 whole genome amplification conditions

PCR amplicons were generated with High Fidelity Taq polymerase (Cat #11732650001, Roche). A mixture of dNTPs (0.4mM), forward and reverse primers (0.2μM of each), 5μl of cDNA, plus 0.38 units of enzyme (Tgo and Taq DNA polymerase) in a 25 μl PCR reaction

including 10 x High Fidelity Taq buffer and 1.25 µl DMSO was run for 35 cycles. To amplify each fragment, thermo cycling began at 94°C for 2 min, followed by 10 cycles with different PCR annealing temperature conditions according to the primer pairs' characteristics. Fragment 1 and 2 underwent 54°C annealing temperature, and 57°C for fragment 3. Elongation was achieved at 72°C for 3 min. For the last 25 cycles, after each cycle, 5s of elongation were added. PCR products were analysed by 1% agarose gel electrophoresis. The remaining PCR products were stored at –20°C for shipment to the Sanger Centre (UK). Whole genome amplification procedure and thermo cycling conditions for DENV-2 are shown in **Table 2.5**.

Whole genome amplification in 3 fragments procedure			
Reagents (working concentration)	µl/reaction	Final Concentration	
FW Primer (10 µM)	0.5	0.2 µM	
RV Primer (10 µM)	0.5	0.2 µM	
DMSO	1.25	5%	
10x HiFiTaq Buffer	2.5	1x	
dNTPs (10mM)	1	0.4mM	
HiFi Taq (5U/µl)	0.2	1 unit	
cDNA	2		
H_2O	up to 25 µl		
Whole genome amplification in 3 fragments conditions			
Step	T°	Time	Number of cycles
Pretreat	94° C	2 mins	1
Melting	94° C	15 sec	10
Annealing[a]	54° C	30 sec	
Extension	72° C	3 mins	
Melting	94° C	15 sec	25
Annealing[a]	54° C	30 sec	
Extension	72° C	3 mins, plus 5s after each cycle	
Final extension	72° C	7 mins	1
[a]: 57° C for the third fragment			

Table 2.5 Whole genome amplification procedure and thermo-cycling conditions for DENV-2 in 3 overlapping fragments

d. Shotgun sequencing and sequence assembly

The 3 amplicons of the DENV-2 whole genome were subjected to shotgun cloning and sequencing at the Sanger Institute, Cambridge UK, according to the Sanger protocols. The amplimers were first purified then kinase treated. Small Insert Libraries were conducted as described in Finishing Library Methods at http://www.sanger.ac.uk/Teams/Team53/psub/methods.shtml and http://www.sanger.ac.uk/Teams/Team53/psub/reference/vectorinfo.shtml.

Libraries were constructed using pUC19 SmaI-CIP (Q-BIOgene Cat#CVPSM029) then transformed to an *E. coli* host.

d1. Purification of PCR products

Each PCR amplimer was resuspended in 9 µl dye mix (available at the Biochemical Development, Team 53, Sanger Institute), loaded on a 0.8% low-melting point agarose gel with one-track gap between each amplimer, run at 5V for 16hrs or 25V for 2hrs in TE buffer (if running at 25V, after 1 hour, the running buffer was changed). Desired fragments separated by electrophoresis were excised from the gel. The agarose piece was melted at 65°C. When the agarose gel completely melted it was then cooled at 42°C and an aliquot of AgarAce (Promega) was added according to this ratio: 1.25 U (5µl) of AgarAce for 200 µl of agarose gel. Then the content was incubated at 42°C for 30 mins. DNA was collected by phenol extraction and then ethanol precipitation (as described in Finishing Library Methods). DNA was recovered in 5µl TE. Because the amount of each amplimer yielded in 3 individual PCR reactions was different, a different volume of each amplicon was used to give a roughly equal molar concentration. To assess this, 0.5 µl of each amplicon was suspended in 4 µl dye mix then run on 0.8% high gelling temperature (HGT) agarose gel in 1xTBE buffer at 70V for 40 minutes. They were roughly equivalent in molarity based on the appearance in the agarose gel.

This process avoided misrepresentation and also normally yielded a random collection of fragments. The mixed-3 amplicon was made up to 50 µl with di-distilled water.

d2. Library construction

Amplicons were treated with kinase (Polynucleotide Kinase, Cat #174645, Roche, Germany) to transfer the phosphate group from ATP to the 5'-hydroxylated terminus of amplicons (phosphorylation) as follows: 16 µl of mixed-3 amplicon mixture, 2 µl of 10x kinase buffer, 1 µl of 1mM ATP and 1 µl T4 polynucleotide kinase was incubated at 37°C for 15 minutes, then 65°C for 10 minutes to denature enzyme. It was then chilled on ice and poured back to the main tube. The mixture was sonicated at full power for 2-3 mins then 1 µl of the sonicated amplicons was analysed on a 0.8% HGT agarose gel to ascertain the sonication was successful Amplicons were treated with Mung Bean Nuclease (Amersham, 156U/µl) to produce blunt ends (6 µl of 10x Mung Bean buffer, 0.3 µl of Mung Bean nuclease was added then incubated at 30°C for 10 minutes). Then 141 µl H_2O, 20 µl of 1M NaCl, 1 µl pellet paint and 560 µl cold ethanol was added to the mixture and incubated at -20°C overnight or -70°C for 30 minutes for ethanol precipitation. DNA was recovered in ~10 µl TE then run onto LMP agarose gel to yield a set of fragments in a similar size. The excised fragments of 0.8 -1.2 kb were purified again as described above and subjected to ligation using ready-to-use pUC19 SmaI-CIP (40ng/µl) (3 µl of DNA solution, 0.3 µl of pUC19, 0.4 µl 10x ligase buffer and 0.3 µl T4 DNA ligase (Roche, 5U/µl) then incubated at 12-14°C overnight). The content was then made up to 50 µl by 0.5 x TE and proteinase K treated (1 µl) at 50°C for 1 hr.

d3. Transformation

Transformation was achieved with BioRad genepulser system set at 1.9 kV, 200 ohms, 25 µF; E. coli DH10B (Invitrogen) served as host for pUC19 SmaI libraries. Ten µl of 10% glycerol were transferred to a pre-chilled eppendorf tube then 0.2 µl libraries and 40 µl electro

competent cells were added. All the content was mixed well and transferred to a pre-chilled 0.1 cm gap electroporation cuvette and shocked by the genepulser set as described above. Five hundred µl of SOC medium [10 ml SOB medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl) plus 200 µl of 20% glucose] were immediately added and incubated at 37°C for 1 hr, then 50µl of each IPTG (40mg/ml) and X-gal (50mg/ml) were added. Volumes corresponding to 0.02 µl and 0.04 µl libraries were plated on TYE (10g of Bacto Tryptone, 5g of yeast extract, 8g of NaCl, 15 g of Bactor Agar per liter) - ampicillin plate (100µg/ml). Recombinant pUC plasmids presented as white colonies based on blue-white selection. Ideally, the number of clones picked must provide adequate coverage spanning the entire genome and be equally representative for the PCR amplimers being sequenced in 8 – 10 times. To meet these criteria, this condition should be satisfied: C=N.r/G (C=Coverage, N: Number of reads, r: Mean read length, G: Length of the genome) [141]. In each shotgun library, between 96 and 192 clones with the average read length of 0.8 – 1.2 kb were picked. Subsequently, plasmid DNA was harvested in 96-Well Miniprep as described at http://www.sanger.ac.uk/Teams/Team53/psub/methods.shtml, in which pellet cells were suspended in GTE (50 mM glucose, 25 mM Tris-HCl (pH8), 1 mM EDTA) with RNaseA (1.2ml of 50mg/ml RNaseA to 100ml GTE), SDS/NaOH (0.2M NaOH, 1%SDS), and 3M KOAc. Biochemical development is described at http://www.sanger.ac.uk/Teams/Team51/MicroPrep.shtml and http://www.sanger.ac.uk/Teams/Team51/vacsol.shtml?;decor=printable. DNA was re-suspended in 50 µl ddH_2O. Double-stranded DNA was dideoxy sequenced using universal primer (M13F) (5'-TGTAAAACGACGGCCAGT-3') and reverse primers (pUC19R) (5'GCGGATAACAATTTCACACAGGA-3') according to the Sanger automated ABI 3700 sequencer system (http://www.sanger.ac.uk/Teams/sequencing/). Sequence assembly was

performed by Team 21-Sanger using Gap4 program. The incomplete sequences or thin coverage was solved by walking PCR.

2.2.1.5. Whole DENV genome amplification and sequencing in collaboration with the Broad Institute

In collaboration with the Broad Institute, a second whole genome amplification approach was deployed for amplification of DENV genomes in 14 overlapping fragments (**Fig.2.1**). DENV-1, -2, and DENV-3 genome were first amplified in 14 overlapping fragments in the first round PCR (conducted in the OUCRU). Each fragment was ~ 2 kb in length. This PCR strategy created 2-3x physical coverage for each nucleotide site. The fourteen amplimers were pooled in one tube and shipped to the Broad Institute – USA. Each genome was dispensed into one quadrant of a 384-well plate containing PCR amplification reaction for second round PCR with M13-tailed primer pairs. The amplification strategy for the whole genome in 14 fragments is illustrated in **Fig.2.2**. The process of amplification (at OUCRU) and sequencing strategy (at the Broad Institute) is illustrated in **Fig.2.3.** All primer sequences (designed by the Broad Institute) for DENV-1, -2, and DENV-3 are shown in **Table 2.6 (A), (B), and (C)**, respectively. PCR procedure and conditions are respectively shown in **Table 2.7**.

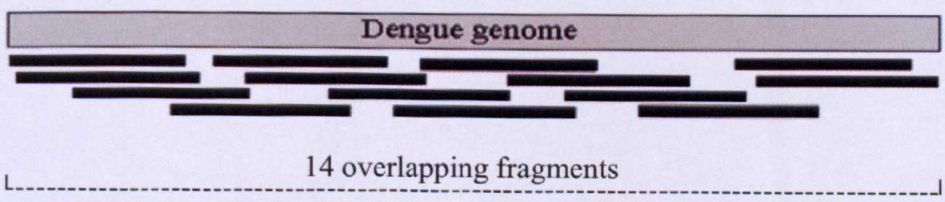


Fig.2.2. Schematic of RT-PCR amplification strategy for the DENV genome in 14 overlapping fragments (designed by the Broad Institute). Each amplimer is ~ 2kb in length ranging from 1.901 (fragment 4) to 2.213 kb (fragment 13).

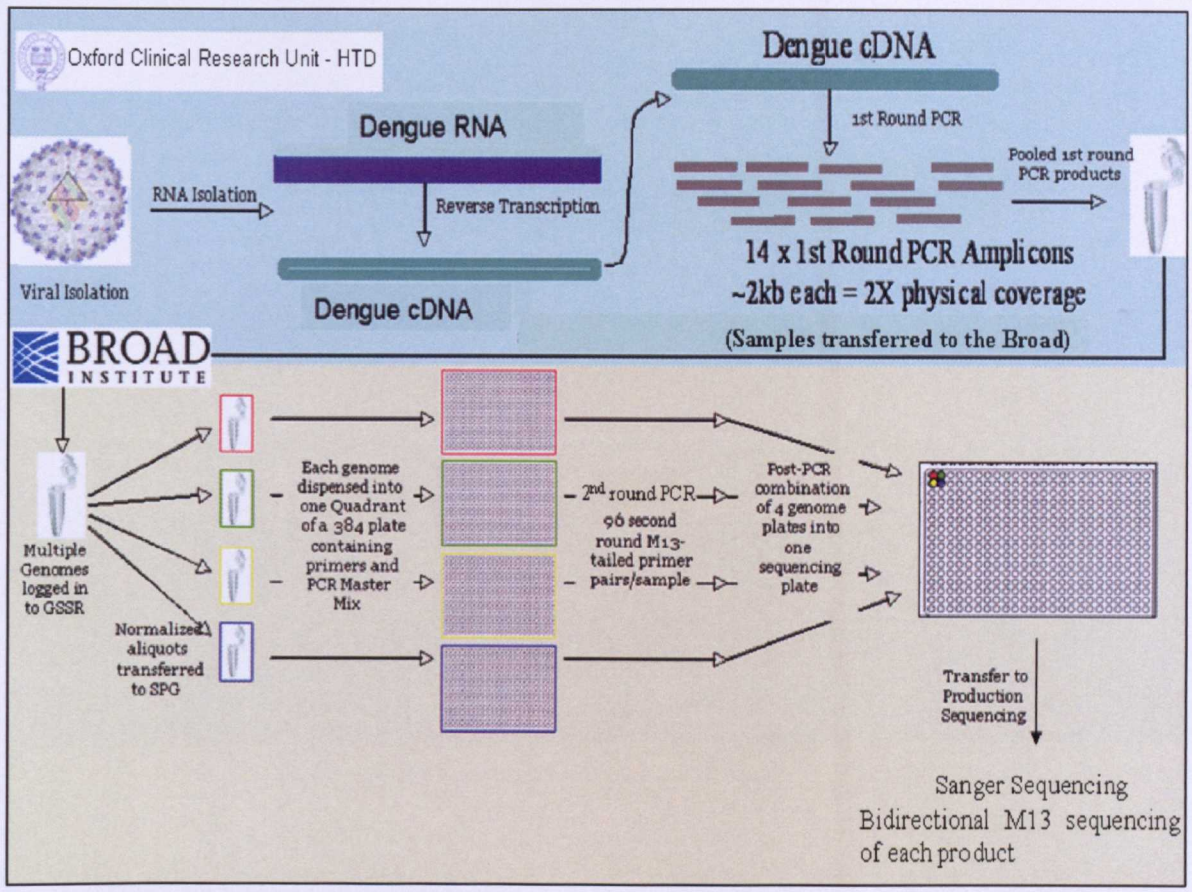


Fig.2.3. Procedure of whole genome amplification (WGA) in 14 fragments (Oxford Clinical Research Unit-HTD) and whole genome sequencing (WGS) of DENVs (The Broad Institute).

DENV-1 Forward Primer	DENV-1 Reverse Primer	Region
AGTTGTTAGTCTACGTGGACCG	AYCACGATGTARCTCTCACCAA	1-2073
TTCTAGCCATACCYCCAACAGC	CTTCCACATTTGAGTTCTCTRCC	261 - 2469
AGCACATGCCATAGGAACATCC	AYTTGGGTGTAGGAGTCACGCA	856 - 2953
TGAGACCCAGCATGGAACYGT	YAGCACCGGAAGCCATGTTGTT	1873 - 4060
CYTGGACCATGAAAATAGGAATAGG	ATRGTTCCATCATCTTGGACCTC	2289 - 4380
CATATGGYTGAAATTGCGTGACTC	AACACCTCGTCYTCAATCTCTGG	2917- 5070
ATGGACTTGCAATGGGYATYATG	TGCTTCTGTCCAATGRGCGTGRT	3849 - 5995
AGTGYTATGGGACACACCYAG	TCCACACTGGCCATCCATARCA	4525 - 6678
GCYCAAGCTAAAGCATCACARG	AAGGCGAGAAGTGGAACTCCTA	5015 - 7116
CACGCYCAYTGGACAGAAGCAAA	TCYACCACACTTGGCATGTARG	5975 - 8145
TCYGAACAAGGAGGAARAGCCTA	TCTGTTGTCCRAAGGGTGTGGT	6476 - 8627
AAAYTGAGGTGGTTTGTGGAGAG	ACGGCTGAACAGATRGCATTAGC	7754 - 9918
AGTGGAACCAGAGGTAGCCAAC	GGTTTTTACATCCCCACGATGG	8368- 10465
ATYCCCATGGTCACACAAATAGC	AGAACCTGTTGATTCAACAGCAC	8573 - 10735

Table 2.6.(A) Oligonucleotide primer sequences used for DENV-1 amplification in 14 fragments. (Y stands for C/T and R for A/G nucleotide)

DENV-2 Forward Primer	DENV-2 Reverse Primer	Region
AGTAGTTAGTCTACGTGGACCG	GATCCRAAATCCCARGCTGTGTC	1-2207
CAGATCTCTGATGAATAACCAACG	GATCCRAAATCCCARGCTGTGTC	87-2207
CCAGAAGACATAGAYTGTTGGTG	TRCCTGCATGATTCCTTTRATGTC	619-2718
TGCCCAACACAAGGRGAACCYA	ATCTTCCATGTRTCATTGAGTGC	1156-3056
ATGGTGCARGCYGATAGTGGTT	TAGGCTCCRTCTTCCAGTTCRG	2410-4589
YATGACAGGAGACATYAAAGGAATC	CTTCCARCCTCCTCCATAYGATA	2685-4773
ATGCTYAGGACCCGAGTAGGAA	AGRCAAGCTGCTATRTCATTTCC	3541-5645
GCATGGAARGTGAGYTGCACAA	TATTCRCCRTCAATGGCATCCAC	3967-6089
GTYACAAGGAGTGGARCATATGT	AGRAGGGGAACTCCRATGTCCA	4984-7106
GCAGCYGGGATTTTYATGACAG	GTYTCTCCTRTGTTGCCAGTTC	5446-7592
AGATGGYTGGAYGCTAGGATCTA	TARTGCCATGAYGTTTCATGCTC	6301-8450
CTAGAWCCAATACCYTATGATCC	GTGATTCTTGTGTCCCAKCCTG	7288-9197
GTGACATAGGGGARTCRTCACC	GTARTCTGTGTATTCCTCATTGCC	8003-10215
GCCATATTCACYGATGAGAACAAR	AGAACCTGTTGATTCAACAGCAC	8800-10723

Table 2.6.(B) Oligonucleotide primer sequences used for DENV-2 amplification in 14 fragments. (Y stands for C/T, R for A/G, W for A /T and K for G/T nucleotide)

DENV-3 Forward Primer	DENV-3 Reverse Primer	Region
TGGACCGACAAGAACAGTTTC	GGCTTTGTCTCCAATTCCAA	16-2086
GCATGATTGTGGGGAAGAAT	TGTCGACCTGATTCCGCACA	468-2593
AGCCCTATTTCTTGCCCATT	GTGAAGACTCCGAACCCGTA	844-2904
TTTCATGCATTGTGATAGGA	GGGTTTGCTTTTGCCAAGTG	2358-4508
ATGGAGTGTGCGGAATCAGG	TCTCCATCCTCCTCCGTATG	2568-4765
ACATGATTGCAGGGGTTCTC	ATTGCCTGAATTCCATGAGC	3561-5560
TGGAGAACAGCCACCCTGATT	TCACCCCTCCTCATGAGTTC	3962-6138
GTGGGGGTTGGAGTACAAAA	TTGCTTGTCTTGGGGAGTTC	4619-6769
CATTGAAAGGGCTCCCAATA	TCCAGCAGCTGTCCTTTTTT	5217-7240
GATGTCTCAAGCCAGTGATT	CACACTTTTCAGGTGGAAGA	5796-7985
GAGACCTAGGTGGCTTGATG	ATGGTAAGCCCACGTTTTGTA	6289-8479
CCCTAGCCACAGGACCAATA	GTTCTCGAGGTCTGCCTTCG	7404-9469
CATGCCAACTGTGATTGAGC	CAAATGGCTCCCTCTGACTC	8119-10263
ACGAAACCATGGGATGTGGT	CATTTTCTGGCGTTCTGTGC	8546-10679

Table 2.6.(C) Oligonucleotide primer sequences used for DENV-3 amplification in 14 fragments. (Y= C/T and R= A/G nucleotide)

Procedure for Whole Genome Amplification in 14 fragments		
Reagents	**µl/reaction**	**Final concentration**
FW Primer (10 µM)	0.5	0.2 µM
RV Primer (10 µM	0.5	0.2 µM
DMSO	1.25	5%
10x HiFiTaq Buffer	2.5	1x
dNTPs 10mM	1	0.4mM
HiFi Taq (5U/µl)	0.2	1 unit
cDNA	2	
H_2O	up to 25 µl	
PCR conditions for Whole Genome Amplification in 14 fragments		
T° (C)	**Time**	**Number of cycles**
94	2 mins	1 cycle
94	30 sec	25 cycles
60	1 min	
72	2 mins	
72	10 mins	1 cycle
4	hold	

Table 2.7 Amplification procedure and thermo-cycling conditions for DENV whole genome in 14 overlapping fragments

2.2.2. Methods for serological assays

2.2.2.1. IgM/IgG capture ELISA procedure

Serological testing was performed for paired plasma samples (collected ~5-7 days apart). Plasma was diluted 1:100 in diluents [0.1% BSA in 1xPBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24 g KH_2PO_4 /1 liter H_2O, pH 7.4)].

a. Coating plates

100 µl per well of anti-human µ (DakoCytomation, Cat # A0425) or anti-human γ chain [Sigma, Cat # I 2136) diluted 1:2000 in 0.05M coating buffer (1 carbonate bicarbonate buffer tablet (Sigma)/100ml dH_2O, pH 9.6; filter through 0.22 µm filter)] was dispensed into 96-well ELISA Plates (Nunc Immuno plate, Cat # 442404) and incubated at 4°C overnight (16-20 hrs).

b. Blocking plates

Plates were washed 3 times with washing buffer (1ml Tween20/2000ml PBS), and then 200µl of blocking buffer (3% BSA in 1xPBS) was added to each well of the plate, incubated at room temperature (RT) for 1 hr, and washed 3 times with washing buffer.

c. ELISA assay

Briefly, 100 µl plasma/sera diluted at 1:100 (in diluent) were dispensed into each well of a plate and the plate was incubated at RT for 2 hrs. After washing 6 times, 100 µl per well of DENVs antigen mixture of four serotypes (supplied by Venture Technologies, Sarawak, Malaysia) diluted at 6:10 in PBS were added and incubated at 4°C overnight. After incubation plates were washed 6 times and 100µl of mAbs (specific for DENV or JEV E protein, supplied by Venture Technologies, Sarawak, Malaysia) diluted at 1:5 in diluent were added to wells, then incubated for 1 hr at RT. After incubation the plate was washed 6 times before 100 µl of conjugate solution (Polyclonal Rabbit Anti-Mouse immunoglobulins/HRP, Dako Cytomation, Cat # P0260) diluted 1:2000 in diluent were dispensed into each well and incubated at RT for 1 hr in the dark. After incubation, the plate was washed 6 times and 100 µl of substrate solution [1 tablet of Buffer with urea (Sigma Fast^{TM}), 1 OPD tablet (Sigma Fast^{TM}), 20ml dH_2O] was added to each well; colour development was stopped with 50 µl of stopping solution (10% H_2SO_4). The optical density was read at 490 nm on a spectrophotometer. Positive controls (positive control sera), negative controls (negative control sera) and background (positive sera and PBS instead of DENV antigen) in triplicate were run together with samples on each plate. The ELISA assay is summarised in **Table 2.8.**

Assay procedure	Reagent added	µl per well	Incubation t°	Time
1- Coating plate	Anti-human (µ/γ)	100µl	4°C	16-20 hrs
	3x washing with washing buffer			
2- Blocking plate	Blocking buffer	200µl	RT	1 hr
	3x washing with washing buffer			
3- ELISA	Diluted plasma	100µl	RT	2hrs
	6x washing			
	DENVs antigen	100µl	4°C	16-20 hrs
	6x washing with washing buffer			
	DENVs mAb-E	100µl	RT	1hr
	6x washing with washing buffer			
	Conjugate solution	100µl	RT/dark	1hr
	6x washing with washing buffer			
	Substrate solution	100µl	RT/dark	30 min
	Stopping solution	50µl		
	Reading absorbent at 490 nm			

Table 2.8 IgM/IgG capture ELISA procedure. RT: Room temperature

d. Calculation and interpretation of results

Negative control value (OD_{NC}) was defined by mean OD value of 3 negative controls minus mean OD of 3 background wells (OD_B). The assay cut-off (OD_{CO}) was defined as 5x OD_{NC}. The sample ratio (R_S) was calculated by taking the sample OD (OD_S) minus mean background (OD_B) and dividing by the assay cut-off [I.e.: $R_S = (OD_S - OD_B)/ OD_{CO}$]. The interpretation of results was as follows: - If $R_S < 0.8$, result was interpreted as negative

- If $R_S > 1.2$, result was interpreted as positive

- If R_S from 0.8 – 1.2, result was interpreted as equivocal

2.2.2.2. NS1 antigen-capture ELISA

a. Assay procedure

The Platelia Dengue NS1 Ag kit is a one step sandwich – format microplate enzyme immunoassay for the qualitative or semi-quantitative detection of DENV-NS1 antigen in

human plasma or serum. Tests were carried out according to the manufacturer's instructions. Briefly, 50 µl of plasma or control sera [including cut-off (in duplicates), one negative and one positive control sera] were incubated directly and simultaneously with 50 µl of diluent and 100 µl of diluted conjugate at 37°C for 90 min in microplate wells. The plate was washed six times with 1x washing solution (TRIS-NaCl – pH 7.4, 1% Tween 20), then immune-complexes were detected by using a color development reaction [160µl, Mix of TMB Substrate Solution (citric acid and sodium acetate pH 4.0, H_2O_2 - 0.015%, and DMSO - 4%), and TMB Chromogen (0.25% of 3,3',5,5' tetramethylbenzidine)]. After 30 mins of incubation in the dark at RT, the enzymatic reaction was stopped by 100 µl of 1N sulfuric acid solution. The OD was read at dual 450/620 nm. The presence of NS1 antigen was based on comparison of the OD of the sample with the cut-off of control. All samples were tested in a single well.

b. Calculation and interpretation of results

The sample ratio (R_S) was expressed by the following formula: $R_S = OD_S/OD_{CO}$, where

- OD_S: Optical density (OD) obtained on the sample
- OD_{CO}: Cut-off value corresponding to the mean OD values of the duplicate control wells, and which must be >0.2.

Result interpretation:

* If sample ratio was <0.5: sample was non-reactive for dengue NS1 antigen, and interpreted as negative
* If sample ratio ranged between 0.5 and 1.0: sample was considered equivocal for dengue NS1 antigen, and interpreted as equivocal.
* If sample ratio was >1.0: sample was reactive for dengue NS1 antigen, and interpreted as positive.

2.2.2.3. Dengue NS1 Lateral Flow Rapid Test (NS1-LFRT) (Bio-Rad Laboratories)

The NS1-LFRT is an individual test for qualitative detection of NS1 antigen in human plasma

or serum, marketed by Bio-Rad Laboratories as Dengue NS1 Ag STRIP. The assay is based on the principle of lateral flow immunochromatography. Tests were performed according to the manufacture's instructions. Briefly, 50 µl plasma was diluted in 1 drop of migration buffer (dropper and migration buffer are provided in the test kit) in the test tube. The NS1 strip was vertically placed in the test tube and the result was read after 15 min. Tests were interpreted as positive if the Test Line and Control Line appeared clearly. If the Test Line appeared in faint colour, the strip was read after a further 15 min. The test was considered positive if the Test Line appeared clearly at this time. Tests were considered valid if the Control Line appeared clearly, otherwise it must be repeated.

2.3. Result analyses

2.3.1. Sensitivity, Specificity, Positive predictive value (PPV) and Negative predictive value (NPV) calculation

Sensitivity was calculated by: TP/TP+FN (TP: True positive, FN: False negative)

Specificity was calculated by: TN/TN+FP (TN: True negative, FP: False positive)

NPV was calculated by: NPV= TN/TN+FN

PPV was calculated by: PPV= TP/TP+FP

2.3.2. Sequence analysis

2.3.2.1. Building maximum likelihood (ML) phylogenetic tree

In collaboration with Dr Eddie Holmes (Penn State University - State College-Pennsylvania, USA), Maximum Likelihood (ML) phylogenetic trees for DENV complete CDS were inferred for each of the serotype data sets using the PAUP* package (PAUP* version 4.0b10, Swofford 2003). Aligned sequence data set in nexus format was run in PAUP to calculate likelihoods using 56 different models of evolution. In each case, the best-fit model of nucleotide substitution was identified by MODELTEST [142] as the general time-reversible (REV or GTR) model. In all cases, (GTR+I+Γ) model was determined. GTR indicates unequal base

frequencies ($\pi A \neq \pi T \neq \pi C \neq \pi G$) and all 6 substitution types occurring at different rates (A-G, C-T, A-C, A-T, G-T, and G-C). I is the proportion of invariant sites and Γ is the gamma distribution indicating mutation rates are different among-sites. A bootstrap re-sampling process (1,000 replications) using the neighbor-joining method was used to assess the robustness of individual nodes on the phylogeny. All reference sequence data used in this study are available from GenBank (shown in List 1, Appendix 2). All complete sequences were manually aligned using the Se-Al program (http://tree.bio.ed.ac.uk/software/seal/). ML tree for 266 DENV-2 E sequences was performed at the phylogeny section, OUCRU/VN using the PAUP* package also. Neighbour joining (NJ) phylogenetic trees were inferred for DENV-2 E/NS1 and NS5 and DENV-3 E sequences using *MEGA* version 4 (Tamura, Dudley, Nei, and Kumar 2007).

2.3.2.2. Estimate of *dN/dS* at every codon site

To understand what codon sites are under positive or negative selection, selection pressures at every codon site across genes were measured as the ratio of nonsynonymous (*dN*) to synonymous substitutions (*dS*) expressed as *dN/dS* using Fixed Effects Likelihood (FEL). The FEL method is a maximum-likelihood method available at Datamonkey Web-server, HYPHY package (http://www.datamonkey.org/) [143]. The procedure includes 3 phases. In phase 1, nucleotide model from the time-reversible class (GTR or REV) that fits the input aligment data set and NJ tree will be determined. The "best-fitting" model can be determined automatically or by a model selection procedure. In phase 2, codon model was obtained by crossing MG94 and the nucleotide model from phase 1 (MG94xREV) and global *dN/dS* (single *dN/dS* for the entire tree) was estimated. Phase 3 will estimate site-by-site *dN/dS* using parameters from phase 1 and phase 2. In all cases, site with $dN/dS > 1$ with a significance set at $P<0.05$ was declared to be under positive selection.

2.3.2.3. Sequence divergence analysis

Sequence divergences in nucleotide and amino acid sequences were calculated as overall mean p-distance (proportion of nucleotide difference amongst compared sequences) using *MEGA* version 4 [123, 136].

2.3.3. Statistical analysis

Indices of sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) with 95% confidence intervals (CIs) were assessed for Platelia-NS1 and NS1-LFRT DIAGNOSTIC TESTS. All statistical analysis was performed by using Intercooled Stata 9.2 (Stata, College station, TX, USA). Significance was assigned at $P<0.05$ for all parameters. The distribution of categorical variables was analysed using the Fisher's exact test/Pearson chi square test to compare proportions between/amongst groups. The Mann-Whitney U test/Kruskal-Wallis was used for comparing continuous, non-parametric variables between groups (e.g. viraemia level, day of illness).

Chapter 3

3. Dengue virus evolution over the last ten years in Southern Vietnam

3.1. Introduction

DENV, like other RNA viruses, lack efficient proofreading or mismatch repair system and therefore exhibit a degree of genetic diversity within DENV serotypes. Differences in viral genotype can affect viral virulence and this can be reflected by disease phenotype or by fitness in the mosquito vector or can also impact on susceptibility to neutralization by pre-existing anti-DENV antibodies. For example, replacement of DENV-2 American viruses with Asian viruses (Am/As genotype) in the Americas is associated with an increased prevalence of DHF [54]. *In vitro*, DENV-2 Asian viruses have greater replicative potential in *Aedes* mosquitoes and human dendritic cells than American lineage viruses [61]. Furthermore, DENV-2 American viruses are neutralized by anti-DENV-1 antibody more effectively than Asian DENV-2 lineages [144]. The ongoing evolution of DENV has inevitably led to viruses with different biological characteristics, therefore a better understanding of DENV diversity and how this changes through time, is central to both vaccine design and drug development efforts. Continuous sampling and phylogenetic analyses of viruses prevalent in dengue endemic regions has revealed patterns of evolutionary processes which encompass continuous inter-serotype change and intra-serotype lineage (clade) replacement [122, 130] even including whole genotypes [133]. Viral lineage turnovers have been temporally associated with changes in serotype [118]. Many factors contribute to phylogenetic replacement; they may be stochastic factors such as the oscillation of mosquito populations, changes in the weather [130, 133], or under the impact of selection [129]. Changes in herd-immunity might also drive serotype lineage replacement. Preexisting antibody at a certain level may be favorable for specific strains of virus in sequential infection [128] and consequently these strains obtain advantages in replication in human and mosquito host [61], and therefore become more successful in transmission than others.

Although dengue is hyper-endemic in Vietnam with the most prevalent DENV serotype changing over time, identification of how serotype/genotype prevalence patterns change with time in this setting and how Vietnamese strains relate to their regional counterparts remains poorly understood. The aim of the work described in this chapter is to document changes in DENV serotypes and genotypes in and around HCMC over the last 10 years and their relationship to disease incidence. Rather than focusing on a single gene in isolation, we carried out an expansive genomic approach, utilizing the complete coding region sequences of 386 DENV from 3 prevalent viral serotypes sampled in and around HCMC. We were particularly interested in better understanding the population of DENV-2 viruses as these were the most prevalent when this study began. The selection pressure acting on DENV-1, -2, DENV-3, and sequence divergence were also investigated.

3.2. Methods

3.2.1. Study subjects

All viral sequences were directly obtained from plasma samples (n=150) or from viral cultures that had undergone $\leq$ 3 passages in vitro (n=44). Patients were recruited into prospective studies at HTD (2001-2008) and Children's Hospital #1 (CH#1), Children's Hospital #2 (CH#2) (2006-2007). Inclusion criteria were: **a)** children (<15 years) with suspected "mild" dengue (MD study at HTD) or **b)** children with severe disease (DHF grade III and IV, *DF* study at HTD) or **c)** children with suspected dengue with less than 7 days of illness (DENCO study at HTD, CH#1 and CH#2) and **d)** adult patients with suspected dengue and less than 3 days of fever (FR study at HTD). Exclusion criteria were patients with signs and symptoms of other acute infectious diseases or lack of written informed consent.

3.2.2. Strategy for sampling viral genomes

For better documenting changes in DENV, especially in DENV-2, from the time when it was less significant in causing clinical disease to when it became the most prevalent viral

serotype in patients, a strategy for recovery of DENV-2 sampled from 1999 to 2005 was made by retrospectively amplifying DENV-2 from dengue patient samples collected in HTD. Retrospective specimens were grouped by period of sampling for the purposes of showing the ratio of successful recovery (1999-2000, 2001-2002, 2003-2004 and 2005). Number of amplification attempts in each period was as follows: 100% in 1999-2000 (5/5), 34% in 2001-2002 (20/59), 46% in 2003-2004 (99/213) and 40% in 2005 (22/55). There is a bias in some of our sampling; for example no viruses from the 1999-2000 period (n=5) were successfully recovered or there was a low rate of success e.g. only 10% (6/59) in the 2001-2002 period. Overall (1999-2005), 21% (70/332) of DENV-2 viruses were retrospectively recovered. Details of virus sequence recovery rate are summarised in **Chart 1**.

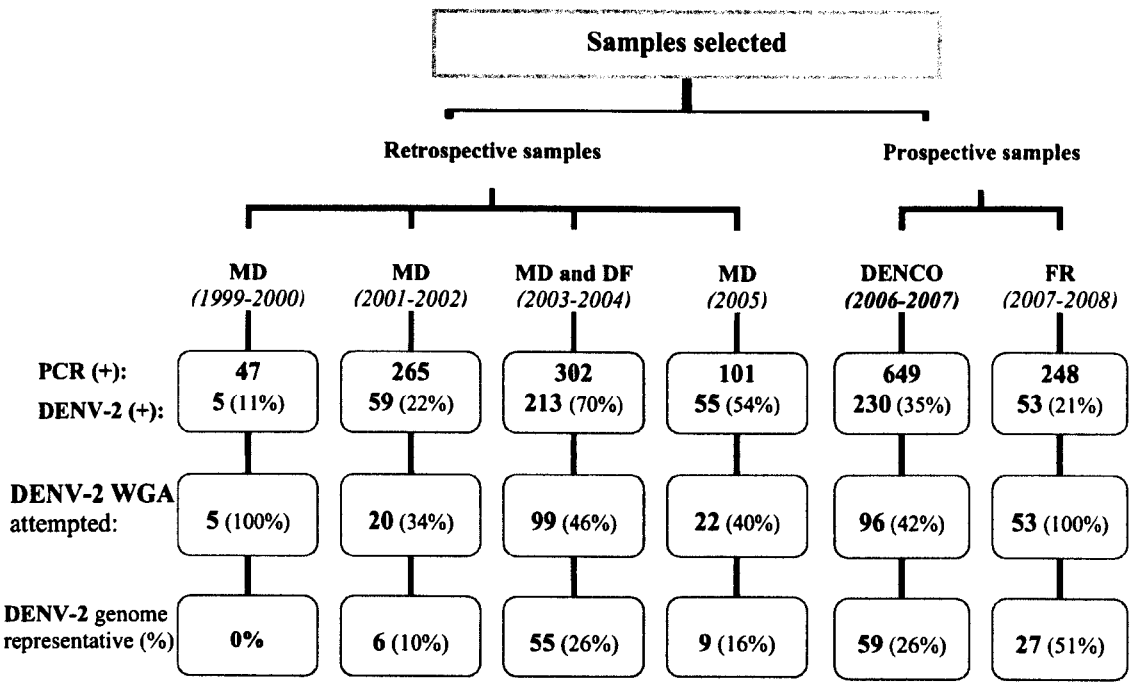


Chart 1. Strategy of sample selection for DENV-2 whole genome sequencing. Showing is sample selection strategy for whole genome sequencing of DENV-2 sampled between 1999 to 2008 and possible biases caused by failure (1999-2000 period, n=0) or low success rate in sample recovery (2001-2002; 10%, 6/59). Shown in the first box line is total PCR (+) and DENV-2 detected in each period. Numbers of DENV-2 attempted for whole genome amplification and sequencing is shown in the second box line. % DENV-2 representative in each period is shown in the third box line at the bottom.

3.2.3. Whole genome amplification and sequencing strategies

Two approaches were employed for genomic sequencing. In the first approach, PCR amplification of the DENV-2 genome (n=54) from position 13 to 10,704 was performed in 3 overlapping amplimers (2.4kb, 4.5kb and 4.5kb). Details of oligonucleotide primers and amplification strategy were summarised in Chapter 2. Amplification procedures and conditions were performed as described in Chapter 2. An example of a DENV-2 whole genome amplified in 3 overlapping PCR amplimers is shown in **Fig.3.1**. These amplimers were then subjected to shotgun cloning in the *pUC19* vector (described in Chapter 2).

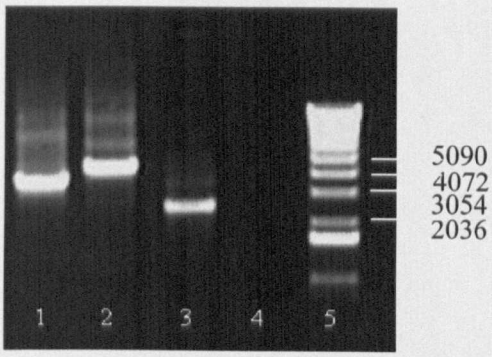


Fig.3.1. A representative of DENV2 genome amplification results in 3 overlapping fragments. Lane 1 is 4.179 kb long; lane 2 is 4.448 kb long; lane 3 is 2.349 kb long; lane 4 is negative control; lane 5 is 1kb ladder

In the second approach (n=332 viruses; 199 DENV-1, 102 DENV-2, 31 DENV-3), viral genomes were first amplified in 14 overlapping fragments as described in Chapter 2. Genomes were sequenced from position 1 to 10,735 (DENV-1), 10,723 (DENV-2) and 10,696 (DENV-3). An example of a DENV-2 whole genome amplified in 14 overlapping PCR amplimers is shown in **Fig.3.2**.

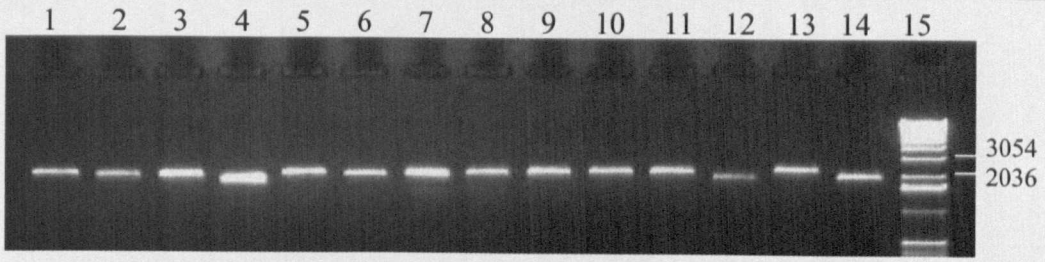


Fig.3.2. A representative result of DENV-2 WGA in 14 amplimers. The length of these amplimers ranges from 1901 bp (lane 4) to 2213 bp (lane 13). Lane 15 is 1kb ladder.

3.2.4. Additional strategy for partial coding sequencing in retrospective DENV-2 samples

Since the numbers of DENV-2 whole genome sequences between 2001-2005 and between 2006-2008 were rather small (n=70 and 86, respectively), further efforts to obtain sequence information on DENV-2 viruses were made to compensate for this limitation. To this end, partial sequence of DENV-2 viruses (177 nt, position 9,937-10,113; specific forward primer: 5'-ACAAGTCGAACAACCTGGTCCAT-3', specific reverse primer: 5'-GCCGCACCATTGGTCTTCTC-3') was obtained. PCR products were sent to Macrogen Korea for double strand sequencing. This strategy covered 335 DENV-2 viruses sampled from 2001 to 2008 (2001, n=2; 2002, n=10; 2003, n=75; 2004, n=51; 2005, n=29; 2006, n=89; 2007, n=66 and 2008, n=13).

3.2.5. Sequence analyses

Sequence alignments were manually constructed using Se-Al program (http://tree.bio.ed.ac.uk/software/seal/) for the complete coding regions for 199 Vietnamese DENV-1 sequences (10,176 nt), 156 Vietnamese DENV-2 (10,173 nt) and 31 Vietnamese DENV-3 (10,170 nt) data sets combined with genome sequences available in GenBank (352 DENV-1, including 176 Dong Thap strains; 368 DENV-2, including 38 Dong Thap strains; and 203 DENV-3). ML tree for Vietnamese DENV-2 E sequences available from GenBank was inferred using PAUP* under the assistance of the OUCRU phylogeny group. Sequence alignments and analyses for the partial coding regions of 335 Vietnamese DENV-2 generated in this study, Vietnamese DENV-2 and DENV-3 (E and E/NS1) sequences available from GenBank were constructed using *MEGA* (V4, Tamura, Dudley, Nei and Kumar- 2007). Methods for building ML phylogenetic trees were described in Chapter 2.

3.3. Results

3.3.1. Sample data

The first dataset from dengue patients admitted to the study hospitals between 2001 to 2008

comprised genome length sequences [nt 24-10715 in DENV-1; nt 32-10680 (the Sanger project) or nt 23-10700 (the Broad project) in DENV-2 and nt 22-10673 in DENV-3] from 199 DENV-1, 156 DENV-2 and 31 DENV-3. The patients from whom these viruses were sampled were resident in HCMC (n=292) or surrounding provinces (n=69). There were 25 patients where the address was unknown.

The second data set from dengue patients admitted to HTD between 2001 to 2008 comprised sequences of 78 nt ($NS5_{2419-2496}$) from 335 DENV-2. Of these, 244 patients lived in HCMC, 59 patients lived in neighboring provinces. The breakdown of sequences by time of sampling and method of sequencing is summarised in **Table 3.1.**

Sequence type / Spatial distribution	Complete CDS			Partial CDS	Total
	DENV-1 (2006-2008)	DENV-2 (2001-2008)	DENV-3 (2006-2008)	DENV-2 (2001-2008)	
HCMC	155	112	25	244	536
Other provinces	28	37	4	59	128
Unknown	16	7	2	32	57
Total	199	156	31	335	721

Table 3.1 Table of number of sequences derived from genome sequencing (complete coding sequence- CDS) and partial sequencing by serotype, time of sampling and geographic location of the patients' home address.

Shown in **Fig.3.3** are HCMC and 13 provinces from which viruses were sampled and sequenced. Number of viral genomes sequenced per year is shown in the table at the bottom.

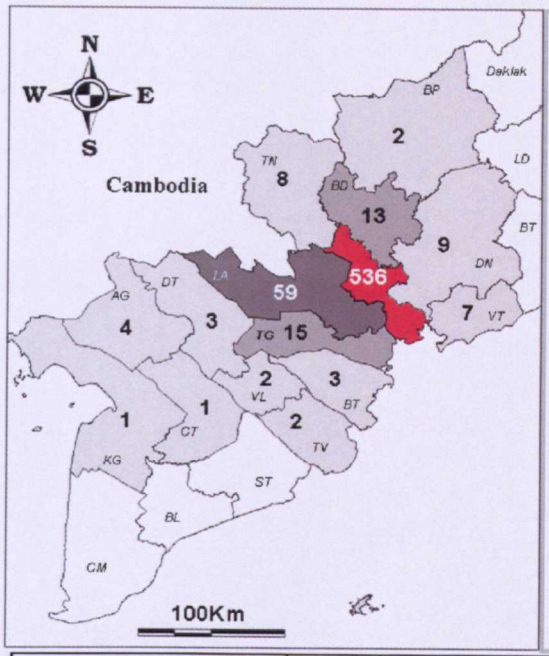


Fig.3.3. Map of patient distribution in southern Vietnam. Map shows provinces where dengue patients lived and from whom viruses were sampled. Number labelling is total patients sampled from that province. Different levels of shading reflect the intensity of sampling. Most patients were living in HCMC when they presented with dengue (74%, n=536/721). Patients from surrounding provinces account for 18%, (n=128/721). Patients with unknown address were 8% of total (n=57/721).

Year	2001	2002	2003	2004	2005	2006	2007	2008	Total
Number of whole genomes sequenced	3	3	35	25	9	138	100	73	386
Number of partial genomes sequenced	2	10	74	51	29	89	66	14	335

3.3.2. Serotype shift events in DENV population in Southern Vietnam

Four DENV serotypes co-circulate in Vietnam. In this study, we documented DENV serotype frequencies determined by TaqMan RT-PCR from 3,465 acute dengue plasma samples collected from dengue patients admitted to HTD between 1999 and 2008, CH#1 and CH#2 in 2006 and 2007, and collated them to determine whether the patterns of serotype abundance matched that seen in the Dengue Virus surveillance program conducted by the Pasteur Institute (HCMC) (**Fig.3.4**). The predominant dengue serotype varied with time. Before 2000, DENV-3 was dominant; in 2001, DENV-4 displaced DENV-3 and was the most prevalent until 2002. DENV-4 was subsequently replaced by DENV-2 from 2003 – 2005, followed by the emergence of DENV-1 since 2006 which has remained the most prevalent serotype until

2008 (referred to **Fig.1.3**). This current serotype shift from DENV-2 to DENV-1 was associated with substantially increased DENV incidence **(Fig.1.3 and Fig. 3.4)**. Shown in **Fig.3.5** are serotype shift events over the last 10 years deduced from patient samples admitted to HTD, CH#1 and CH#2. Generally, our results of serotype prevalence (spanning from 1999-2007) were in agreement with data courtesy of Pasteur Institute (**Fig.3.4**).

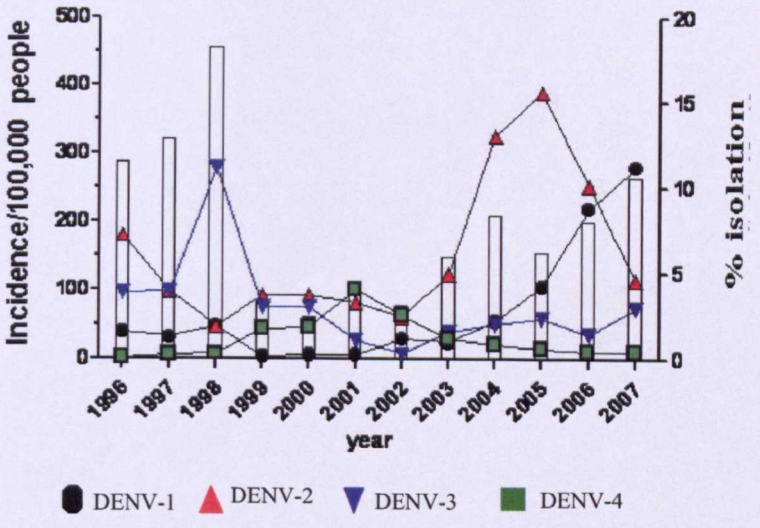


Fig.3.4. Disease incidence and DENV serotype prevalence in southern Vietnam from 1996 to 2007 reported by the Dengue Control Programme for the 20 provinces of Southern VN (Pasteur Institute-based).

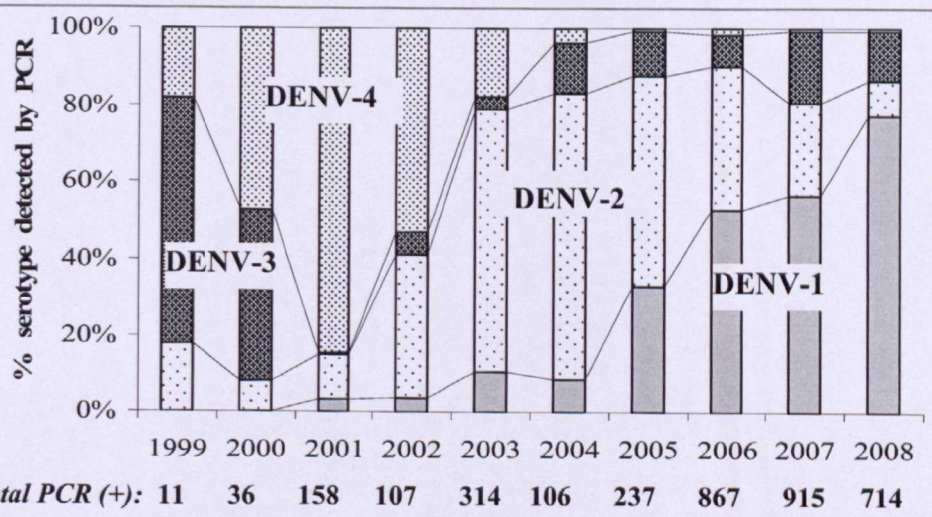


Fig.3.5. Pattern of DENV serotype abundance in patients admitted to HTD, CH #1 and CH #2 over the last 10 years. The predominant dengue serotype changed through time, before 2000: DENV-3; 2000-2002: DENV-4; 2003-2005: DENV-2; 2006 to up till now: DENV-1. Number of total RT-PCR positive in each year is shown under the graph (n=3,465). Serotype prevalence patterns from 1999-2007 match DENV surveillance data reported by the Dengue Control Program for the 20 provinces of Southern Vietnam (**Fig.3.4**).

3.3.3. Molecular epidemiology of DENV-2

3.3.3.1. Sampling of DENV-2

Of 491 complete and partial DENV-2 coding sequences derived from patients admitted to HCMC hospitals (as described in **Table 3.1**), 72% patients resided in HCMC (n=356), 20% patients came from 11 surrounding provinces (n=96) and 8% patients were of unknown address (n=39). Shown in **Fig.3.6** is a map showing the distribution of sampled patients in HCMC and other 11 provinces. The 156 DENV-2 complete genome sequences generated from this project have been logged into public databases (http://www.ncbi.nlm.nih.gov/sites/entrez) under the accession numbers shown in Appendix 3.

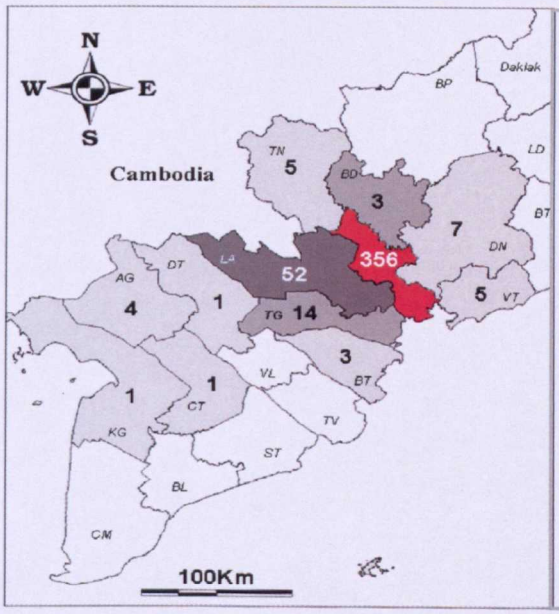


Fig.3.6. Map of southern Vietnam and the distribution of residential addresses of patients from whom DENV-2 viruses were sequenced. Each number indicates number of DENV-2 sampled in the province/city. Patients derived from HCMC accounted for 72 % (356/491). Ninety-six patients came from 11 other surrounding provinces (20%). Province name is displayed in abbreviations and italics

3.3.3.2. Phylogenetic analyses of DENV-2

The ML tree of 156 Vietnamese DENV-2 complete coding sequences (10,173 nt) generated in this study plus 368 complete coding sequences available from GenBank (Accession numbers shown in List 1, Appendix 2) is shown in **Fig.3.7**. The tree shows 2 distinct phylogenetic relationships: the sylvatic strains from Asia and Africa (monkey or sylvatic mosquito or human sera) and the human-related lineages defined as 5 genotypes (American, Asian 1, Asian

2, Am/As and Cosmopolitan genotypes). The Vietnamese strains fell into 3 phylogenetically distinct groups referred to as Asian 1, Am/As, and Cosmopolitan genotypes. The Asian 1 genotype, of which 103 sequences from this study belonged, was first sampled in Vietnam in 2003. ML tree of these Vietnamese strains revealed that this genotype was genetically close to Thai strains (e.g. ThD2_0078_01, ThD2_0017_98). The Vietnamese Am/As (n=51) genotype clustered closely with Thai (ThD2_0284_90) and Chinese (China 04) strains. This lineage split into 2 distinct groups: group A (n=31) consisted of virus sequences sampled between 2001-2005, whilst group B (n=20) comprised sequences sampled between 2001 and 2006. When taken together, there was an obvious temporal structure in the tree; Am/As genotype was only sampled between 2001-2006 period and not thereafter whilst the Asian 1 genotype (clustered closely to Thai strains) was first sampled only after 2002. Only 2 samples fell into the Cosmopolitan genotype (2%). The Vietnamese Cosmopolitan strains were phylogenetically related to Indonesian (BA05i, 2004) and Singaporean (D2/SG/05K4155DK1/2005) strains.

To understand better whether DENV-2 Asian 1 genotype viruses circulated in and around HCMC prior to 2003, we analyzed DENV-2 sequences available from GenBank and sampled from HCMC and other provinces [n=59, 38 E gene sequences (1,485nt) (1995, n=1; 1996, n=10; 1997, n=9; 1998, n=8; 2002, n=1; 2003, n=1; 2004, n=2; 2005, n=3; 2006, n=1; 2007, n=2) and 21 E/NS1 sequences (240nt) (1987, n=2; 1995, n=2; and 1996, n=17). GenBank accession numbers are shown in List 2, Appendix 2]. The ML phylogenetic tree for E gene sequences (**Fig.3.8**) and NJ tree for E/NS1 sequences (**Fig.3.9** in Appendix 1) showed that all viruses sampled prior to 2003 belonged to the Am/As lineage (n=50), whereas viruses sampled from 2003 onwards fell in Am/As (n=4) or Asian 1 (n=4) or Cosmopolitan genotypes (n=1). As a further effort to understand whether Asian 1 genotype viruses circulated before 2003 and whether Am/As viruses circulated after 2006, sequence length of 78 nt ($NS5_{2419-2496}$) of the

DENV-2 genome from 335 patients enrolled into one of the previously described prospective studies at HTD between 2001 and 2008 was analysed using NJ method. Phylogenetic analyses of these short sequences clearly identified them as Am/As or Asian 1 or Cosmopolitan lineages. NJ tree of these sequences is shown in **Fig.3.10** in Appendix 1.

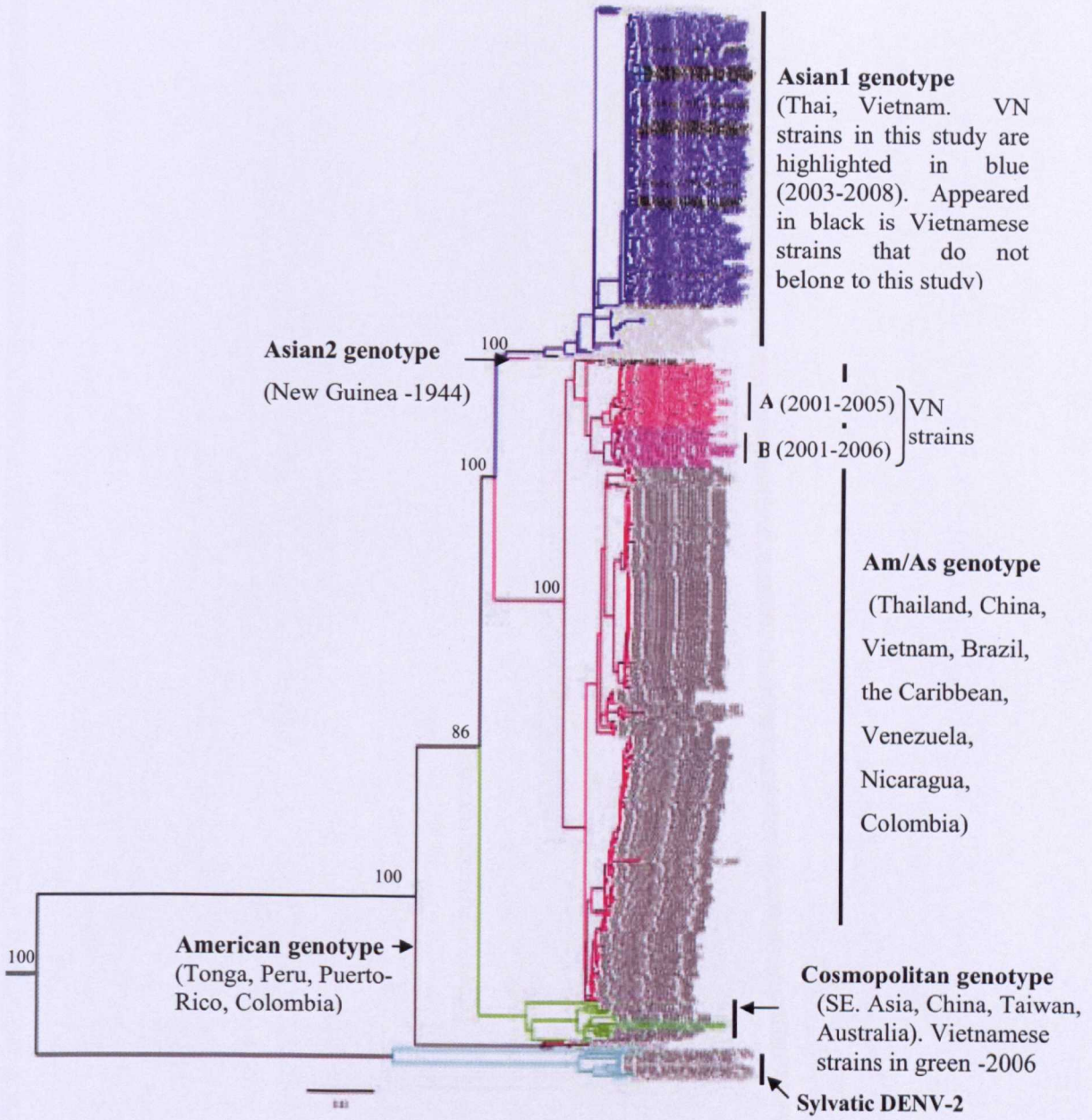


Fig.3.7. ML tree of complete coding sequences from 156 Vietnamese DENV-2 in this study and 368 DENV-2 strains from GenBank (grey). Tree contains 6 DENV-2 genotypes labeled by genotype name. Branches with different colour indicate different genotypes. Geographical distribution of these viruses in each genotype is also noted. Viruses are listed by strain name. All Vietnamese Asian 1 viruses are in blue (2003-2008). Vietnamese Am/As genotype (in red) divides in 2 distinct clades, 2001-2005 (A) and 2001-2006 (B). Vietnamese Cosmopolitan genotype appears in green (n=2). Tree is rooted with sylvatic DENV-2. Viruses listed in black are Vietnamese strains that do not belong to this study.

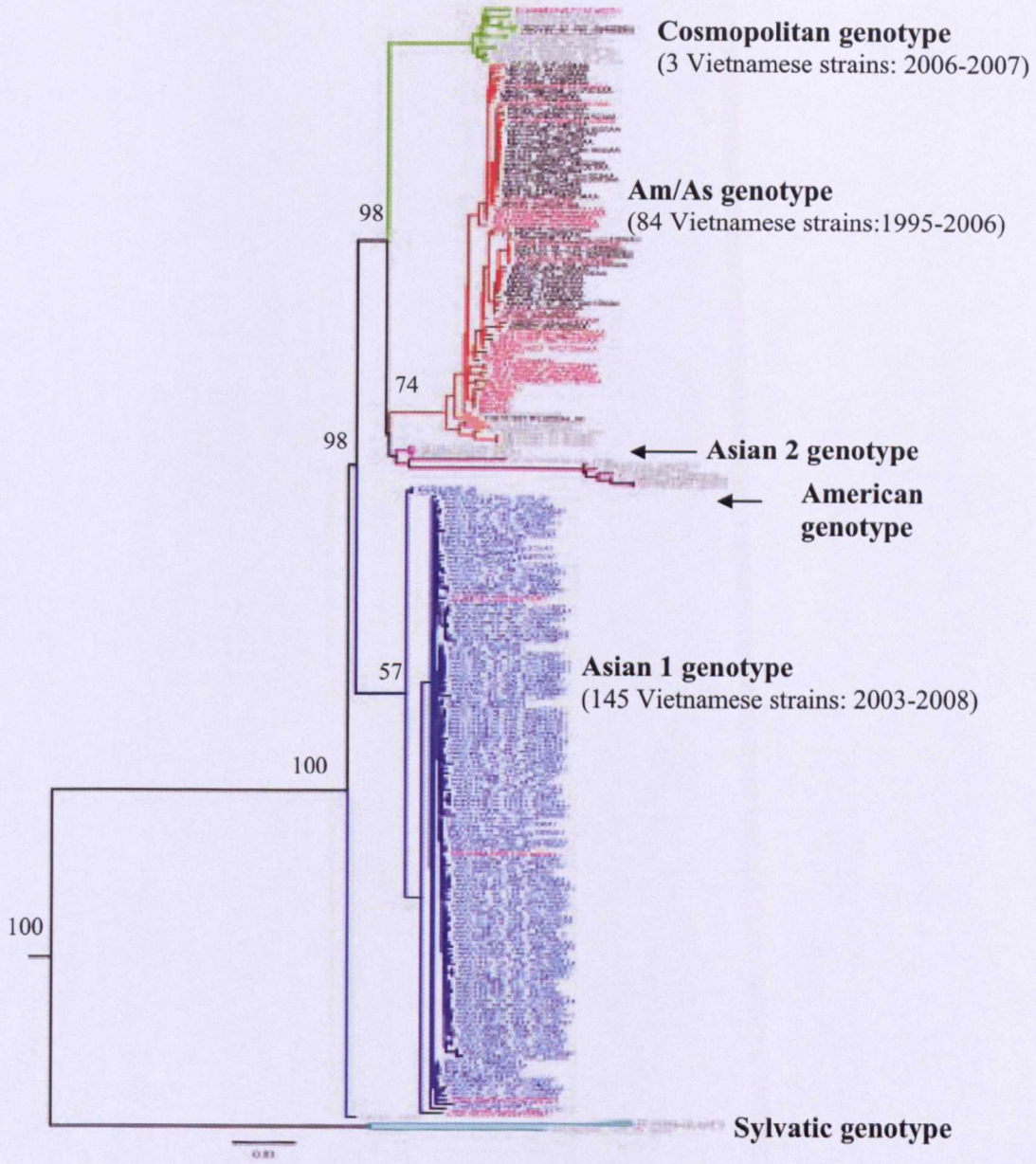
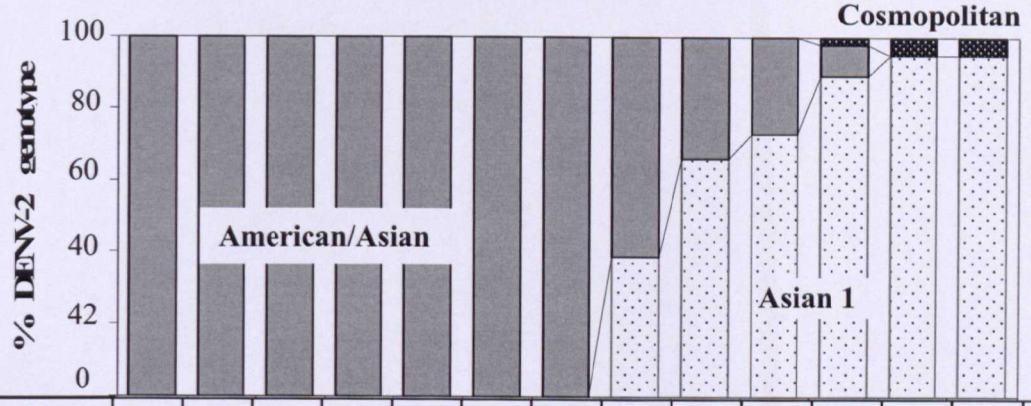


Fig.3.8. ML phylogenetic tree of 72 E gene sequences (1,485 nt) available from GenBank. Thirty-eight sequences were Vietnamese strains sequenced by other investigators highlighted in red, 194 Vietnamese strains were generated in this study (highlighted in black or blue). Sequences from other countries are highlighted in grey. All Vietnamese E sequences available from GenBank collected prior to 2004 fell in Am/As genotype (n=31), sequences collected from 2004 onwards were defined as Am/As (n= 2) or Asian 1 (n=4) or Cosmopolitan (n=1) genotypes. Bootstrap values are shown in main nodes.

To summarise the temporal trend in lineage replacement, the relative prevalence of each lineage was plotted against time in years for the period in which sampling was performed (**Fig.3.11**). Collectively, on the basis of complete and partial sequences from DENV-2 spanning more than 10 years and 550 patients, the results suggest the Asian 1 lineage was probably an introduction that arrived into southern Vietnam between 2002 and 2003 (**Fig.3.11**). This introduction was associated with increased DENV-2 prevalence (**Fig.3.5**).



Year	1987	1995	1996	1997	1998	2001	2002	2003	2004	2005	2006	2007	2008	Total
Partial CDS[a]	2	3	27	9	8		1	1	2	3	1	2		59
Partial CDS[b]						2	10	75	51	29	89	66	13	335
Complete CDS[c]						3	3	30	25	9	59	20	7	156
Total analyses	2	3	27	9	8	5	14	106	78	41	149	88	20	550

[a]: Sequences available from GenBank [b]: Sequences obtained from this study
[c]: Complete CDS generated from this study

Fig.3.11 DENV-2 genotypes detected in southern Vietnam and DENV-2 genotype replacement between 1987 and 2008. Data were integrated by complete CDS (n=156) and partial CDS (n=394 including 59 sequences available from GenBank) from 1987 to 2008 in and around HCMC. Number of complete CDS and partial CDS per year is shown in the table under the graph.

3.3.3.3. DENV-2 lineage replacement associated with increased DENV-2 incidence

The appearance of DENV-2 Asian 1 lineage viruses was associated with increased DENV-2 prevalence and disease incidence relative to previous years (**Fig.3.12**). For example, DENV-2

incidence in 2003 (84 cases/100,000 people) was 185% higher relative to 2002 (29 cases/100,000 people) and DENV-2 incidence in 2004 (150 cases/100,000 people) was 79% higher compared to 2003.

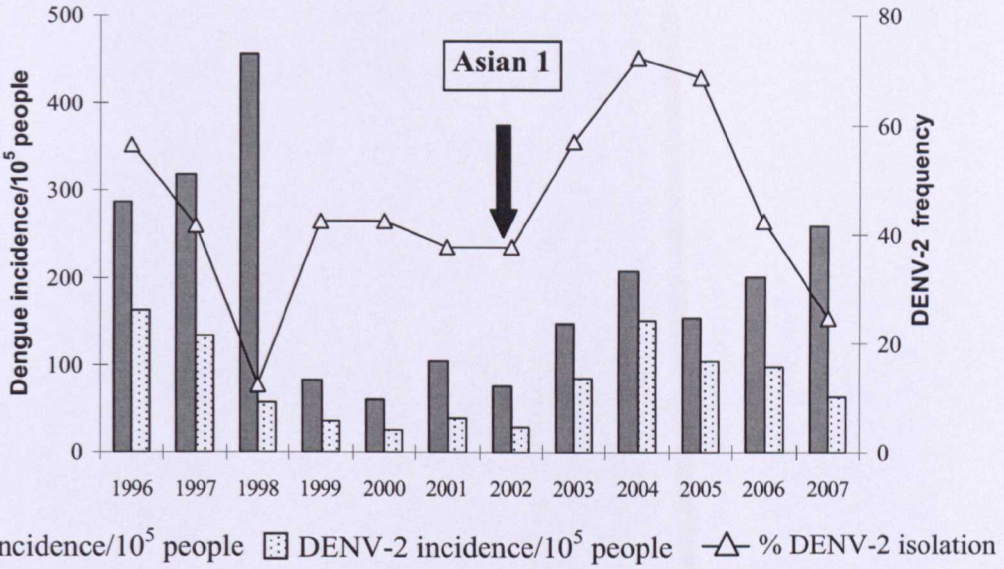


Fig.3.12. The association between DENV-2 Asian 1 introduction and increased DENV-2 incidence. Chart showing the appearance of DENV-2 Asian 1 genotype (2003) is associated with increased disease incidence and DENV-2 incidence (2003-2004). Black bars indicate total disease incidence, dot bars indicate DENV-2 incidence corresponding to numbers in left Y axis.

3.3.3.4. Selection pressure on DENV-2

To determine the extent of the selection pressure acting on DENV-2, an analysis of selection pressures on individual genes amongst lineages was conducted on the data set of genome sequences (CDS only) using Fixed Effects Likelihood (FEL) methods (HYPHY package available at http://www.datamonkey.org/). Selection pressure at nucleotide site was measured by comparing the ratio of non-synonymous (*dN*) and synonymous (*dS*) substitutions per site (*dN/dS*). Sites where $dN/dS > 1$ ($dN > dS$) is evidence for positive selection (the process that fixes non-synonymous mutations that improve an individual's fitness). Sites or where $dN/dS < 1$ is evidence for purifying (negative) selection (purifying selection decreases the prevalence of nonsynonymous mutations that diminish an individuals fitness). Mean *dN/dS* ratios across

genes were very low (ranging from 0.028 to 0.136, **Table 3.2**). The Capsid exhibited the highest level of non-synonymous variation (*dN/dS* = 0.136), implying that this gene was least impacted by purifying selection. Generally, the major evolutionary theme was that of purifying selection. However, 2 positively selected sites were detected in the NS5 gene at position 634 and 644 ($NS5_{634}$, $NS5_{644}$, genome position 3125, 3135; **Table 3.2**). *dN/dS* at these sites were 2.0 and 4.8, respectively. Selection pressure amongst all genes and positively selected sites in this DENV-2 dataset are summarised in **Table 3.2**.

Gene	Length (No of aa)	Global *dN/dS*	Positively selected site	*dN/dS* at selected site	P value for site
Capsid	114	0.136			
prM	166	0.063			
E	495	0.041			
NS1	352	0.082			
NS2A	218	0.098			
NS2B	130	0.028			
NS3	618	0.041			
NS4A	150	0.031			
NS4B	248	0.039			
NS5	900	0.059	634	2.0	0.02
			644	4.77	0.001

Table 3.2 Selection pressure in DENV-2 viruses (n=156) on a gene-by-gene basis. Mean *dN/dS* of all genes is <1, ranging from 0.028 (NS2B) to 0.136 (Capsid). Two positively selected sites were discovered in NS5 gene (position 634 and 644) with *dN/dS* of 2.0 and 4.8 respectively).

3.3.3.5. Sequence divergence between DENV-2 lineages

Sequences were grouped by genotype using MEGA version 4 to explore sequence divergence at the individual gene level. The overall mean sequence divergence (p-distance) between genotypes was 8.2% in nucleotide sequence and 2.6% in amino acid sequence (complete coding sequence) (**Table 3.3**). NS2A had the most divergent sequence with 10.8% difference

at the nucleotide level and 5.2% at the amino acid level (**Table 3.3**). Capsid was the second divergent sequence at amino acid level. At the nucleotide level, the second most divergent gene was NS4B with 9.6% difference, but at the amino acid level, this was the least divergent sequence (1.4%) (**Table 3.3**).

The relatively higher level of amino acid diversity in the NS2A and Capsid genes may suggest that these genes were less constrained by purifying selection pressure than other genes. This is compatible with the results shown previously in that these 2 genes exhibiting higher *dN/dS* ratio than the other genes (*dN/dS* = 0.136 and 0.096, Capsid and NS2A respectively). Conversely, the high sequence identity in amino acid sequence in NS4B sequences may be due to stronger functional constraints on this gene product. Sequence divergence at the nucleotide and amino acid level amongst DENV-2 genotypes is shown in **Table 3.3.**

Gene	Length (No of amino acid)	Mean divergence in nucleotide sequence (%)	Mean divergence in amino acid sequence (%)
Capsid	114	7.6	4.2
M	166	8.4	3.9
E	495	7.8	2.4
NS1	352	8.9	3.9
NS2A	218	**10.8**	**5.2**
NS2B	130	8.6	2.4
NS3	618	8	1.5
NS4A	150	9.4	1.8
NS4B	248	9.6	1.4
NS5	900	7.9	2.5
Complete CDS	3391	8.2	2.6

Table 3.3 Sequence divergence between Am/As and Asian 1 genotypes in individual genes and in complete coding sequence (CDS). Shown in bold is the gene with highest divergence in nucleotide and amino acid sequence (NS2A -10.5% and 5.2%, respectively).

3.3.3.6. Characteristics of the amino acid changes between the Am/As and Asian 1 lineages

ClustalW (MEGA version 4) was used to align amino acid sequences from Am/As (n=51) and Asian 1 (n=103) data sets. From a comparison of consensus amino acid sequences, a total of 41 amino acid changes were consistently detected between the 2 genotypes. Of them, 15 amino acid differences were associated with physicochemical properties. Eight of these changes resulted in a charge difference and 7 resulted in a side chain difference (polar vs. non-polar) (**Table 3.4**). Collectively, 5 of the 6 amino acid changes detected in structural genes were localized in E gene and 4 of 9 amino acid differences detected in nonstructural genes were in NS5.

Position in genome	Gene	Position in gene	Am/As (VN)	Asian I (VN)	Type of change
130	prM	16	R	I	(+), polar→ non polar
363	E	83	N	K	no charge→ (+)
483	E	203	D	N	(-) → no charge
506	E	226	T	K	no charge→ (+)
508	E	228	G	E	no charge→ (-)
626	E	346	H	Y	(+) → no charge
825	NS1	50	H	Q	(+) → no charge
855	NS1	80	S	A	no charge, polar → non-polar
1280	NS2A	153	S	L	no charge, polar → non-polar
1316	NS2A	189	A	T	non-polar → no charge, polar
2265	NS4B	22	E	Q	(-) → no charge
2626	NS5	135	T	I	no charge, polar →non-polar
2666	NS5	175	D	N	(+) → no charge
2687	NS5	196	T	A	no charge, polar → non-polar
3356	NS5	865	T	A	no charge, polar → non-polar

Table 3.4 Summary of consistent amino acid differences between Vietnamese Am/As and Asian1 genotypes. Eight substitutions related to charge change, 7 substitutions related to side chain change.

E (Glutamic acid), D (Aspartic acid): *Negative charge/polar amino acids.*

H (Histidine), K (Lysine), R (Arginine): *Positive charge/polar amino acids.*

T (Threonine), N (Asparagine), S (Serine), Q (Glutamine), Y (Tyrosine), G (Glycine): *Neutral amino acids with polar side chains.*

I (Isoleucine), L (Leucine), A (Alanine): *Amino acids with non-polar side chains.*

3.3.4. Molecular epidemiology of DENV-1

3.3.4.1. Sampling of DENV-1

One hundred ninety-nine DENV-1 genomes sampled from dengue patients admitted to hospitals in HCMC were investigated. Five viruses were sampled in 2003 and 194 were sampled between 2006 and 2008. Most patients resided in HCMC (155/199) and lived in 21 of 24 districts. Twenty-eight patients came from 10 provinces surrounding HCMC, and 16 patients had no recorded address. Shown in **Fig.3.13** are HCMC and the provinces from which DENV-1 infected patients were resident when they acquired dengue.

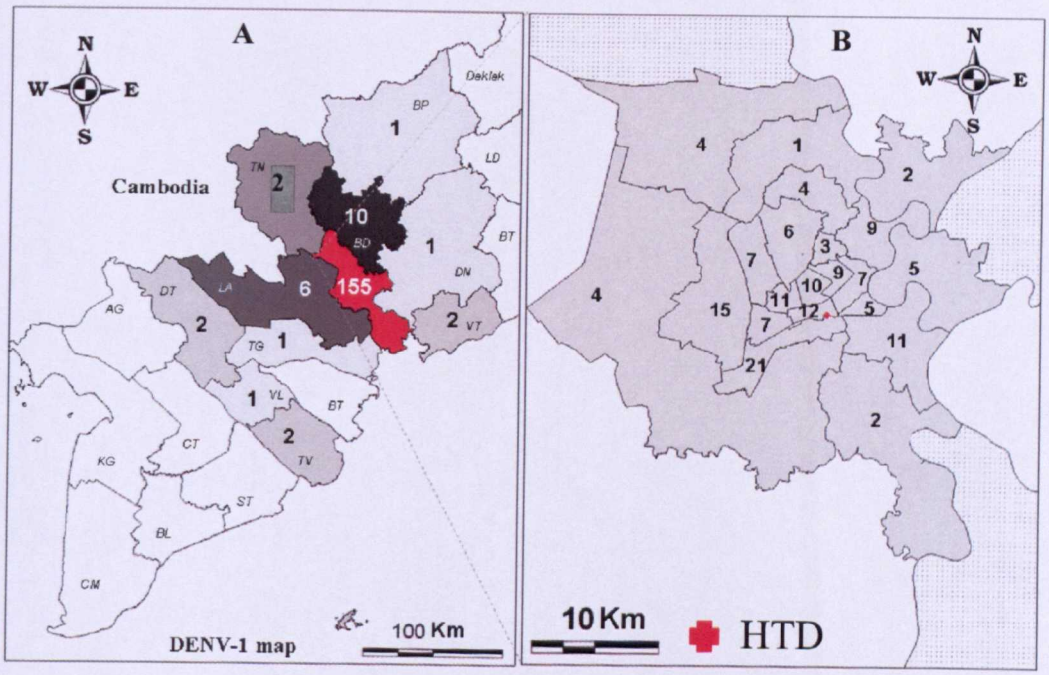


Fig.3.13. Distribution of DENV-1 infected patients in southern provinces/HCMC VN . Shown in **(A)** is the map showing the distribution of patients' addresses in HCMC (in red) and 10 provinces from whom DENV-1 viruses were sampled during admission to HCMC hospitals (n=199). Labeled with numbers indicates amount of patients in each city/province. Shown in (**B**) is a map showing the distribution of patient addresses in HCMC accounting for 77.9% (155/199) of total patients. (HTD: Hospital for Tropical Diseases)

3.3.4.2. Phylogenetic analyses of DENV-1

DENV-1 has become increasingly prevalent in and around HCMC since 2005 (referred to **Fig.3.5**). The ML tree of 199 Vietnamese DENV-1 complete coding sequences generated in this study, plus 352 DENV-1 sequences available from GenBank (Accession number shown in List 1, Appendix 2), including 176 DENV-1 Vietnamese sequences sampled in Dong Thap province (Accession number shown in Appendix 3) is shown in **Fig.3.14**. The tree shows 2 distinct phylogenetic relationships: the sylvatic strain (sentinel monkey isolate, Malaysia, 1972) and the human related strains consisting of genotype I (Asian countries such as Thailand, Myanmar, China, Singapore, Japan, Cambodia, Vietnam), genotype II (Indonesia, Japan, China, Pacific Ocean: Hawaii, French Polynesia) and genotype III (The Americas, Caribbean, Thailand, Singapore, Myanmar). All the Vietnamese DENV-1 viruses in this study fell into genotype I and were closely related to isolations in Thailand, Myanmar (D1.Myanmar.059/01), Cambodia (AF309641-1998), and Singapore (D1/SG/05K4621DK1/2005). This population exhibited sequence diversity with 6 clades, herein defined as groups 1-6 evident. The percentage representation of each DENV-1 phylogenetic group is illustrated in **Fig.3.15.** Interestingly, there were no strong spatial features in the ML tree, with strains sampled from patients living in and around HCMC being phylogenetically related to strains sampled from Dong Thap (**Fig.3.14**). Four of the five DENV-1 viruses sampled in 2003 clustered in group 1, one clustered in group 6. This suggested that DENV-1 genotype I, group I was the dominant group when this serotype was not the most prevalent in circulation.

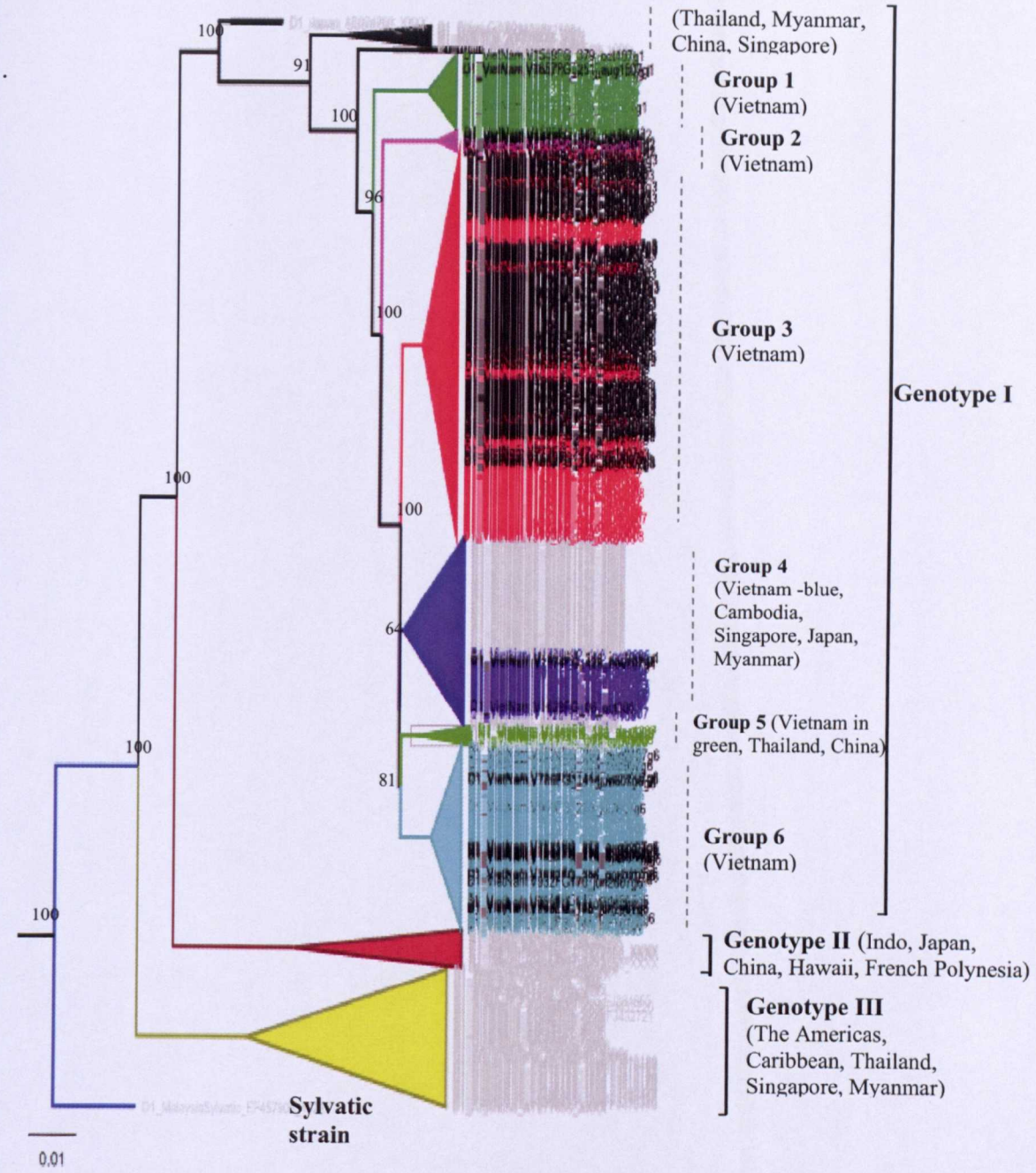


Fig.3.14. ML tree of complete coding sequences from 551 strains of DENV-1. Tree includes 199 Vietnamese strains in this study and 352 complete CDS available from GenBank (grey and black). Strains marked by color indicate Vietnamese viruses. Tree contains 3 human-related genotypes and 1 sylvatic. All Vietnamese strains fall into Genotype I and diverge in 6 groups labeled from 1 to 6. Group1 (n=34) appears in dark green, group 2 is in pink (n=2), group 3 is in red (n=67), group 4 is in blue (n=25), group 5 is in green (n=6) and group 6 is in turquoise (n=65). Tip labels in black are Vietnamese strains sampled in Dong Thap.

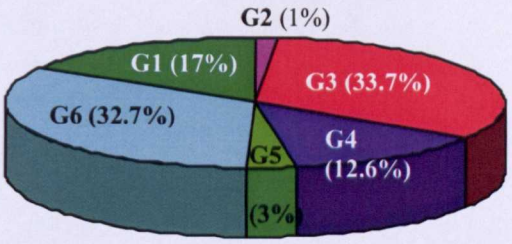


Fig.3.15. Percentage contribution of each clade to the total DENV-1 virus population sampled in HCMC (n=199).

3.3.4.3. Selection pressure on DENV-1

To help understand whether positive selection acted on the DENV-1 population, a selection pressure analysis was carried out in individual genes of all groups. The mean *dN/dS* ratios across genes were low, ranging from 0.046 (NS3) to 0.182 (NS2A) (**Table 3.5**). Here, NS2A and Capsid also exhibited higher *dN/dS* than other genes (0.182 and 0.177, respectively). We found no evidence for adaptive evolution in any sites, or genes in DENV-1. The overall theme was of purifying selection.

Gene	Capsid	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Length (No of aa)	114	166	495	352	218	130	619	150	249	899
Global *dN/dS*	0.177	0.110	0.075	0.075	0.182	0.069	0.046	0.059	0.046	0.064

Table 3.5 Selection pressure in Vietnamese DENV-1 sequences (n=199) on a gene-by-gene basis. Mean *dN/dS* of all genes is <1, ranging from 0.046 (NS3, NS4B) to 0.182 (NS2A).

3.3.4.4. Sequence divergence at nucleotide and amino acid level amongst DENV-1 groups

The overall mean sequence difference (complete CDS) amongst 6 lineages of DENV-1 genotype I was 2.02% in nucleotide sequence and 0.66% in amino acid sequence. NS2A was the most divergent sequence with 2.6% difference at the nucleotide level and 1.2% at the amino acid level. The least divergent in nucleotide sequence was Capsid (1.29%). At the amino acid level, the least divergent sequence was NS4B (0.29%). Collectively, at the

nucleotide level, some of the non-structural genes (NS2A, NS1 and NS5) exhibited higher level of divergence than structural genes (Capsid, prM and E gene). Similar to what was observed in Vietnamese DENV-2 sequences, NS2A also exhibited the highest sequence divergence at the amino acid level (1.2%). Sequence divergence in individual genes for DENV-1 is shown in **Table 3.6.**

Gene	Length (No of amino acid)	Mean divergence in nucleotide sequence (%)	Mean divergence in amino acid sequence (%)
Capsid	114	1.29	0.76
prM	166	2.02	0.96
E	495	2.07	0.65
NS1	352	2.22	0.89
NS2A	218	**2.60**	**1.20**
NS2B	130	1.81	0.43
NS3	619	1.94	0.29
NS4A	150	1.86	0.19
NS4B	249	1.49	0.29
NS5	899	2.13	0.84
Complete CDS	3392	2.02	0.66

Table 3.6. Sequence divergence in DENV-1 viruses sampled in HCMC at individual gene level and complete CDS. Shown in bold is the highest sequence divergence at nt and amino acid level.

3.3.5. Sequence analysis of DENV-3

3.3.5.1. Sampling of DENV-3

DENV-3 was first detected in HCMC in 1987 [14]. It increased in prevalence in southern Vietnam in 1994 and was the serotype associated with the 1998 DHF epidemic where it accounted for 71% of the serotypes detected [14]. It gradually declined in prevalence in 1999. Following was a lull in DENV-3 endemic for many years. Its prevalence slightly increased in 2007 accounting for ~20% of all serotypes now. History of DENV-3 activity in southern Vietnam and its incidence between 1996 and 2007 is shown in **Fig.3.16**. Thirty-one DENV-3 sequences sampled between 2006 and 2008 were analyzed hereafter, of which 25 originated in

HCMC (**Fig.3.17**), 4 were from other provinces and 2 sequences were from patients of unknown address.

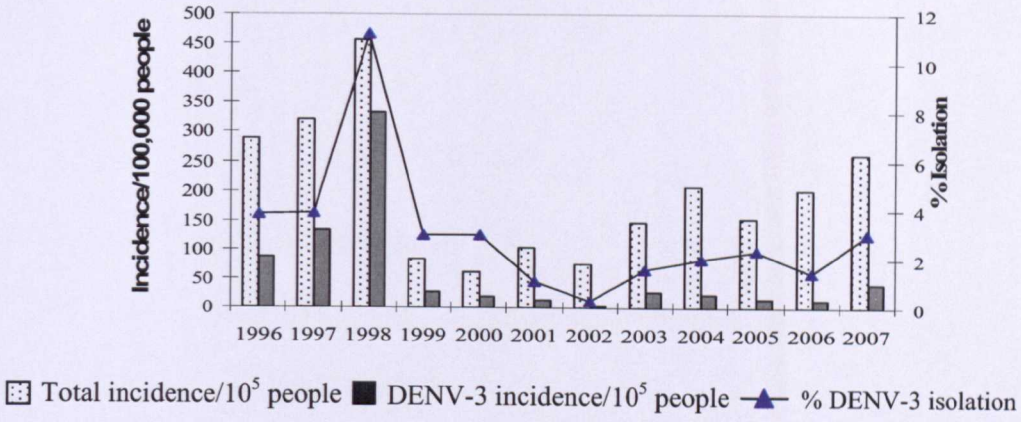


Fig.3.16. History of DENV-3 activity in southern Vietnam between 1996 - 2007. Increase in DENV-3 frequency in 1998 is associated with the incidence in1998-outbreak. After that DENV-3 exists as minor population (Source: Pasteur Institute-HCMC). DENV-3 frequency increased from 2007.

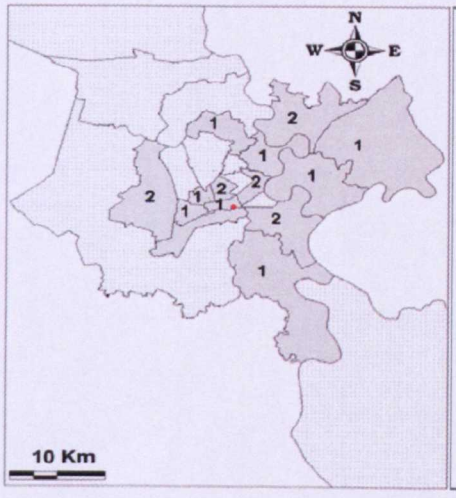


Fig.3.17. Distribution of DENV-3 infected patients in HCMC. Shown in the map is the distribution of patients addresses in HCMC from whom DENV-3 viruses were sampled during admission to HCMC hospitals (n=25). Labeled with numbers indicates amount of patients in each district.

3.3.5.2. Phylogenetic analyses of DENV-3

The ML tree of 31 Vietnamese DENV-3 complete CDS generated in this study (Accession numbers shown in Appendix 3) plus 203 sequences available from GenBank (Accession numbers shown in List 1, Appendix 2) is shown in **Fig.3.18**. The tree shows 3 distinct genotypes designated I, II and III. Genotype I is widely distributed in many regions across the

Americas, Asia, and Pacific region. Genotype II viruses were commonly sampled in Asian countries (e.g. Thailand, China, Singapore, Taiwan, Bangladesh). Genotype III segments in 2 distinct clades, with one clade common in some Asian countries (Singapore, Taiwan, Sri Lanka) and the other clade found in the Americas (Venezuela, Brazil), Caribbean (Puerto-Rico). All Vietnamese DENV-3 fell in genotype II and clustered together with Thai and Taiwan isolations. To understand better DENV-3 historical phylogeny in and around HCMC, we analyzed DENV-3 E and E/NS1 sequences (n=21) available from GenBank in 1996 (n=15), 1998 (n=1), 2003 (n=2), 2004 (n=1), 2005 (n=1) and 2006 (n=1) (GenBank accession numbers shown in List 2, Appendix 2). Phylogenetic analyses of these partial genomic sequences by NJ method indicated that they all belonged to Genotype II (**Fig.3.19**). Thus DENV-3 genotype II has circulated in and around HCMC since at least 1996.

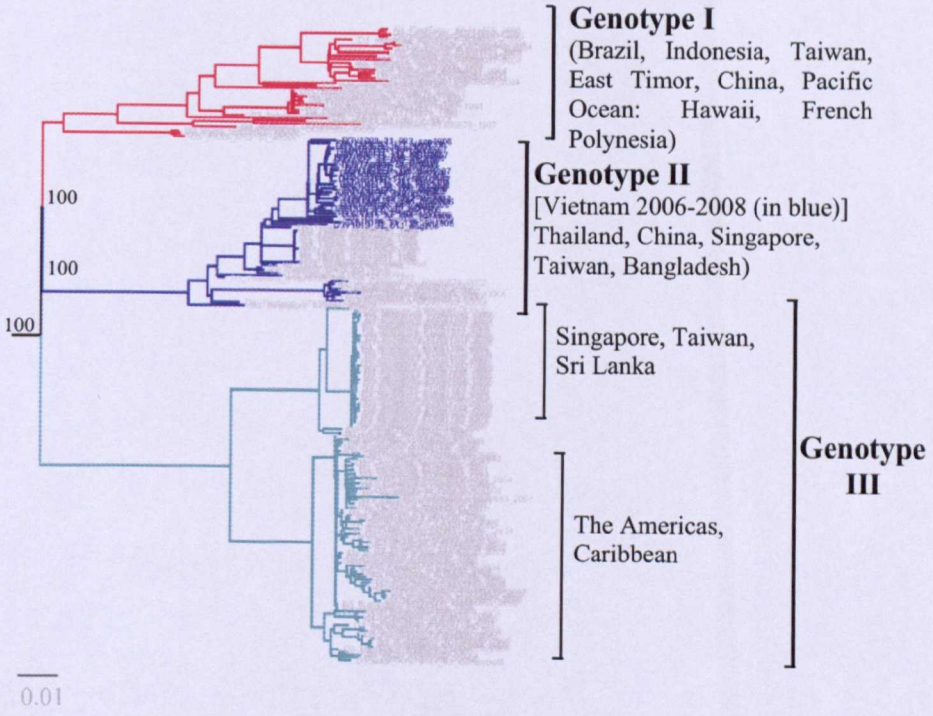


Fig.3.18. ML tree of complete CDS from 234 strains of DENV-3. Tree contains 31 Vietnamese DENV-3 sampled from 2006 to 2008 and 203 complete CDS available from GenBank (in grey). Branches with different colour indicate different genotypes. Tree contains 3 human related genotypes marked as I, II and III. Strains highlighted in blue indicate Vietnamese DENV-3 sequences generated in this study. All Vietnamese strains fall into Genotype II.

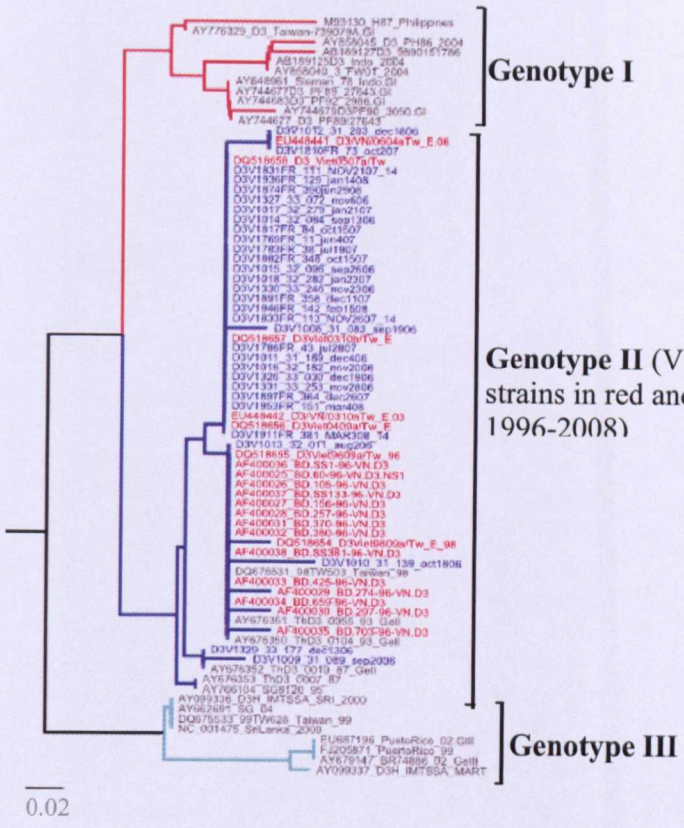


Fig.3.19. NJ tree of partial E gene from 77 strains of DENV-3. Thirty-one Vietnamese DENV-3 sequences generated in this study (blue) and 21 Vietnamese sequences available from GenBank (red) sampled between 1996 and 2008 fall in genotype II. Tree was built with 25 DENV-3 references from GenBank (grey).

3.3.5.3. Sequence divergence in nucleotide and amino acid sequence in DENV-3

Overall sequence difference (complete CDS) in 31 DENV-3 viruses was 0.70% in nucleotide sequence and 0.24% in amino acid sequence. At the nucleotide level, NS2A, once more, showed the highest sequence divergence (0.85%), though NS4A was the most divergent sequence (0.67%) at the amino acid level. The least divergent in nucleotide sequence was NS1 (0.62%) and NS4B (0.63%). At the amino acid level, the least sequence divergence was NS4B (0.08%). Collectively, sequence difference amongst Vietnamese DENV-3 viruses was very low at nucleotide as well as amino acid level. This could be due to sampling time of these

viruses was relative short (spanning from September-2006 to March-2008). Sequence divergence in nucleotide and amino acid sequence in 31 DENV-3 are shown in **Table 3.7.**

Gene	Length (No of amino acid)	Mean divergence in nucleotide sequence (%)	Mean divergence in amino acid sequence (%)
Capsid	114	0.65	0.34
M	166	0.64	0.19
E	493	0.69	0.28
NS1	352	0.62	0.41
NS2A	218	**0.85**	0.32
NS2B	130	0.74	0.20
NS3	619	0.68	0.14
NS4A	150	0.76	**0.67**
NS4B	248	0.63	0.08
NS5	900	0.70	0.16
Complete CDS	3390	0.70	0.24

Table 3.7 Sequence divergence in DENV-3 complete CDS and in individual genes based on nucleotide and amino acid sequence. Shown in bold indicates sequence with highest divergence in nucleotide sequence and amino acid sequence (NS2A: 0.85% and NS4A: 0.67%, respectively).

3.3.5.4. Selection pressure on DENV-3

The mean *dN/dS* ratios across genes were low, ranging from 0.027 (NS4B) to 0.161 (Capsid) (**Table 3.8**). Capsid and NS4A exhibited higher *dN/dS* than other genes (0.161 and 0.157, respectively). We found no evidence for adaptive evolution in all sites, or genes in DENV-3.

Gene	Capsid	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Length (No of aa)	114	166	493	352	218	130	619	150	248	900
Global *dN/dS*	0.161	0.065	0.093	0.112	0.085	0.081	0.039	0.157	0.027	0.033

Table 3.8 Selection pressure in Vietnamese DENV-3 sequences (n=31) on a gene-by-gene basis.

3.4. Discussion

To our knowledge, this is the largest data set of genome sequences assembled to examine the changing evolution of DENV in a defined geographic setting and time. We report here DENV serotype prevalence patterns over the last 10 years (1999-2008) and molecular epidemiology of the three DENV serotypes in and around HCMC. To provide a more comprehensive and expansive history of DENV-2 molecular epidemiology, particularly the emergence of the Asian 1 lineage, we integrated complete DENV-2 CDS with partial CDS generated in this study and partial Vietnamese CDS available from GenBank.

In DENV-2, a major finding was the replacement of DENV-2 Am/As lineage with the Asian 1 lineage during 2003-2006. This event was associated with recent increased in dengue incidence. These suggest the possibility of that DENV-2 Asian 1 lineage viruses have a fitness advantage over Am/As lineage viruses. In DENV-1, an interesting finding was the extent of within-genotype nucleotide sequence diversity present amongst viruses sampled in HCMC (and Dong Thap Province also). This suggests the virus population fills the "sequence space" available to it but that purifying selection removes less fit viruses in either the human or mosquito host. In DENV-3, the limited amount of sequence information available did not allow major conclusions beyond understanding how these Vietnamese viruses were related to others in the region. Consistent with previous findings [118, 132], the overall evolutionary pressure was purifying selection.

As shown previously, NS2A consistently contained the highest levels of nucleotide sequence divergence within each of DENV-1, DENV-2 and DENV-3 (2.58%, 10.8% and 0.85%, respectively). At the amino acid level, NS2A was also the most divergent sequence within DENV-1 and DENV-2 serotype (1.2% and 5.2%, respectively). NS2A also had higher ratio of *dN/dS* than other genes. Previous studies have reported similar results in DENV-1, DENV-2 and DENV-4 [118, 122, 136]. Taken together, the results imply that NS2A was less impacted

by purifying selection than other genes and that structural or functional flexibility is permitted for this gene. The basis for greater flexibility in the sequence of NS2A is unclear since little is known about the role of NS2A in the viral life cycle or in infected hosts. It has been suggested NS2A plays a vital role in virus assembly [145]. Thus, further research would be needed to investigate why this gene can tolerate greater sequence diversity than others.

Regular changes in serotype prevalence are well documented in many dengue endemic settings [9, 127, 146]. The serotype identification results from patients admitted to HTD between 2001 and 2007 are in line with data from the Pasteur Institute virus surveillance program that suggests DENV-1, -2 and -3 are on 6-8 year cycles in southern Vietnam. Presumably, development of widespread herd immunity to an individual serotype is an important event that mediates regular turnover of viral serotypes in endemic settings. Our knowledge of what serotypes have been prevalent in previous years allows us to make predictions about what might happen in the future. We would predict that DENV-3 will be the next serotype to become prevalent in southern Vietnam as the last time it circulated at high prevalence was in 1998 when it was associated with one of Vietnam's most severe DHF epidemics. The 10-year interval since DENV-3 has circulated widely means that a considerable number of children born in the last 10 years will have no or limited immunity to this serotype.

The most striking feature in the DENV-2 genome dataset was the DENV-2 lineage replacement event (**Fig.3.11**). The Am/As lineage appears to have been the only DENV-2 lineage present in southern Vietnam between 1987 (strain 24H, 57S, [119]) and 2003 when we first sampled viruses belong to the Asian 1 lineage. Our best estimate is that between 2002-2003, the Asian 1 lineage was introduced to Vietnam. On the basis of phylogenetic relationship the likely sources of this introduction were Thailand, Cambodia and China. Over the space of ~4 years, the Asian 1 lineage has completely displaced the Am/As lineage to such

an extent that the Am/As lineage has not been sampled from dengue patients at the HTD since 2006.

There are several potentially overlapping explanations for this replacement event. First, it could be due to the inherent fitness of Asian 1 lineage viruses for the mosquito vector such that they outcompete Am/As viruses by either **a)** having a lower infectious dose for *Ae. aegypti* mosquitoes, **b)** have a shorter extrinsic incubation period or **c)** replicate to higher titres in salivary glands and are therefore more infectious per biting episode. Perhaps less likely is that Asian 1 viruses confer a survival advantage to *Ae. aegypti* mosquitoes such that they persist in the environment for longer. The fitness advantage may also occur in the human whereby Asian 1 viruses might achieve higher or longer viraemias in vivo and thereby provide more opportunity for human to mosquito transmission. In any of these scenarios, the fitness advantage might be mediated solely by features inherent in the virus, e.g. a set of sequences in Asian 1 viruses that confer greater infectivity to *Ae. aegypti* mosquitoes, or possibly, the features of the virus and host in combination, e.g. a set of sequences in the E gene of Asian 1 viruses that allow a certain level of immune escape from neutralising antibody elicited by previous DENV-2 (Am/As) infection. DENV-2 Asian has been proven to be fitter than the American lineage [61]. From our epidemiological results and observed differences between Am/As and Asian 1, we assume that the Asian 1 genotype could have a greater potential fitness than the Am/As and has successfully replaced the Am/As lineage. However, this hypothesis has not yet been experimentally assessed in this study and needs to be examined further.

Of the 15 amino acids that differed between Asian 1 and Am/As and which were also different in their physicochemical properties, 4 changes occurred in the NS5 gene, 1 in NS4B, 2 in NS2A, 2 in NS1, 5 in E, and 1 in prM. Five of the amino acid differences were in the E gene which is exposed on the surface of the virion and associated with a number of biologic

activities including haemagglutination [102], receptor binding [103], low-pH mediated endosomal fusion [104]. In addition, the E protein is the major antigenic target of the humoral immune response and neutralising antibodies map to this protein [105]. Amino acid differences in E between the two 2 lineages could potentially result in altered binding of neutralising antibody; particularly antibody elicited by previous DENV-2 (Am/As) infections. These hypotheses will have to be confirmed through further experiments such as side-by-side comparisons of the ability of antibodies (mAb and polyclonal) to neutralize Asian 1 and Am/As lineage viruses.

An alternative explanation for DENV-2 lineage change is that this event simply resulted from stochastic process. Irrespective of the mechanism that drives this replacement, vaccine and drug development must take into account the increasing footprint of Asian 1 DENV-2 lineages as these viruses are now firmly rooted in Thailand [147], Cambodia and Vietnam.

Shortly after the DENV-2 Asian 1 lineage was first sampled, a change in serotype prevalence occurred, with DENV-1 becoming the dominant serotype. Whether the change in serotype prevalence was a consequence of DENV-2 lineage replacement remains unknown. There have been similar previous reports of lineage turnover at times of serotype replacement in Thailand [118]. The first time was in the early 1980s (around 1983). The predominant serotype changed from DENV-1 to DENV-4 and was associated with DENV-1 genotype III replacement. The second occasion was in the early 1990s (around 1994). DENV-1 clade "1980-1994" was replaced by clade "1990-2002", then DENV-1 decreased in prevalence and DENV-4 emerged as a dominant serotype. Similar events have been reported in Paraguay when introduction of a new DENV-2 clade was associated with a shift of dominant serotype from DENV-3 to DENV-2 in 2005 [127]. In the Philippines, DENV-2 Cosmopolitan genotype viruses have gradually replaced Asian 2 genotype viruses since 1999 and this occurrence has been associated with an increase in the number of severe cases [133].

To our knowledge, our sequence data on DENV-1 is the most comprehensive assessment of sequence diversity in a single serotype in a single setting ever performed. DENV-1 viruses fell into 6 distinct phylogenetic clades. The large genetic diversity in DENV-1 lineages could have resulted from the introduction of foreign clades from elsewhere. For example, DENV-1 group 4 viruses were related to Japanese, Chinese and Singaporean (2005) strains; DENV-1 group 5 viruses were closely related to Thai (2001) and Chinese (2001) strains. The diversity amongst the DENV viruses sampled here was primarily at the nucleotide level, with little variation present at the amino acid level. Once again this suggests purifying selection is a dominant feature of DENV-1 viral evolution in and around HCMC. The existence of such significant diversity in a single genotype of DENV-1 in a single geographic location underlines the ability of DENV to fill the allowable sequence space within the confines of purifying selection. It will be of great interest to see how the DENV-1 virus population changes over time, particularly to see if some virus lineages have a selective advantage over others when (inevitably) serotype turnover occurs in southern Vietnam.

Chapter 4

4. Dengue virus molecular epidemiology in an outbreak in Dong Thap Province in the Mekong Delta region of southern Vietnam

4.1. Introduction

Dengue was first recognized in southern Vietnam in 1960 and has since become a major public health problem. Since 1963, the incidence of DHF has steadily increased in Vietnam [14]. During the last 30 years, epidemic peaks of dengue occurred regularly at 3-4 year intervals (1975, 1978/79, 1983, 1987/88, 2001, 2005) (Source: Pasteur Institute HCMC: http://www.pasteur-hcm.org.vn/english/). In 1999, the National Dengue Control Program for southern Vietnam was established and based in the Pasteur Institute, HCMC. Dengue is recorded all year round with peak transmission between June and November during the rainy season.

Dong Thap Hospital (herein abbreviated to DTH) is a 750 bed general hospital in Cao Lanh City - capital town of Dong Thap, and is the designated provincial hospital for Dong Thap Province (162 km from south-west HCMC, **Fig.4.1**). Cao Lanh district is adjacent to Cao Lanh City. Most of the patients in this study resided in these 2 areas. OUCRU has been collaborating with DTH since 1993 and on dengue related activities since 2006.

Since 2000, the number of dengue cases has increased annually in Dong Thap (**Fig.4.2**. Source: Centre of Precautionary Measures against Epidemic - Dong Thap province). In 2007, a dengue outbreak occurred in Dong Thap resulting in 12,943 cases ($773/10^5$ population), of which there were 1,635 DSS cases and 9 deaths. (Source: Centre of Precautionary Measures against Epidemic - Dong Thap province). During this outbreak DTH and OUCRU enrolled suspected dengue patients into an ongoing prospective descriptive study examining early virological, immunological and host genetic risk factors for DSS and viral molecular epidemiology. Study enrolment took place in the outpatient clinic of the infectious diseases department. All study participants were admitted to DTH.

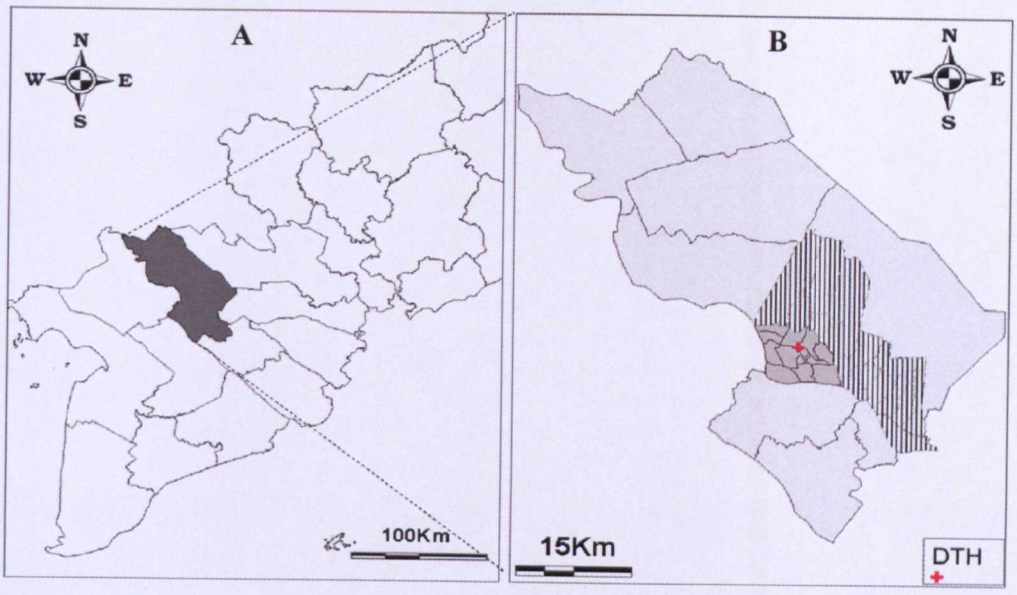


Fig.4.1. Maps of southern VN and Dong Thap province. (**A**) is southern Vietnam; the shaded area is Dong Thap province. Shown in (**B**) is Cao Lanh district (hachured) and Cao Lanh City (dark grey). DTH is located in Cao Lanh City (the red cross).

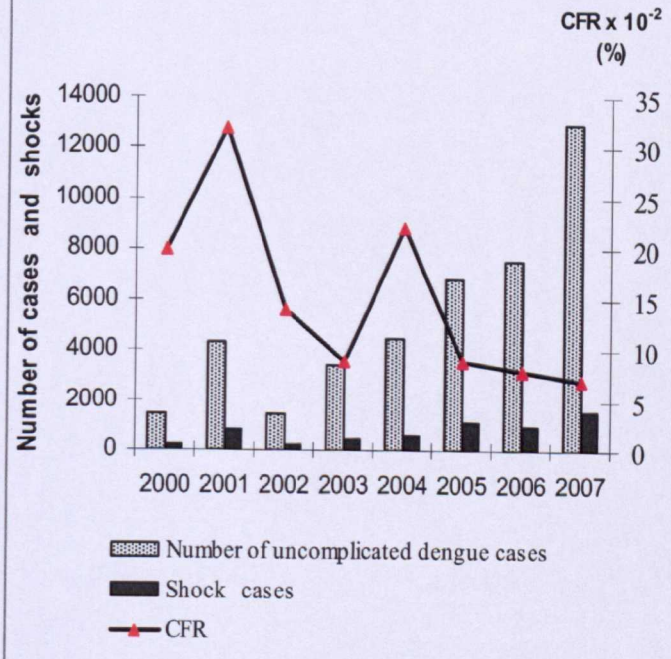


Fig.4.2. Number of uncomplicated dengue cases, DSS cases, and the case fatality rate (CFR) in Dong Thap province from 2000 to 2007. An increasing number of dengue cases has been reported annually, though the CFR has declined over time, it dropped from 0.32% (2001) to 0.07% (2007). (Source: Report of Precautionary Measures against Epidemic Department - Dong Thap province).

4.2. Aims of the study

The aim of this study was to describe the molecular epidemiological patterns of DENV-1 and DENV-2 from high transmission periods in 2006 and 2007 in Dong Thap Province in southern Vietnam. We particularly focused on an outbreak in 2007 by utilizing sequence analyses of the complete coding sequences of 176 DENV-1 and 38 DENV-2. Additionally, the availability of well documented clinical outcomes in the study population offered us an opportunity to study the association between the viral genetic characteristics of DENV-1 clades with clinical outcome and laboratory variables (viraemia and platelet nadir). Finally, we planned to look for any selection pressure acting on DENV-1 and DENV-2 viruses.

4.3. Patient recruitment and sampling method

Study inclusion criteria were; **1)** clinical suspicion of dengue and/or NS1 positive with NS1 ELISA assay, **2)** history of fever less than 72 hrs and **3)** written informed consent obtained. Exclusion criteria were patients with dual acute infections, or history of fever more than 72 hrs, or lack of informed consent. Details of procedure of sample collection were described in Chapter 2. All viral sequences were obtained from the plasma of patients enrolled in this study at DTH between October-December/2006 and May-November/2007. Paired specimens from enrollment and convalescence phase were tested for diagnostic IgM and IgG capture ELISA. DENV viraemia was detected with a serotype specific TaqMan RT-PCR.

4.4. Study Methods

4.4.1. Whole genome amplification and sequencing strategies

Consensus viral genome sequences were determined directly from plasma samples using the Broad Institute's ABI directed amplification viral sequencing pipeline. Details of these methods are described in Chapter 2. The DENV-2 complete genome sequences generated from this project have been logged into public databases (http://www.ncbi.nlm.nih.gov/sites/entrez) under the accession numbers shown in Appendix 3.

4.4.2. Sequence analyses

Sequence alignments were constructed (using Se-Al program http://evolve.zoo.ox.ac.uk/software.html) for 176 DENV-1 (10176 nt) and 38 DENV-2 complete coding sequences (10173 nt). Data sets were combined with complete genome sequence references from GenBank (176 DENV-1, 330 DENV-2) and genome sequences from patients' blood sampled in HCMC (199 DENV-1 and 156 DENV-2).

4.4.3. Mapping of households

The household of each patient who contributed a DENV genome sequence was mapped using a hand-held global positioning system (GPS) device. MapInfo (version 9.0 package, ESRI, Redlands CA, USA) was used to digitalize the households onto a digital administrative map of Vietnam. DENV sequences were then matched with household cases to investigate viral spatial distributions.

4.5. Results

4.5.1. A dengue outbreak in 2007 in Dong Thap Province

Dong Thap province experienced a major dengue outbreak in 2007. The peak of the epidemic was from June to October. **Fig. 4.3** shows the number of dengue and shock cases admitted to DTH in 2007 relative to the previous year and the year after. Hospitalised patients had clinical manifestations ranging from DF to DHF and DSS. Interestingly, the number of dengue cases declined dramatically in 2008. It is uncertain whether this was due to increased vector control or a level of herd immunity obtained by the high levels of transmission during 2007. However, the ratio of shock cases in 2008 was as high as in 2006 or 2007 (13.2% versus 11.8% or 13.4%, $P=0.2$, Fisher's exact).

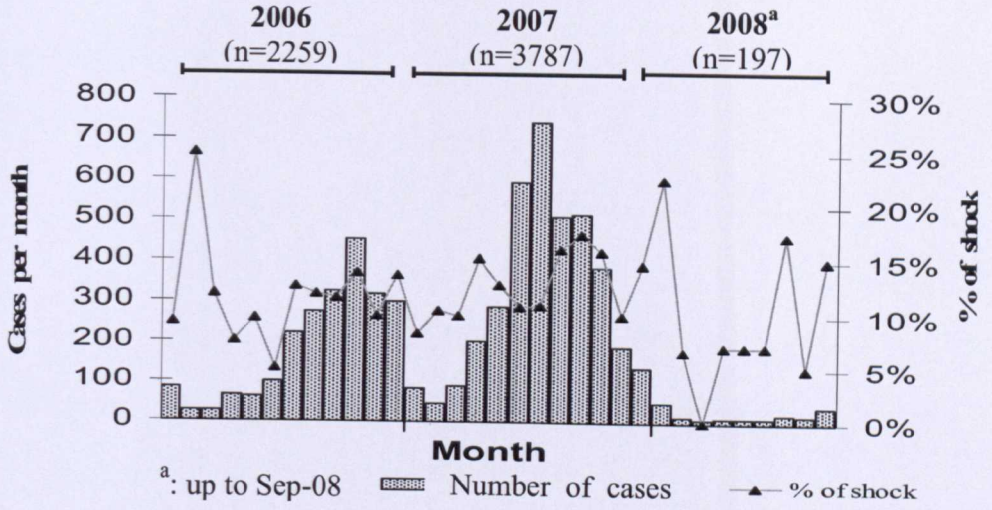


Fig.4.3. Number of dengue and % of shock cases in Dong Thap hospital in 2006, 2007, and 2008. Total cases in 2007 (n=3787) is 1.67 fold greater than 2006 (n=2259) and 19.2 fold greater than the first nine months of 2008 (n=197).

4.5.2. Characteristics of the study population

From October to December/2006 and over the 2007 outbreak, blood samples from 448 dengue patients admitted to DTH were collected (74 in 2006 and 374 in 2007). Most of the patients were ≤ 15 years [76.1%, n=341; median (range): 11 (2-42)]. The ratio between male and female was 1.2:1. Of these 448 patients, DF patients accounted for 46.2%, DHF 44.2% and DSS for 9.6%. Details of sample collection and clinical classification are described in **Table 4.1**.

Sample characteristics		Total	OFI[b]	Acute Dengue	DF (%)	DHF (%)	DSS (%)
Sampling	2006[a]	76	2	74	24 (32.4)	45 (60.8)	5 (6.7)
	2007	376	2	374	183 (48.9)	153 (41.0)	38 (10.2)
Overall		452	4	448	207 (46.2)	198 (44.2)	43 (9.6)
Male (%)		55	75	55	50	54	67
Median age (range)		11(2-42)	11(2-29)	11(2-42)	11(2-42)	11(2-34)	11(2-30)

[a]: Samples collected from October to December [b]: Other febrile illness

Table 4.1 Characteristics of samples collected in 2006 and 2007 in DTH. Acute dengues are 448, of which 46.2% are DF, 44.2% are DHF and 9.6% are DSS.

4.5.3. Infecting serotype prevalence

Table 4.2 shows the DENV serotypes identified in plasma samples by TaqMan-PCR. Of 448 acute dengue cases, 391 were RT-PCR positive (58 sampled in 2006 and 333 in 2007). Similar to the recent DENV picture in southern Vietnam, DENV-1 was the dominant serotype during the sampling period accounting for 81.3% of all DENV detected. Second was DENV-2, 16.4% then DENV-3 at 1.8% and DENV-4 was 0.5%. There were no differences in the proportion of DF, DHF and DSS between DENV-1 and DENV-2 (*P=1*, *P=0.8* and *P=0.7*, respectively; Fisher's exact). The ratio of the infecting serotype relative to disease severity is summarised in **Table 4.2**.

Serotype (n=391) / Year	DENV-1 (81.3%, n=318)			DENV-2 (16.4%, n=64)			DENV-3 (1.8%, n=7)		DENV-4 (0.5%, n=2)	
	DF	DHF	DSS	DF	DHF	DSS	DF	DHF	DF	DHF
2006	13	35	2	2	2	3	0	0	1	0
2007	125	111	32	26	26	5	6	1	0	1
Total (%)	138 (43.4)	146 (46.0)	34 (10.7)	28 (43.7)	28 (43.7)	8 (12.5)	6	1	1	1

Table 4.2. Serotype prevalence relative to DF, DHF and DSS patients enrolled in the study in Dong Thap Hospital in 2006 and 2007. The predominant serotype is DENV-1 (81.3%), then DENV-2 (16.4%). DENV-3 and DENV-4 are present at 1.8% and 0.5%. There was no significant difference between proportion of DSS cases in DENV-2 and DENV-1 (12.5% versus 10.7%, *P=0.7*, Fisher's exact).

4.5.4. Patient distributions

Using a Global Positioning System navigation device, 278 (44 were 2006 sampling, 234 were 2007 sampling) case households were plotted on a digital map. Each dot represents the household of 1 patient. Forty-one percent of patients (114/278) lived in Cao Lanh city (dark

grey), 41.4% (115/278) lived in Cao Lanh district and 17.6% (49/278) lived in neighboring districts. Households of dengue patients are shown in **Fig.4.4**.

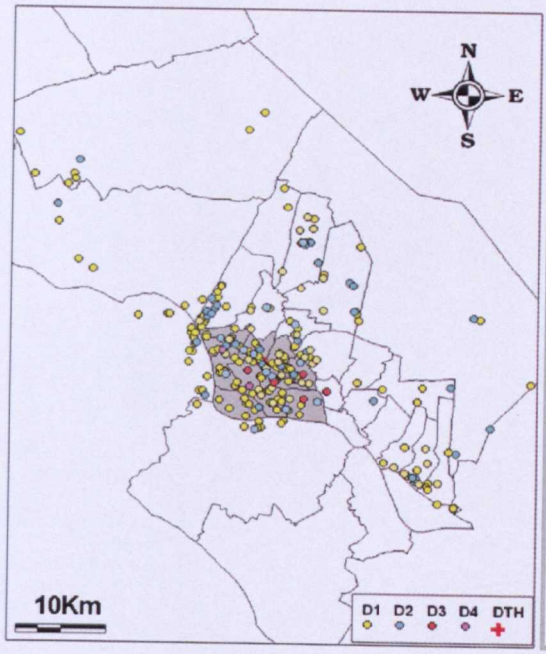


Fig.4.4. Location of mapped households of 278 patients in Dong Thap province. Yellow dots are DENV-1 infected patients (n=219), turquoise dots are DENV-2 (n=55), red dots are DENV-3 (n=5) and pink, DENV-4 (n=2).

4.5.5. Molecular epidemiology of DENV-1 and DENV-2 in Dong Thap province

4.5.5.1. Viral genome sequences

To better reveal the accuracy of DENV molecular epidemiology patterns, efforts were made to obtain the highest number of viral genome sequences. To this end, 288 DENV-1 (90% of total DENV-1), 55 DENV-2 (85% of total DENV-2), all DENV-3 and DENV-4 available were attempted for the whole genome sequencing. Shown in **Fig. 4.5** is a chart showing the number of samples attempted whole genome amplification and the number of successful genomic sequences in individual DENV serotypes.

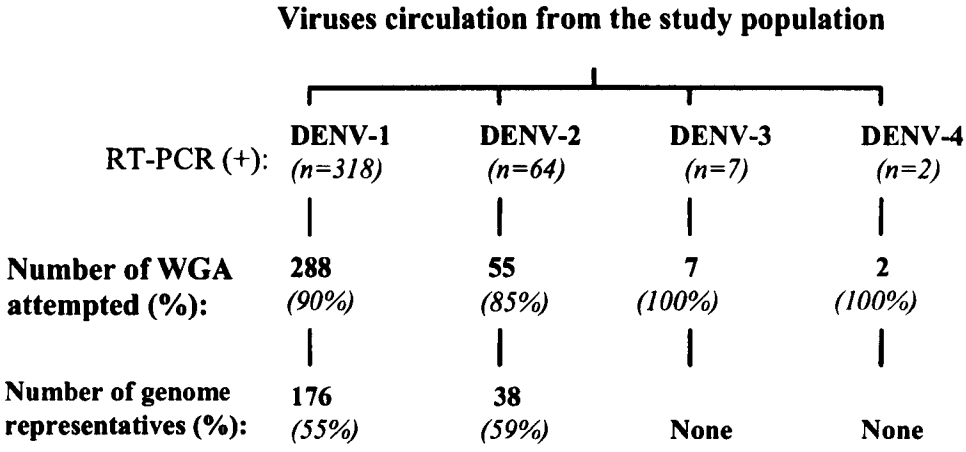


Fig.4.5. Sample selection in DTH for DENV whole genome sequencing. The chart shows the process of sample attempts and the success rate for whole genome sequencing in the study population. Shown in the first box line is the total of DENV serotypes detected by RT-PCR. Numbers of individual DENV serotypes attempted for the whole genome amplification are shown in the second box line. The overall number of genome sequences in each DENV serotype is shown in the third box line. The final representation of DENV-1 was 55% (n=176) and 59% for DENV-2 (n-38). No genome sequence of DENV-3 and DENV-4 was successfully amplified.

Finally, 214 clinical samples [74 DF, 109 DHF (grade I to grade II) and 31 DSS] comprising 176 DENV-1 (representative of 55% DENV-1) and 38 DENV-2 (59%) were successful for whole genome sequencing. Of those, 181 (85%) were ≤18 years (school age group). No DENV-3 or DENV-4 genomes were successfully recovered. Details of the number of genomic sequences are summarised in **Table 4.3.** Patients who were resident in Cao Lanh city accounted for 40% (n=86) of total sequences, 37% (n=80) from Cao Lanh district, and 23% (n=48) from other adjacent districts. Seventy-eight percent of viral sequences (n=167) were collected during the peak of transmission between June to the end of October/2007.

Year	Total of genomic sequences								
	DENV-1 (n=176)			DENV-2 (n=38)			Age		Total
	DF	DHF	DSS	DF	DHF	DSS	≤18	>18	
2006[a]	2	11	0	0	0	1	14	0	14
2007	54	82	27	18	16	3	167	33	200
Total	56	93	27	18	16	4	181	33	214

[a]: October to December -2006

Table 4.3 Details of virus source and total number of genome sequences in Dong Thap province from October/2006 to November/2007. Samples from the 2007-outbreak represented 93% (200/214) of the total. School-age samples represented 85% (181/214) of the total

Shown in **Fig. 4.6** is a map on which the household residence of each patient from whom a DENV genome sequence was determined. Each spot represents 1 virus.

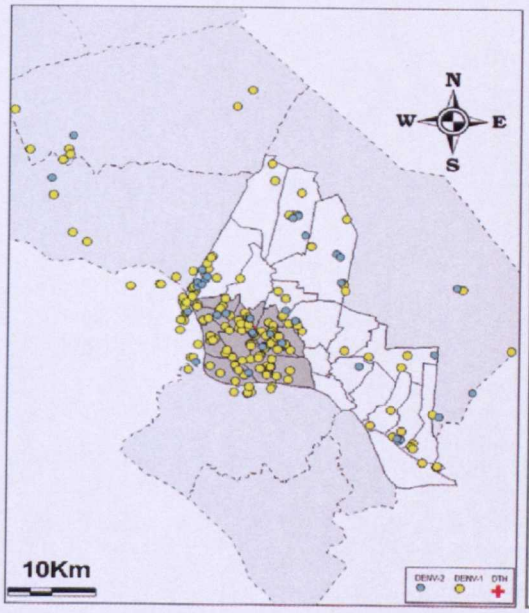


Fig.4.6. Distribution of 214 DENV genomes. Map showing distribution of residential addresses of patients from whom DENV viruses were sequenced, Cao Lanh district (white area), Cao Lanh city (dark grey area), and other adjacent district (grey). Each dot corresponds to 1 virus. Shown here are 214 households representing 214 genome sequences. Yellow dots are DENV-1 (n=176).

4.5.5.2. Molecular epidemiology of DENV-1

a. Sequence analyses of DENV-1 in Dong Thap

Phylogenetic analysis of 176 DENV-1 coding region sequences from Dong Thap, 199 from in and around HCMC and 176 from NCBI is shown in **Fig. 4.7**. The maximum likelihood tree contained 3 DENV-1 genotypes labeled as genotype I, II and III and was rooted by the sylvatic virus. Each branch of the tree was supported by high bootstrap (shown on main nodes). All DENV-1 in Dong Thap (n=176) belonged in Genotype I. There was significant diversity in Genotype I with 9 lineages represented; however viruses from Dong Thap belonged to five of these. As shown in Chapter 3, DENV-1 in HCMC diverged into 6 lineages (or clades) in Genotype I numbered from 1 to 6. In order to keep the numbering consistent in the DENV-1 phylogenetic tree, 5 DENV-1 lineages in Dong Thap province were numbered as 1, 2, 3, 4 and 6. No strain fell in lineage #5. In Dong Thap, lineage #3 was the most prevalent virus sampled (73.4%, 129/176). Lineage #6, which existed nearly equal to lineage #3 in HCMC (lineage #6 versus lineage #3: 32.7% versus 33.7%), was present in Dong Thap at 15.3% (27/176) of the sampled population. Lineage #1, #2, and #4 accounted for 2.8%, (5/176) 5.1% (9/176), and 3.4% (6/176), respectively. Lineage 1, 2, 3 and 6 were purely of Vietnamese origin whilst lineage #4 contained DENV-1 sampled from other Asian countries (Cambodia, Singapore, China, and Japan). Amongst these lineages, lineage #3, #4 and #6 contained a number of clusters in which 2007-viruses were originally associated with 2006-viruses. When sequences were stratified by year; lineage #3 was still the dominant lineage in 2006 (54%, 7/13), then lineage #6 (31%, 4/13) and lineage #4 (15%, 2/13). Thus, lineage #1 and #2 were either newly introduced to DT in the 2007 outbreak or existed at low frequency before the outbreak. The ratio of each DENV-1 lineage relative to the overall population is shown in **Fig. 4.8**.

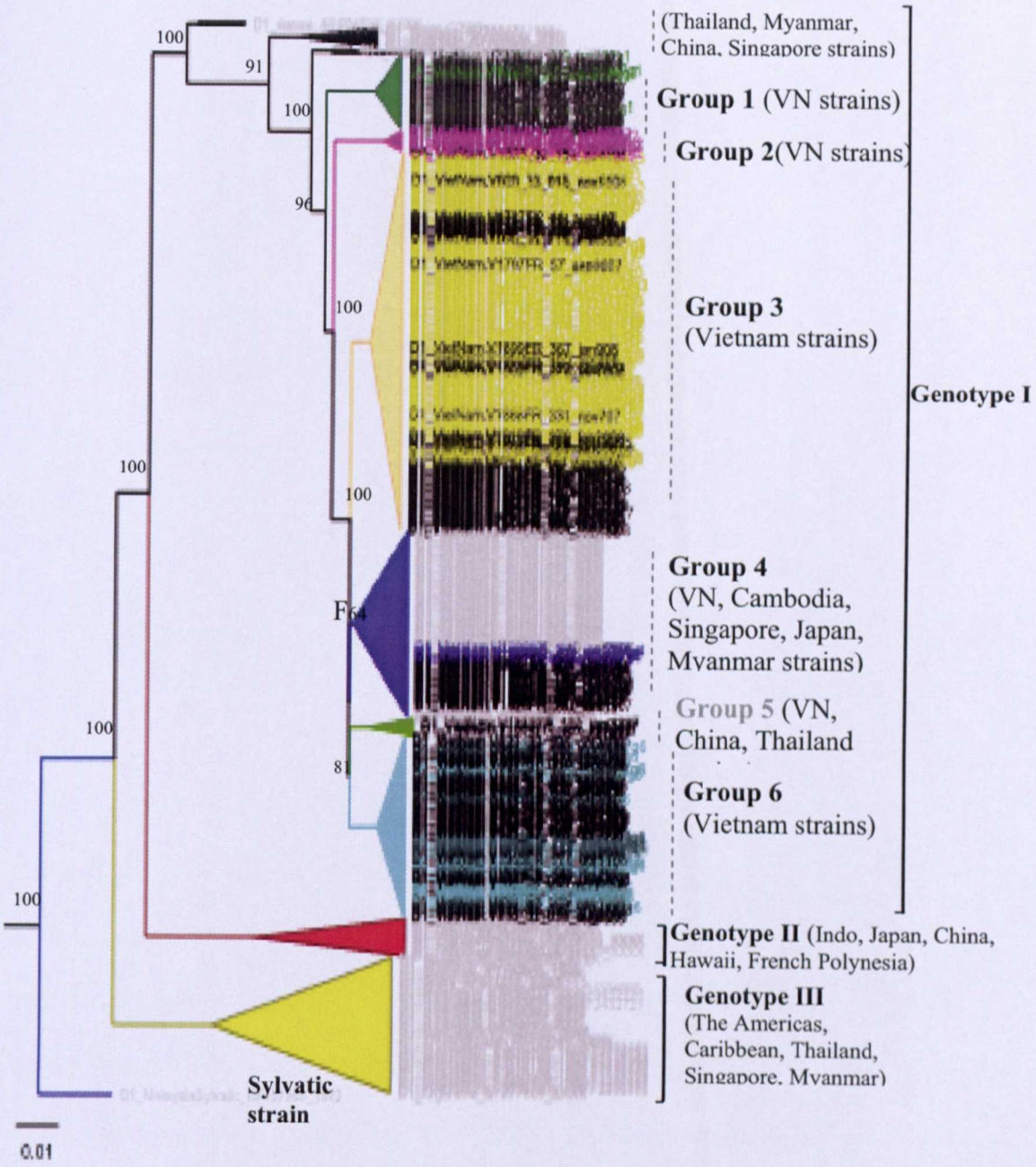


Fig.4.7. ML tree of complete coding sequence from 551 DENV-1 strains. The tree includes 176 Dong Thap strains, 176 strains available from GenBank (highlighted in grey) and 199 Vietnamese strains in HCMC and other provinces (highlighted in black) that do not belong to this study. The tree is rooted by DENV-1 sylvatic genotype. Bootstrap support is shown on main nodes. Each genotype branch is painted in a different colour and labeled with a genotype name. All Dong Thap strains fall in Genotype I and diverge in 5 lineages (#1, #2, #3, #4 and #6). Branches in Genotype I that have Dong Thap strains are highlighted in different colours corresponding to different groups.

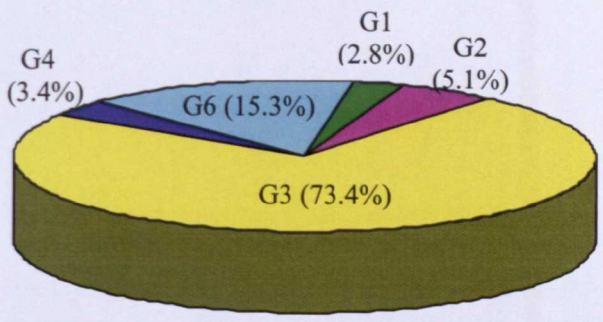
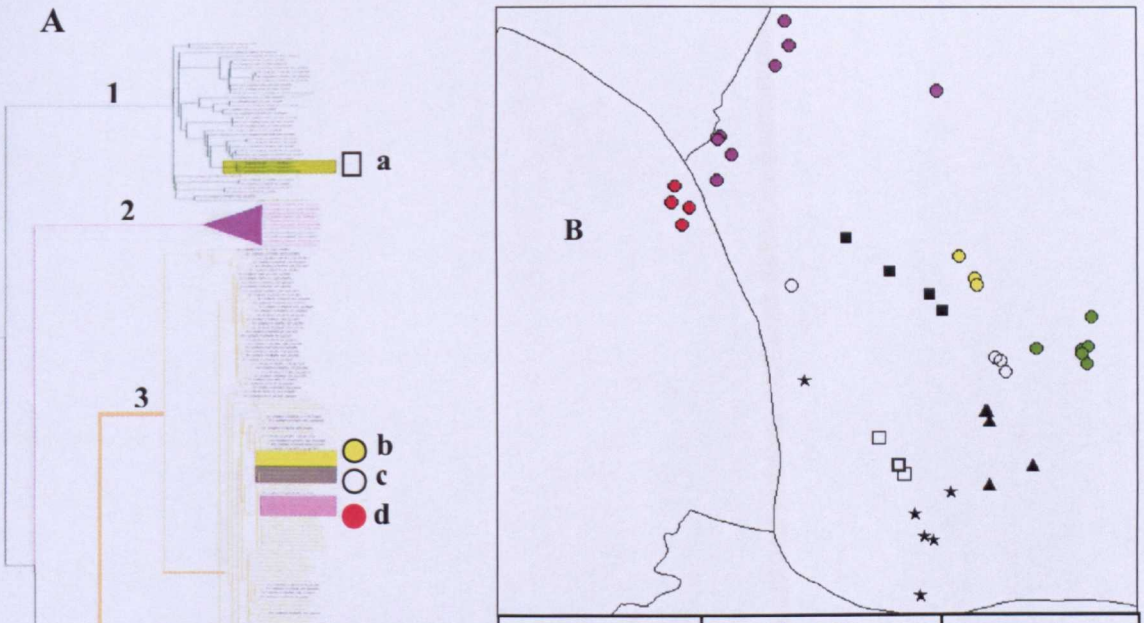


Fig.4.8. DENV-1 lineages sampled in Dong Thap province. Pie graph shows the percentage of each clade relative to the overall number of DENV-1 sampled from Dong Thap province (n=176). Lineage #3 was the most prevalent (73.4%) and then lineage #6 (15.3%). Lineage #1, #2, and #4 appear as a minority.

When the different lineages were compared to the digital map of household locations in Dong Thap, there was no apparent spatial relatedness amongst lineages. All five lineages scattered in Cao Lanh city, Cao Lanh district and other neighboring districts. However, within some individual lineages, there was a number of genetically distinct clusters showing temporal and spatial structure. These clusters are highlighted (labeled **a, b, c, d, e, f, g, h** and **i**) and shown in **Fig.4.9.A**. Geographical distributions of distinct clusters, details of nucleotide difference, sampling time difference, and physical distance within clusters are also shown in **Fig.4.9.B**.



	nt difference (range)	Physical distance (range) m	Temporal distance (day)
□	2-7	35-1200	2-17
○	1-4	300-900	10-17
○	4-6	200-5600	11-28
●	1-7	500-1000	3-16
●	1-9	150-1600	1-43
●	0-8	0-6000	1-48
★	1-14	200-6000	6-24
■	3-10	400-3200	8-11
▲	0-6	100-2000	7-20

Fig.4.9. Relationship between phylogenetic cluster and temporal and spatial distribution in DENV-1 lineages in Dong Thap province. **(A)** is 6 lineages of DENV-1 genotype I indicated by different branch colours. Five lineages contain viruses collected in Dong Thap (1, 2, 3, 4 and 6). Clusters highlighted in different colours (labeled from **a** to **i**) are genetically distinct viruses distributed within a different limitted area and correspondingly displayed in **B**. Shown in **(B)** are spatial distributions of clustering viruses. Individual clusters are indicated by different colours or symbols. Number of nucleotide differences, physical distance, and temporal distance are shown in table under (**B**).

b. Highly active transmission periods associated with a number of identical sequences

Shown in **Fig.4.10.(A)** are 5 pairs of identical sequences found during the peaks of transmission (Oct/2006 and Jul-Sep/2007). Pair #1 which was 10,690 nt in length (position 23-10,712), ~200 metres apart and collected 3 days apart and sampled at the high transmission period of 2006 (October). Pair #2 was 10,691 nt in length (23-10,713), ~1km apart, 7 days apart and sampled at high transmission time in the 2007 outbreak (August). The remaining 3 pairs, namely #3, #4 and #5 were 10,685 nt (position 23-10,707), 10,690 nt (23-10,712) and 10,684 nt (23-10,706) in length, respectively. All were sampled during high transmission time in the 2007 outbreak. The identical viruses #3 were the interesting ones which were sampled from 2 patients (7 and 15 years) living in the same house with a history of illness starting 1 day apart. The identical sequences #4 appeared 4 days apart and were ~70 metres distant. Interestingly, the identical viruses #5 were collected 27 days apart and at a distance of ~2.5km. All patients from whom the identical sequences derived were from 7-18 years. **Fig.4.10.(B)** shows numbers of cases per month and high transmission time (black bars) in DT in 2006 and 2007 at which identical sequences were collected. Collectively, the identical sequences obtained in a restricted area over the epidemic were associated with the highest transmission periods; particularly the twin #5 (lineage #3) collected 27 days and ~2.5km apart.

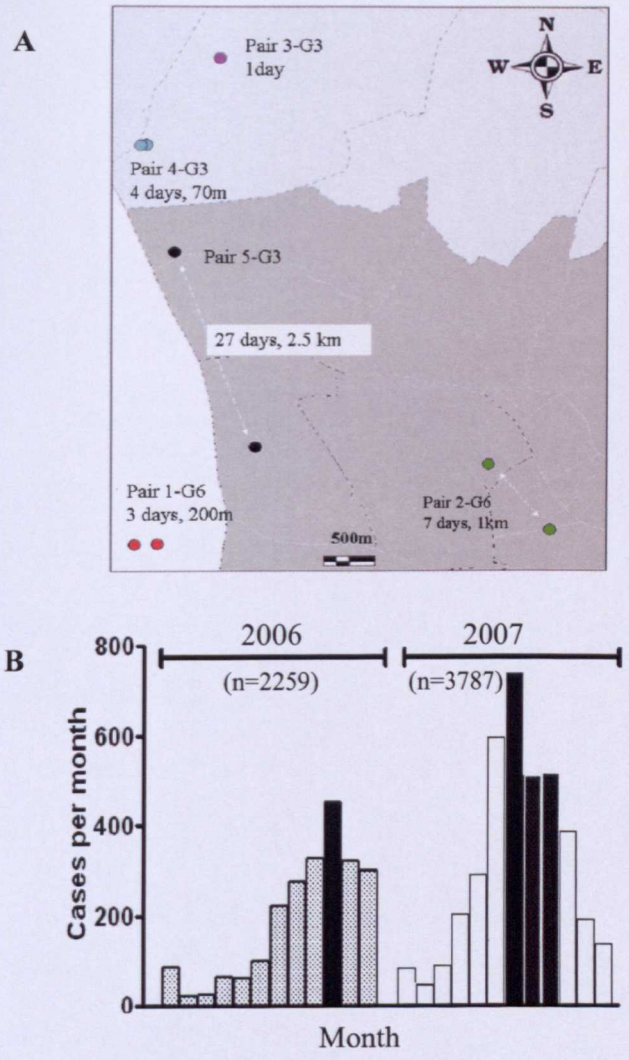


Fig.4.10. Geospatial distribution of identical DENV-1 genome sequences. Shown in (**A**) is 5 pairs of identical sequences obtained during the peaks of tranmission in 2006 and 2007 with different time of collection (day, d). Pair #1 (red dots, ~200 metres apart) and #2 (green dots, ~1km apart) belonged to lineage #6. Pair #3 (pink, the same location), #4 (turquoise, 70 metres apart) and #5 (black, ~2.5km) belonged to lineage #3. Shown in (**B**) is number of dengue cases per month in Dong Thap in 2006 and 2007. Identical sequences were obtained at time when number of cases was highest (black bars).

c. Selection pressure on DENV-1

We found no positive selection in any gene or site amongst DENV-1 lineages #1 and #6. *dN/dS* in every gene was low (<1), ranging from 0.029 (NS4B) to 0,335 (Capsid). This, again, suggested that purifying selection had a major impact on the DENV population structure. Similar to DENV-1 virus population sampled in and around HCMC, here, Capsid and NS2A also exhibited higher *dN/dS* ratio than other genes (0.335 and 0.134, respectively). *dN/dS* results on a gene-by-gene basis are presented in **Table 4.4**.

Gene	Capsid	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Length (No of aa)	114	166	495	352	218	130	619	150	249	899
Global *dN/dS*	0.335	0.110	0.101	0.099	0.134	0.052	0.030	0.068	0.029	0.053

Table 4.4 Selection pressures in DENV-1 collected in Dong Thap on a gene-by-gene basis in DENV-1 data set (n=176). No site is under positive selection pressure. All *dN/dS* ratios were < 1, ranging from 0.029 (NS4B) to 0.335 (Capsid).

d. Sequence divergence in nucleotide and amino acid sequence amongst DENV-1 lineages

The overall mean sequence divergence (complete CDS) amongst 5 DENV-1 clades was 1.25% in nucleotide sequence and 0.42% in amino acid sequence. The divergence in nucleotide and amino acid sequences on a gene by gene basis is shown in **Table 4.5**. Collectively, in this virus population, Capsid and M protein showed the highest level of sequence divergence amongst the Vietnamese viruses sampled in this study (1% and 0.81%, respectively).

Gene	Length (No of amino acid)	Mean divergence in nucleotide sequence (%)	Mean divergence in amino acid sequence (%)
Capsid	114	0.92	**1.00**
M	166	1.59	0.81
E	495	1.31	0.48
NS1	352	1.34	0.54
NS2A	218	**1.68**	0.70
NS2B	130	1.31	0.10
NS3	619	1.17	0.17
NS4A	150	1.08	0.15
NS4B	249	0.88	0.52
NS5	899	1.24	0.52
Complete CDS	3392	1.25	0.42

Table 4.5 Sequence divergence amongst 5 DENV-1 clades on a gene by gene basis. Shown in bold are the genes with the highest divergence in nucleotide sequence (NS2A: 1.68%) and in amino acid sequence (M protein: 1.00%). The lowest divergence in nucleotide sequence is NS4B (0.88%); in amino acid sequence is NS2B (0.10%). The overall sequence divergence (complete CDS) in 5 DENV-1 clades is shown in the bottom row (1.25 and 0.42 at nucleotide and amino acid level, respectively).

e. Investigation of the association between clinical outcome and DENV-1 clades

Thirty-one patients, from whom DENV genome were sequenced, developed DSS, of which 4 cases was DENV-2 (1 sampled in 2006, 3 in 2007), 27 were DENV-1 (all sampled in 2007). Though DF and DHF were found in patients infected with any one of the 5 DENV-1 lineages, 4 of 5 lineages contained DSS patients. Lineage #3 accounted for 77.8% (21/27) of total DSS; DSS in lineage #6 was 14.8% (4/27), lineage #1 and lineage #2 was 3.7% of each (1/27). No DSS case was associated with lineage #4. The proportion of DSS cases in each of the 5 lineages is as follows: lineage #1: 1/5 (20%), lineage #2: 1/9 (11.1%), lineage #3: 21/128 (16.4%), lineage #4: 0/6 (0%), lineage #6: 4/27 (14.8%). To explore whether genetic differences amongst 5 DENV-1 clades may cause differences in clinical outcome, a further analysis was made. Viraemia levels and platelet nadirs were compared amongst the 5 lineages and between lineage #4 (in which no patients developed DSS) and each of the other 4 lineages (1, 2, 3, and 6). Median viraemia values (log10 cDNA equivalents/ml) and platelet nadirs at the time of enrolment in each of the 5 lineages were compared (**Table 4.6**). No significant differences in viraemia levels or platelet nadirs were found in enrolment amongst 5 lineages (*P=0.8*, and *P=0.3*, respectively, Kruskal–Wallis test). Paired comparison of viraemia and platelet nadir between group 4 versus each of other 4 groups (1, 2, 3 and 6) also showed no significant differences (*P=0.2; 0.5; 0.9; 0.7* and *P=0.5; 0.2; 0.2; 0.1*; respectively). Summary of laboratory variables (medium log10cDNA equivalents, medium platelet nadirs) and demographic information on DENV-1 clades are described in **Table 4.6**. Platelet nadirs and viraemia levels in DENV-1 clades are shown in **Fig.4.11**.

Clade	Median log10cDNA equivalents (SD)	Median platelet nadirs (SD)	DOI [Median (range)]	Male (%)	Median age (SD)	Number of shock
1 (n=5)	7.7 (0.82)	80,000 (60,076)	3 (1-3)	8	13 (5.5)	1
2 (n=9)	8.2 (0.63)	88,000 (38,069)	3 (2-3)	66	10 (3.5)	1
3 (n=128)	8.11 (1.04)	83,750 (39,679)	3 (1-5)	54	11 (6.5)	21
4 (n=6)	8.2 (0.53)	55,500 (39,043)	2.5 (2-4)	50	10 (2.5)	None
6 (n=27)	7.9 (1.01)	93,500 (54,946)	3 (1-4)	50	12 (7.5)	4
P value[a]	*0.8*	*0.3*	*0.8*			

[a]: Kruskal–Wallis DOI: Day of illness

Table 4.6 Viraemia levels, platelet nadirs and demographic information in patients infected with different DENV-1 lineages. No association between viraemia level or platelet nadir with a particular lineage was found.

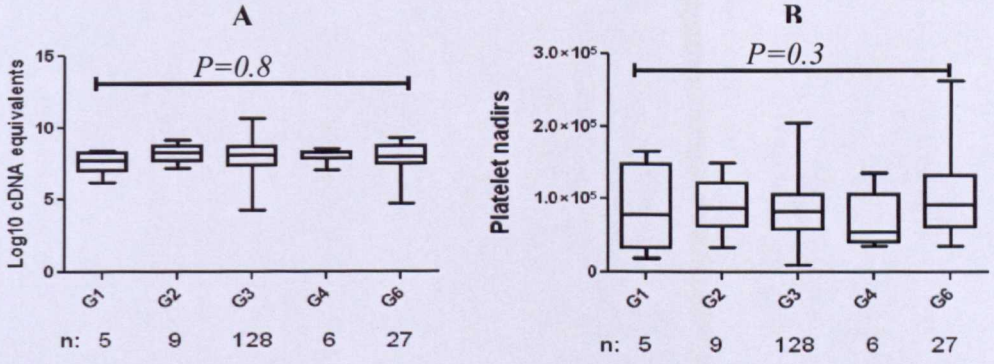


Fig.4.11. Viraemia levels and platelet nadirs in different DENV-1 clades. Shown in **A** is the median (interquartile and range) of viraemia values at the time of enrolment for each of 5 DENV-1 lineages. **B** is platelet nadirs in 5 clades. There is no significant difference in viraemia level and platelet nadir amongst 5 groups.

4.5.5.3. Molecular epidemiology of DENV-2 in Dong Thap

a. Phylogenetic analysis of DENV-2

DENV-2 accounted for 16.4% (64/391; 57 in 2007, 7 in 2006) of all serotypes during sampling time. The genome sequences of 38 DENV-2 were determined. A maximum likelihood tree of 38 DENV-2 revealed that they all belonged to the Asian 1 genotype (**Fig.4.12**).

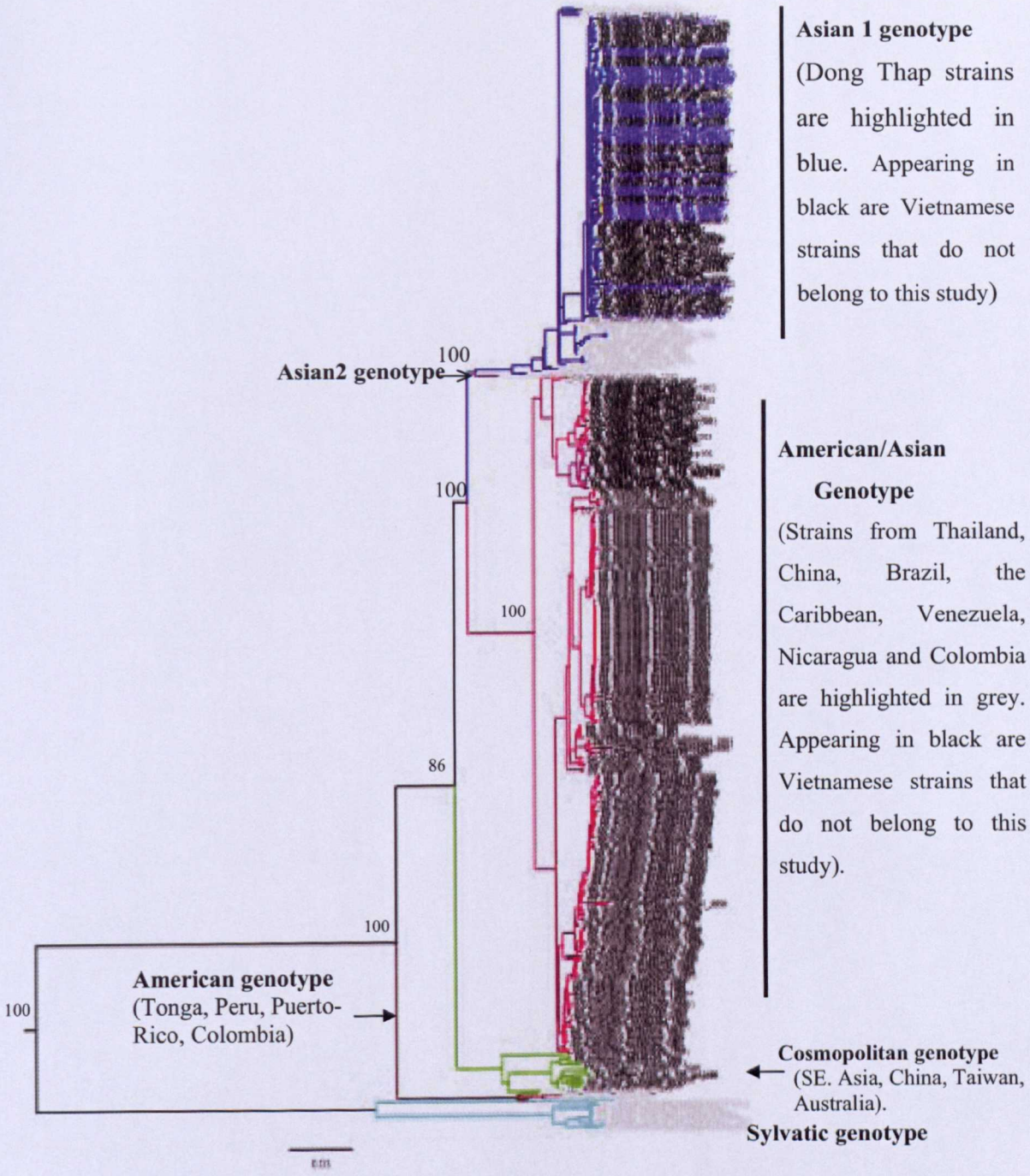
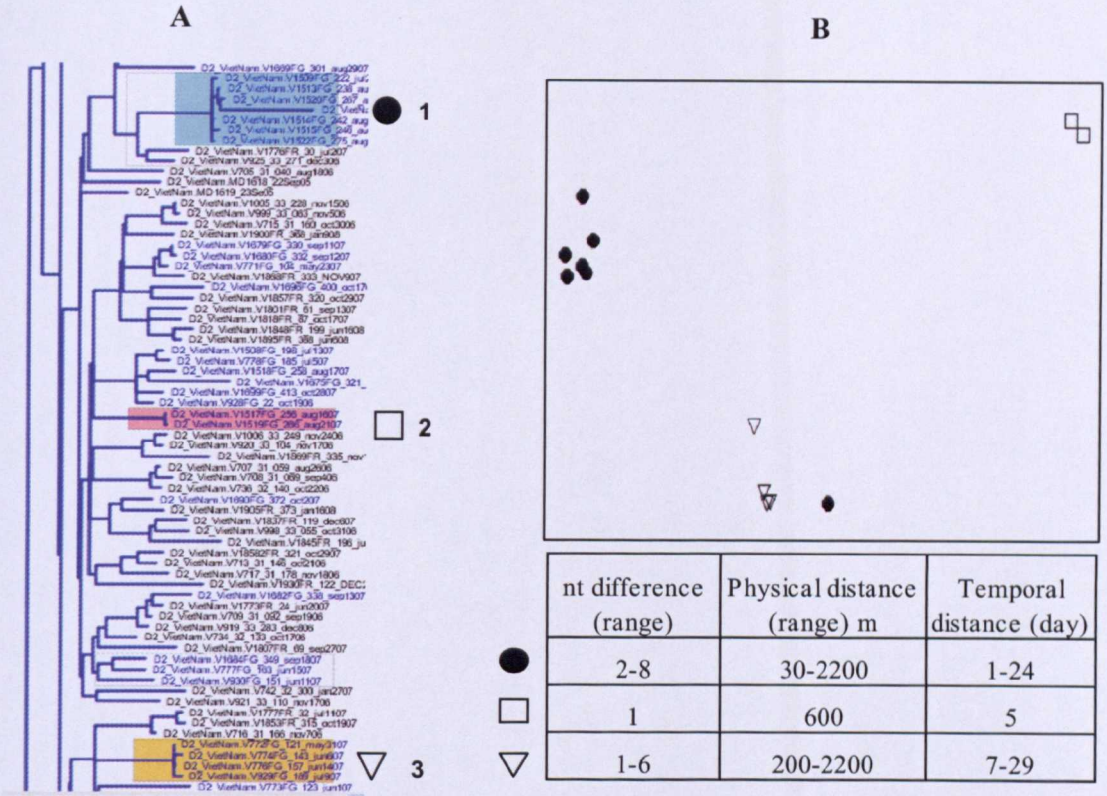


Fig.4.12. ML tree of complete coding sequence from 524 DENV-2 strains. The tree includes 38 Dong Thap strains, 330 strains available from GenBank (highlighted in grey) and 156 Vietnamese strains sampled in HCMC and other provinces (highlighted in black). The tree is rooted with DENV-2 sylvatic strains. Branches defining genotypes are highlighted in different colours and labeled with their genotype name. Nodes that separate branches defining different genotypes are supported with high bootstraps shown at all main nodes. All Dong Thap strains fall into Asian 1 genotype (highlighted in blue).

When different sub-lineages within Asian 1 genotype were compared to the digital map of household locations, a spatially and temporally clustering pattern was observed in several genetically distinct clusters. Shown in **Fig.4.13** are 3 distinct Asian 1 DENV-2 clusters (**A**) and areas (**B**) within which these distinct clusters were distributed.



	nt difference (range)	Physical distance (range) m	Temporal distance (day)
●	2-8	30-2200	1-24
□	1	600	5
▽	1-6	200-2200	7-29

Fig.4.13. Temporally and spatially genetic association in DENV-2 lineages. Shown in (**A**) are DENV-2 Asian 1 viruses described in the DENV-2 ML tree above. Highlighted clusters are viruses showing temporal and spatial association (numbered from 1 to 3). Shown in (**B**) is geographical distribution of distinct clusters. Different clusters are indicated by different symbols. Shown in table under (**B**) is information of nucleotide difference, physical distance and temporal distance within individual clusters. In cluster 1, 6 of 7 viruses showed genetically temporal association and were distributed between 30-2200m.

b. Sequence divergence in nucleotide and amino acid sequences of DENV-2

The overall mean sequence divergence (complete CDS) in 38 DENV-2 was 0.77% in the nucleotide sequence and 0.37% in the amino acid sequence and is summarised in **Table 4.7**.

Generally, sequence divergence in this DENV-2 population is rather low, probably because they were sampled in a short period of time in the same location and belonged to the same genotype.

Gene	Length (No of amino acid)	Mean divergence in nucleotide sequence (%)	Mean divergence in amino acid sequence (%)
Capsid	114	0.49	0.23
M	166	0.78	0.13
E	495	0.75	0.32
NS1	352	0.77	0.43
NS2A	218	0.84	**0.87**
NS2B	130	0.44	0.00
NS3	618	0.86	0.27
NS4A	150	0.82	0.41
NS4B	248	**1.02**	0.33
NS5	900	0.72	0.45
Complete CDS	3391	0.77	0.37

Table 4.7 The sequence divergence amongst 38 DENV-2 sampled in Dong Thap. Shown in bold are the highest divergence in nucleotide sequence (NS4B: 1.02%) and in amino acid sequence (NS2A: 0.87%). The overall sequence divergence in nucleotide and amino acid sequence (complete CDS) is 0.77 and 0.37%, respectively.

c- Selection pressure on DENV-2

Shown in **Table 4.8** is the *dN/dS* result on a gene-by-gene basis of DENV-2 in Dong Thap. In the same context of the evolutionary process in DENV, the overall selection pressure impacted on DENV-2 was that of purifying selection. *dN/dS* ratio across genes was <1 ranging from 0.002 (NS2B) to 0.227 (NS2A). NS2A and Capsid again exhibited higher *dN/dS* ratio than other genes (0.227 and 0.171, respectively).

Gene	Capsid	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
Length (No of aa)	114	166	495	352	218	130	618	150	248	900
Global dN/dS	0.171	0.042	0.051	0.117	0.227	0.002	0.071	0.133	0.094	0.118

Table 4.8 Selection pressures on a gene-by-gene basis of DENV-2 (n=38) collected in Dong Thap. None site is under positive selection pressure. All *dN/dS* ratios were < 1, ranging from 0.002 (NS2B) to 0.227 (NS2A)

4.6. Discussion

All four DENV serotypes were identified during the study period (October-December/2006 and the 2007 dengue outbreak) in Dong Thap province, but DENV-1 and DENV-2 were the major serotypes associated with the outbreak. Although both mild and severe diseases have been found to derive from any one of the 4 serotypes, the proportion of severe disease (DHF versus DF) in DENV-1 was greater than the other DENV serotypes. With our greatest effort, DENV-1 and DENV-2 were the most easily available viruses for sequencing. This study therefore, describes the molecular epidemiological pattern of DENV-1 and DENV-2 in this setting, covering the periods of high DENV activity, and investigates the association between virus genetics and clinical outcome and laboratory variables.

In DENV-1, a notable finding was the extent of genetic variation (but not amino acid diversity) within a single genotype, although these viruses were collected during a short time in a restricted area. Purifying selection was the overall evolutionary pressure shaping the genetic diversity in this viral population. In DENV-2, Asian 1 genotype was the unique lineage detected over the outbreak, compatible with what was observed in and around HCMC during this period. This, again, suggests a possibility of DENV-2 Asian 1 genotype having a competitive advantage over Am/As genotype.

The extent of genetic diversity of DENV on a localized scale utilizing E gene sequences has been previously measured in primary schools in Kamphaeng Phet, Thailand and showed little evidence for in situ evolution, and that viral migration was the major mechanism shaping the genetic diversity of DENV in the region [147, 148]. We exploited the full genome approach to study the structure of genetic variation in this DENV population in relation to temporal and geographic distribution on a background of households mapped within a ~10km radius around DTH. Our phylogenetic analysis showed that the 2007-viruses were originally associated with 2006-strains in DENV-1 lineage #3 and #6 clusters. In addition, a number of phylogenetic clusters were observed within which genetic distances ranged from 0 to over 10 nucleotides in samples separated by days to ~month of sampling and distributed in close vicinity to one another. As such, with whole genome data derived from a large number of samples, our results provide strong evidences for in situ evolution in this fine spatial scale. However, viral introduction is the main mechanism shaping the genetic diversity in this study region, which is shown by the breadth of viral lineages sampled over the study area.

Investigation of DENV molecular epidemiology at the full genome level has demonstrated the utility of this approach in giving higher phylogenetic resolution that can be coupled with spatial and temporal epidemiological data [137, 149]. A previous report has shown the existence of clearly distinct spatial clusters in DENV-1 and DENV-3 in Singapore [137]. Consistent with this previous study, our results revealed spatially and temporally constrained phylogenetic clusters in both DENV-1 and DENV-2. This could be a reflection of focal transmission patterns (e.g. within a household or between neighbouring households).

Amongst the DENV-1 population in Dong Thap, a number of pairs of identical sequences at the level of the whole viral genome were detected. This could be a reflection of a shared exposure between these individuals (for example an infected mosquito in the same living accommodation, neighborhood, school, workplace or place of entertainment), or, perhaps less

likely it just occurs by chance in a DENV population where identical sequences co-circulate and could be revealed when a large enough number of viruses is sampled. Pairs of identical sequences had a spatial range of 0-1,000m distance within a collection period of 1-7 days apart. One pair of identical sequences that was detected in 2 patients living in the same house with 1 day difference between collections strongly suggests that they were infected by the same mosquito in their residence. Interestingly, our results show a pair of identical sequences sampled ~2.5km apart in distance and 27 days apart in time. The simplest explanation for this is that this represents a consensus virus sequence that has not accumulated any mutations from the consensus after going though several rounds of mosquito-human infection. A less probable explanation is that this is a consequence of shared exposure to a long-lived infected mosquito, since the lifespan of *Ae. aegypti* mosquitoes is usually only a few weeks. Also possible is trans-ovarial transmission such that the progeny of an infected mosquito carry a virus with an identical genome sequence. Vertical transmission has been proven previously for several mosquito-borne viruses, such as La Crosse virus [150], West Nile virus [151] and Yellow Fever virus [152]. Recent studies have also shown this transmission pattern occurring in DENV [88, 94, 153] but the role of it in dengue epidemics remains unknown [153, 154]. It would be particularly interesting to carry out further studies to determine how this transmission pattern contributes to dengue epidemiology in Vietnam.

Within the DENV-1 virus population in and around HCMC, viruses belonging to lineage #6 were as abundant as viruses in lineage #3 (33.7% versus 32.7%). In Dong Thap however, lineage #6 was sampled at a much lower rate than lineage #3 (15.3% versus 73.4%) suggesting that this lineage was first introduced into HCMC where it has become established with then occasional introductions into Dong Thap. Alternatively, but less likely is that herd immunity status in Dong Thap might be more favorable for lineage #3 than lineage #6 relative to HCMC.

Our results indicated that the number of dengue cases in Dong Thap was relatively high between July-December/2006 (referred to **Fig.4.3**). The number of cases suddenly dropped during the first 3 months of 2007, and then the outbreak was triggered in the early rainy season (April). Interestingly, after the 2007 outbreak, a remarkable drop in dengue cases in 2008 was observed in Dong Thap. The basis of this phenomenon remains uncertain. A possible explanation is that the population-wide immunity level probably has reached a threshold that curtails DENV-1 transmisison. This could also result from the Vector Control Program having been launched after the outbreak (Source: Dong Thap Committee of Precautionary Measures against Epidemic). Training courses for Prevention, Management and Control of Dengue had been organized in 2008 in Dong Thap province (Source: Dong Thap Committee of Precautionary Measures against Epidemic).

The same genetic diversity was observed in 2006 and 2007 in that DENV-1 clade #3 was the major lineage. In addition, sequence analyses showed no evidence for adaptive selection impacting on this viral population over the outbreak. In summary, in agreement with previous studies [130, 137, 155], our results imply that stochastic factors, vector control or herd immunity or a combination of these aspects were the major contributions driving the end of the dengue outbreak in Dong Thap province.

Chapter 5

5. Evaluation of the Platelia NS1 ELISA assay for diagnosis of acute dengue

5.1. Introduction

Though most DENV infections are uncomplicated and not life-threatening, accurate and timely diagnosis is helpful for clinical management, disease surveillance and research activities. Laboratory diagnosis for acute dengue is currently performed by many approaches: virus isolation in mosquito cells or mammalian cells, viral RNA detection by RT-PCR, or serological assay e.g. MAC ELISA.

Virus isolation is definitive but requires at least seven days for completion. It is expensive, requires specialized techniques and usually accessible only in reference or research laboratories. RT-PCR is a rapid, sensitive, and reliable assay but this technique is expensive and not always feasible in resource poor endemic areas. Moreover, it is less sensitive around the time of defervescence, a time when the clinical complications of vascular leakage are most likely to manifest. The MAC ELISA assay is widely used for dengue diagnosis in many dengue endemic laboratories. However, firstly, the interpretation of the result in some settings is complicated due to cross-reactive antigenic determinants shared by DENV with other flaviviruses; secondly, definitive confirmation requires paired specimens collected within the appropriate time frame (5-7 days apart). This limits its utility, since confirmation of results occur when the patient has recovered or even left hospital. Therefore, an accurate and sensitive diagnostic test that differentiates dengue from other viral infections in the first few days of illness is an important practical requirement.

DENV NS1 protein becomes detectable from the first day after fever onset up to day 9 [21]. Moreover, this protein can be detected even when viral RNA becomes unmeasurable in RT-PCR or in the presence of IgM antibodies [21, 22]. The recent introduction of commercial ELISA assays to detect the dengue virus NS1 antigen in human serum or plasma has provided an additional dengue diagnostic to the existing approaches of RT-PCR, serology and less frequently, virus isolation. Previous studies have described the sensitivity of commercial NS1

assays in patients with dengue [25-28]. These studies have shown this antigen is a promising diagnostic marker but further evaluation in different settings is required. Outstanding questions exist for existing NS1 assays as diagnostic tools and these include their sensitivity across different serotypes, viral burdens and DENV reactive-IgG/IgM status.

5.2. Purposes of the study

The aims of this study was to evaluate the diagnostic utility of a commercial NS1 dengue antigen-capture ELISA test kit produced by BioRad Ltd (Platelia DENGUE NS1 AG, herein referred to as Platelia NS1). Diagnostic accuracy was assessed via specificity and sensitivity across viral burdens, different infecting serotypes, IgG/IgM status and infection status.

5.3. Methods

5.3.1. Patient recruitment

This study was performed in Vietnam as part of the DENCO study (Dengue case classification and case management). The DENCO study was a prospective clinical study characterizing the clinical features of dengue in patients in Asia (Thailand, Vietnam, Philippines, Malaysia) and the Americas (Brazil, Venezuela, Nicaragua). In Vietnam, the study enrolled participants admitted to CH #1, CH #2, or HTD from August 2006 to March 2007. Participants were eligible for entry to the study if they had a history of fever of less than 7 days and there was a clinical suspicion of dengue. Exclusion criteria were patients with signs and symptoms of dual acute infections, or lack of informed consent. Clinical classifications were defined according to WHO criteria. In this study, day of onset of illness was defined as day 0 and was self-reported by the patient or patient's parents/guardian.

5.3.2. Sample collection and study procedures

Details of procedure of sample collection are described in Chapter 2. Patients' blood samples collected at the enrollment day (admission samples) were tested with quantitative serotypic TaqMan RT-PCR, virus isolation using C6/36 cell line and NS1 diagnostic investigations.

Paired specimens from enrollment and convalescence phase were tested in IgM and IgG capture ELISA assays. NS1-ELISA accuracy indexes were assessed based on sensitivity, specificity, negative and positive predictive values (NPV and PPV).

5.3.3. The dengue diagnosis reference algorithm

No single assay can diagnose all dengue patients in the acute, febrile stage of illness. Consequently, we use an algorithm that uses results from 3 assays- **a)** RT-PCR detection of DENV RNA in plasma, **b)** viral culture and **c)** serological changes in DENV reactive IgM and IgG levels in paired plasma specimens. A diagnosis of confirmed dengue was made if the PCR was positive and/or virus culture positive. Acute dengue confirmed by serology if

- IgM sero-conversion was recorded (i.e. from negative to positive), or
- Rising levels of IgM were detected (IgM Units at discharge (IgM2) > 1.2 x IgM Units at admission (IgM1) and IgM2 > 20 Units) or
- IgG sero-conversion and positive for IgM1 or IgM2.

Samples with IgM positive in a single specimen or IgM positive in paired specimens but no significant rise in Units and no IgG sero-conversion were diagnosed as "recent dengue". Patients with paired samples collected at least 3 days apart with the 2nd sample collected ≥7 days after illness onset and with no virological or serological evidence of DENV infection were defined as "no evidence of acute or recent dengue". A schematic showing the reference algorithm for laboratory confirmation of dengue is shown in **Fig.5.1**.

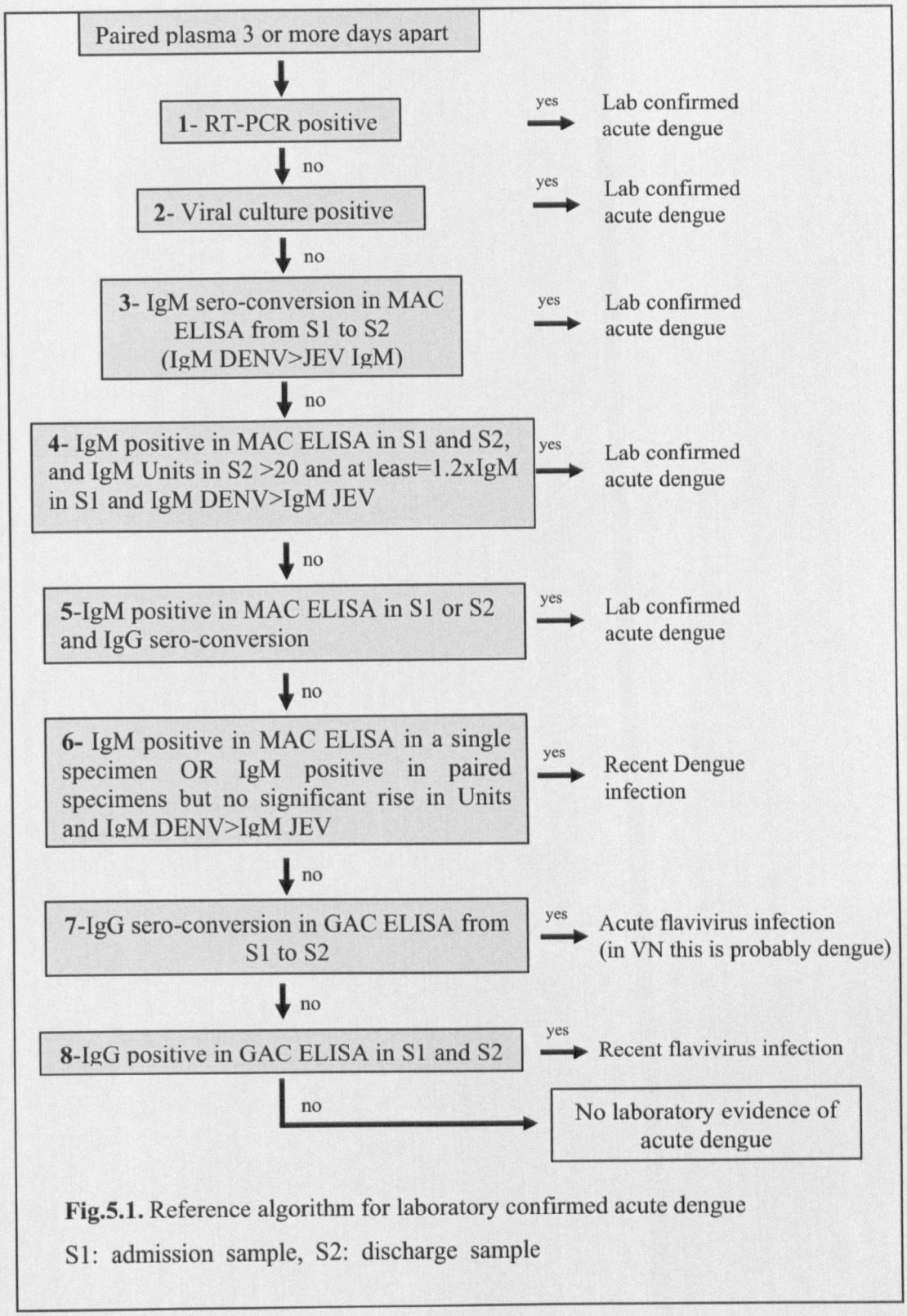


Fig.5.1. Reference algorithm for laboratory confirmed acute dengue

S1: admission sample, S2: discharge sample

Primary and secondary dengue cases were defined according to the level of IgG ELISA Units measured on day 6 or day 7 of illness. The IgG cut-offs for determination of primary versus secondary dengue in this ELISA were determined separately against a well recognized reference ELISA (Dr Sutee Yoksan, Centre for Vaccine development, Thailand). Secondary dengue is determined in patients with IgG ELISA Units >10.7 on day 6 of illness or >18.4 Units on day 7 of illness.

5.3.4. Analysis

The diagnostic accuracy of the Platelia NS1 assay was calculated relative to the final diagnosis determined by the reference algorithm: acute dengue or other febrile illness. Platelia NS1 results that were equivocal and remained equivocal after retesting were considered negative. Statistical analysis was performed with Intercooled Stata 9.2 (Stata, TX, USA). Sensitivity, specificity, PPV and NPV were calculated as described in Chapter 2.

5.4. Results

5.4.1. Characteristics of the study population

From August 2006 to March 2007, paired blood samples from 891 suspected dengue patients were collected from hospitals in HCMC; 289 patients were from the CH #1, 302 patients from the CH #2 and 300 patients from HTD. Of 891 patients, 732 (82.2%) had laboratory confirmation of acute DENV infection according to the reference algorithm, of which 22.2% (n=162) were primary dengue, 75.5% (n=553) were secondary dengue and 2.3% (n=17) were indeterminate. Thirty-seven patients had DENV-reactive IgM and/or IgG positive in paired plasma, but with no significant rise in ELISA Units between samples and were defined as having had "recent dengue". There were 122 patients who had no serological or virological evidence of acute or recent dengue and were defined as "other febrile illness" (OFI). The median age (year) in dengue patients was 12 (range, 1-54). Of 732 acute dengue patients, 65.8% (n=482) were children (<15 yrs) and 34.2% (n=250) were adults (≥15 yrs). The median

day after the onset of fever in confirmed dengue patients that enrolment samples were collected was 4 days (range, 1-6). The characteristics of the patient population are described in **Table 5.1.**

Characteristics	Total	Acute dengue	OFI	Recent dengue
No. of patients	891	732	122	37
Sex (M %)	52	54	41	49
Age (year) median (range)	13 (1-54)	12 (1-54)	15 (1-53)	14 (2-37)
Duration of fever (days) at enrolment				
Day 0	2	2	0	0
Day 1	19	11	8	0
Day 2	109	88	21	0
Day 3	310	263	42	5
Day 4	302	266	23	13
Day 5	139	96	25	18
Day 6	10	6	3	1
Day of illness median (range)	4 (1-6)	4 (1-6)	3 (1-6)	5 (3-5)
Primary infection		162 (22.2%)		
Secondary infection		553 (75.5%)		
Indeterminate		17 (2.3%)		

Table 5.1 Baseline table summarizing mean duration of illness at enrollment, serological profile and demographic features of the study population. 732 (82.2%) had laboratory confirmation of acute DENV infection. OFI: Other febrile illness

The 732 confirmed acute dengue cases based on the described algorithm and the overlap of the 3 diagnostic tests (RT-PCR, virus culture and serological assays) is shown in **Fig.5.2.** Six hundred forty eight cases were positive for RT-PCR, 217 were virus culture positive and 629 were confirmed by serology.

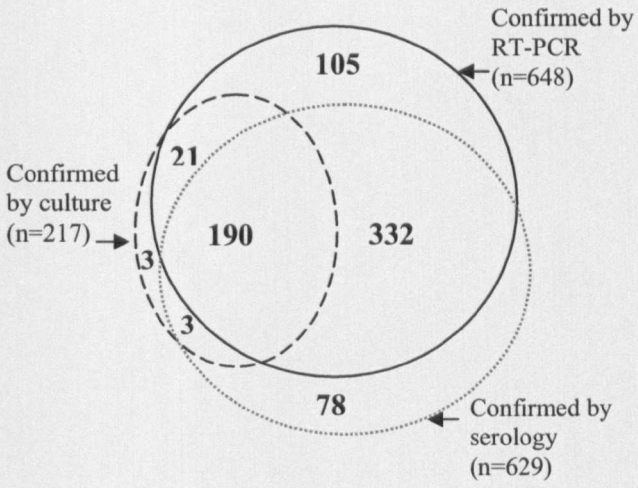


Fig.5.2. Diagram showing dengue cases and the overlap of RT-PCR, virus culture and serology assays. Three cases were confirmed by culture only, 105 by RT-PCR only and 78 by serology only; Three hundred fifty six cases were confirmed by two assays (332 by serology and RT-PCR, 21 by RT-PCR and culture, and 3 by culture and serology) and 190 cases were overlapped by 3 assays.

5.4.2. DENV serotype prevalence

Of 648 DENV infections identified by TaqMan RT-PCR (88.5%), DENV-1 was the dominant serotype, accounting for 53.2% of all RT-PCR confirmed infections (n=345). The second most prevalent was DENV-2 with 35.5% (n=230), then DENV-3 with 10.2% (n=66). DENV-4 was detected in just 1.1 % (n=7) of all RT-PCR confirmed infections. Eighty-four samples (11.5%) were of unknown serotype. A frequency of individual DENV serotype is summarised in **Fig.5.3**.

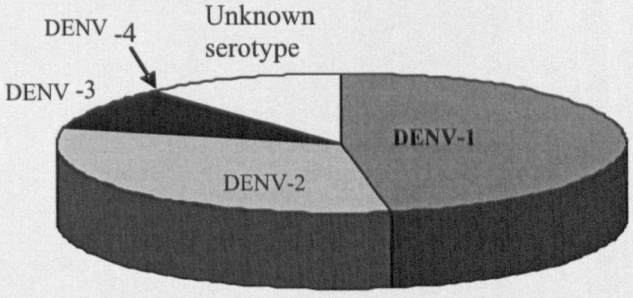


Fig.5.3. Chart of serotype prevalence in the study population detected by RT-PCR positive. DENV-1 was the most prevalent serotype (53.2%); the second most prevalent was DENV-2 (35.5%), then DENV-3 (10.2%). DENV-4 was the least prevalent (1.1%).

5.4.3. Evaluation of the Platelia NS1 accuracy for the diagnosis of dengue

Platelia NS1 antigen-capture ELISA was performed on enrolment samples from all 891 patients in the study. Five-hundred twenty three samples were positive for NS1, 341 were NS1 negative and 27 samples were equivocal. Amongst 27 equivocal samples, 13 samples remained equivocal and 14 samples became negative after a second run; for the purposes of analysis equivocal samples were regarded as negative for NS1. Of 523 NS1 positive samples, 500 were from patients with laboratory confirmed acute dengue (273 were DENV-1, 134 were DENV-2, 53 DENV-3, 4 DENV-4 and 36 of unknown serotype), 23 were from patients with "recent dengue" according to the algorithm. Of 368 NS1 negative samples, 122 were diagnosed as OFI, 232 were acute dengue and 14 were recent dengue.

The sensitivity and specificity of Platelia NS1 was assessed relative to the "acute dengue" classification by the reference algorithm. The overall sensitivity of Platelia NS1 on enrolment specimens was 68.3% (500/732) (95% CI: 64.8–71.7). The NS1 positivity was consistently lower than RT-PCR when matched with day of illness (**Fig.5.4**). Against RT-PCR as a reference test, the overall Platelia NS1 sensitivity was 71.7% (465/648) (95% CI: 68.1-75.2). None of plasma samples from patients with no laboratory evidence of acute dengue (n = 122) were NS1 antigen positive. Specificity of Platelia NS1 in acute plasma was 100% (**Table 5.2**).

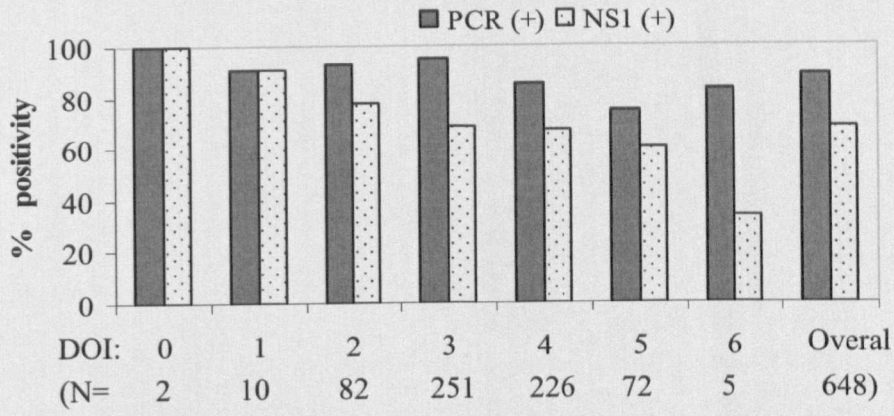


Fig.5.4. PCR and NS1 positivity by day of illness. Graph shows NS1 positivity by day of illness and overall NS1 positivity in test sample comparative to RT-PCR. NS1 positivity was consistently lower than RT-PCR. DOI: Day of illness.

Platelia NS1 sensitivity in samples from patients enrolled on day 0 of illness was 100% (2/2). Sensitivity gradually declined with increasing day of illness (**Table 5.2**). The sensitivity, specificity and PPV, NPV with 95% CI of Platelia NS1 assay against the reference algorithm by day of illness is summarised in **Table 5.2**.

Day of illness	Acute Dengue	NS1 (+)	Sensitivity % (95% CI)	Specificity % (95% CI)*	PPV % (95% CI)*	NPV % (95% CI)
Day 0	2	2	100 (22.3-100,0)		100.0(15.8- 100.0)	
Day 1	11	10	90.9 (58.7-99.7)	100.0 (68.8-100.0)	100.0 (77.9-100.0)	88.9 (51.7-99.7)
Day 2	88	68	77.3 (67.1-85.5)	100.0 (86.7-100.0)	100.0 (95.7-100.0)	51.2 (35.1-67.1)
Day 3	263	181	68.8 (62.8-74.4)	100.0 (93.1-100.0)	100.0 (98.4-100.0)	33.8 (25.6-42.9)
Day 4	266	179	67.3 (61.3-72.9)	100.0 (87.8-100.0)	100.0 (98.3-100.0)	20.9 (13.7-29.7)
Day 5	96	58	60.4 (49.9-70.3)	100.0 (88.7-100.0)	100.0 (94.9-100.0)	39.7 (27.5-52.7)
Day 6	6	2	33.3 (43.3-77.7)	100.0 (36.8-100.0)	100.0 (22.4-100.0)	42.8 (10.0-81.5)
Total	732	500	68.3 (64.8-71.7)	100.0 (97.6-100.0)	100.0 (99.4-100.0)	34.5 (29.5-39.6)

*: One-sided, 95% confidence interval

Table 5.2 Diagnostic accuracy for NS1-ELISA stratified by day of illness. Overall NS1 sensitivity of NS1 is 68.3%.

Sensitivity of Platelia NS1 in patients enrolled within ≤ 72 hrs of illness duration were significantly higher than in patients enrolled at later times [sensitivity (95% CI); 79.2% (69.9–86.6), n=101 versus 66.5% (62.7–70.2), n=631, *P=0.01*]. This difference was strongly associated with viraemia levels, as viraemia level of test sample collected ≤72hrs was significantly greater than >72hrs [median log10 cDNA equivalents (SD); 8.12 (1.67) versus 6.47 (1.77), *P<0.0001*].

5.4.4. NS1 sensitivity in relation to viraemia levels

Of 732 acute DENV infections, viraemia, expressed as cDNA equivalents/ml plasma, was measured in 648 samples. Viraemia was highest at day 1 of illness (n=10) then gradually declined on the following days (2, 3, 4, 5 and 6). The overall decreasing of viraemia level over the acute phase of illness is illustrated in **Fig.5.5.**

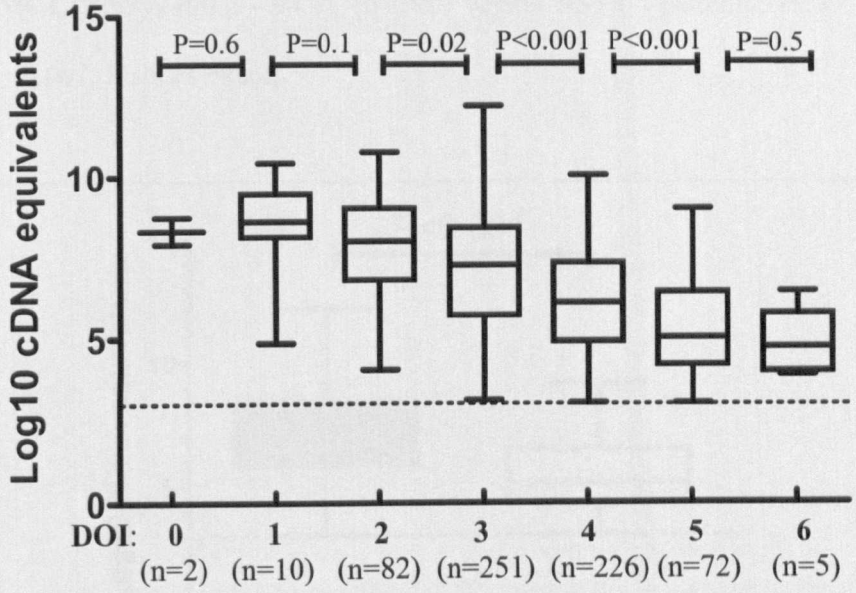


Fig.5.5. Viraemia levels in test samples on enrolment day. Shown is median (interquartile and range) viraemia level in patient samples on enrolment day. The limit of detection of the assay is shown with a dashed line. (DOI: Day of illness)

We hypothesised that plasma viraemia levels would be associated with the detection of plasma NS1, since NS1, like virions, is a product of infected cells. Accordingly, viraemia levels were significantly higher in patients who were NS1-positive at the time of study enrolment (n=464) versus those who were negative for NS1 (n=184) [median (SD): 7.27 (1.72) versus 5.12 (1.45), *P<0.001*, Mann-Whitney test] (**Fig.5.6**). Furthermore, patients who were NS1 positive were also more likely to be RT-PCR positive [ratio of RT-PCR (+) in NS1 positive patients (95% CI); 93% (90.2 - 94.9), (n=500) versus NS1 (-) patients; 79.3% (73.5 - 84.3), (n=232), *P<0.001*, Fisher's exact].

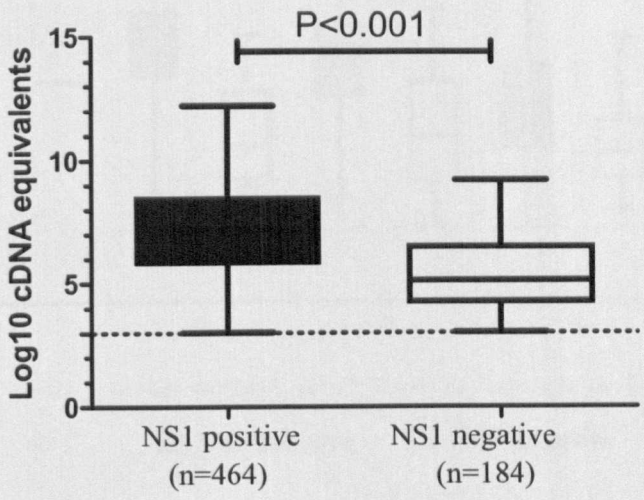


Fig.5.6. Viraemia levels by NS1 status tested by the Platelia ELISA. Shown is the median (interquartile and range) viraemia level in NS1 positive (n=464) and NS1 negative (n=184) patients with a measurable viraemia level at the time of study enrolment. The limit of detection of the assay is shown with a dashed line. Viraemia levels were significantly higher in NS1 positive patients relative to NS1 negative patients (Mann-Whitney test).

The NS1 sensitivity relative to viraemia levels in test sample compared by day of illness (day 1, 2, 3, 4 and 5) was also consistently higher in NS1-positive patients (*P=0.1* for day 1 and *P<0.001* for day 2 to day 5) suggesting that NS1 sensitivity reduced concomitantly with reduced viraemia level and with increased illness duration. Viraemia levels according to NS1 status in dengue patients by day of illness is shown in **Fig.5. 7.**

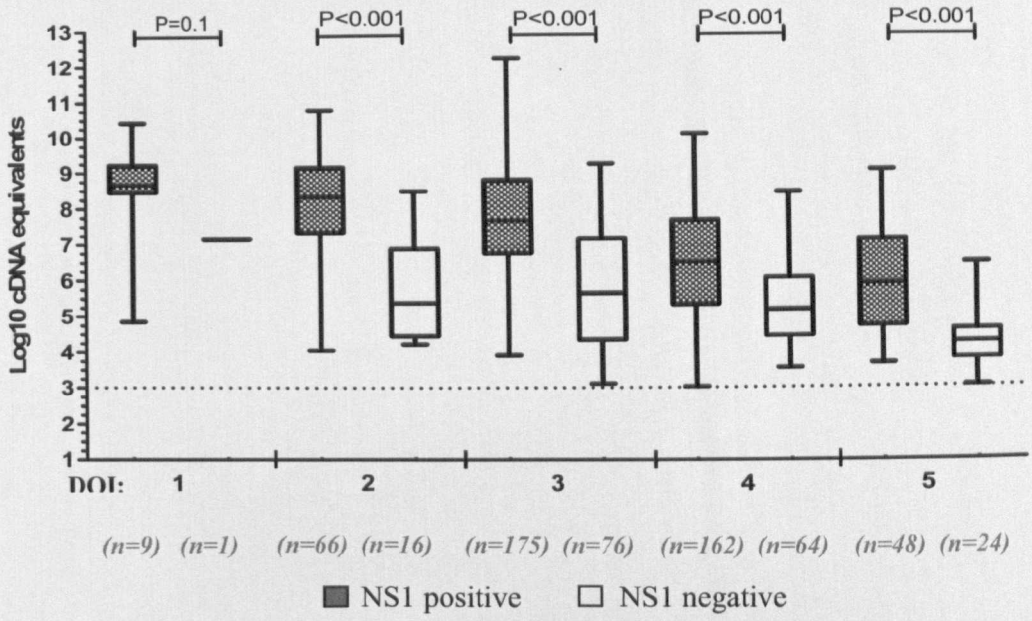


Fig.5.7. Viraemia levels by NS1 status in dengue patients compared by day of illness. Median viraemia level in NS1 positive cases is consistently higher than in NS1 negative cases on the same day of illness. Viraemia levels at day 0 and day 6 were not shown in the graph as sample size was small for statistical analysis (n=2 and 5, respectively). (n= Number of samples in each NS1 status group, DOI: Day of illness)

The association between NS1 sensitivity and viraemia levels was consistently observed in individual infecting serotypes. In patients infected with DENV-1 (n=345), DENV-2 (n=230) or DENV-3 (n=66), viraemia levels in those who were positive for NS1 were significantly higher than in those who were negative for NS1 on the enrolment day (*P<0.001*) (**Fig.5.8**).

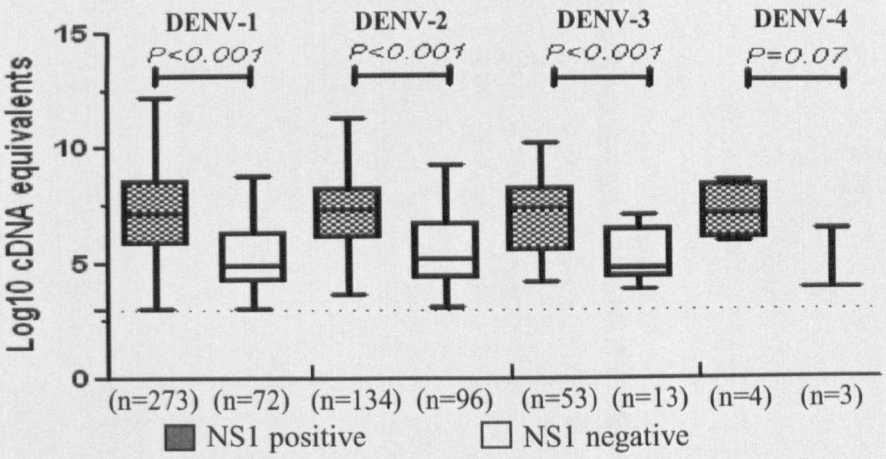


Fig.5.8. Viraemia levels by NS1 status relative to DENV serotypes. Box and whisker plot shows viraemia levels by NS1 status in enrolment sampled from patients infected with each of the 4 DENV serotypes. Viraemia in those who were NS1 positive were consistently higher than in those who were NS1 negative. The difference is statistically significant in patients infected with DEN-1 (n-345), DENV-2 (n=230) and DENV-3 (n=66). Numbers below the chart indicate number of samples in NS1 positive or negative group in individual serotypes. Hatched blocks are NS1 positive, open blocks are NS1 negative.

5.4.5. NS1 sensitivity in relation to immune response status

5.4.5.1. NS1 sensitivity in primary or secondary infection

Shown in **Table 5.3** is NS1 sensitivity relative to immune response status. Overall, NS1 sensitivity was higher in patients with primary dengue (85.2%) than secondary dengue (63.3%). Reduced NS1 sensitivity in secondary infection was not associated with the duration of illness, as at the time of testing, illness day in secondary cases was non-significantly greater than primary cases (3.5 versus 3.4, *P=0.4*, **Table 5.3**). Reduced sensitivity in secondary

infection was also not statistically associated with viraemia levels between primary and secondary cases [secondary log10 viraemia; 6.7 versus primary, 6.8, *P=0.1*, **Table 5.3**].

Sample group	Mean DOI (SD)	*P value* [a]	Viraemia median (SD)	*P value* [a]	NS1 (+)	NS1 sensitivity% (95%CI)	*P value* [b]
Primary infection (n=162)	3.4 (1.0)	*0.4*	6.8 (1.9) (n=147)	*0.1*	138	85.2 (78.8-90.3)	*< 0.0001*
Secondary infection (n=553)	3.5 (0.9)		6.7 (1.7) (n=484)		350	63.3 (59.1-67.3)	

[a]: Mann-Whitney test; [b]: Fisher's exact test; DOI: Day of illness

Table 5.3 Sensitivity of Platelia assays in patients with primary or secondary infection. Sensitivity in secondary cases is significantly lower than in primary cases (63.3% vs. 85.2%, *P<0.0001*). This reduced sensitivity is not associated with day of illness (secondary, 3.5 versus primary, 3.4, *P=0.4*) or viraemia levels in the test samples (secondary dengue, 6.7 versus primary, 6.8, *P=0.1*).

5.4.5.2. Influence of IgM and IgG responses on NS1 sensitivity

In general, NS1 sensitivity in samples from patients with measurable DENV-IgG (in GAC ELISA) was significantly lower than in samples from patients without measurable DENV-IgG (39.7% versus 86.2%, *P<0.001*, Fisher's exact), whereas IgM positivity (MAC ELISA) in test sample was not significantly associated with NS1 sensitivity [NS1 sensitivity in IgM (-), 69.3% versus IgM (+), 67.5%, *P=0.6*, Fisher's exact]. Collectively, IgM responses did not affect detection rate of NS1 in the Platelia assay. In contrast, NS1 sensitivity was significantly reduced in the presence of measurable DENV reactive-IgG in test sample. This significant NS1 sensitivity reduction in non-measurable DENV-IgG was consistently observed when test sample were matched by day of illness [NS1 sensitivity in IgG (+) versus IgG (-), %: 28.5 versus 55.5 for day 2; 40.8 versus 67.6 for day 3; 35.8 versus 81.6 for day 4; and 30.4 versus 85.7 for day 5; *P< 0.005* for all]. No sample on day 0 or day1 was positive for IgG. Results from day 6 were not shown because number of samples was small, n=7. Shown in **Table 5.4** is NS1 sensitivity in relation to IgM/IgG response status.

NS1 result / Immune response	Total	NS1 (+)	Sensitivity % (95% CI)	*P value* (Fisher's exact)
Measurable IgM	419	283	67.5 (62.8 - 72)	
Non-measurable IgM	313	217	69.3 (63.9 - 74.4)	*P=0.6*
Measurable IgG	282	112	39.7 (34.0 - 45.7)	
Non-measurable IgG	450	388	86.2 (82.7 - 89.3)	*P <0.001*

Table 5.4 NS1 sensitivity relative to DENV-reactive IgM/IgG status in the test samples. NS1 sensitivity in IgG (-) samples was significantly greater than in IgG (+) samples while there is no significant difference in NS1 sensitivity by IgM response status.

Reduced NS1 sensitivity in the presence of DENV-reactive IgG was also consistently observed in the context of individual infecting serotypes. NS1 sensitivity in samples from both DENV-1 and DENV-2 infected patients with non-measurable DENV-IgG was significantly greater than in patients with measurable DENV-IgG (NS1 sensitivity in IgG (-) versus IgG (+); DENV-1, 92.8% versus 48.1%, *P<0.001*; DENV-2; 76.5% versus 33.6%, *P<0.001*). This difference was of borderline significance for DENV-3 (85.7% versus 66.6%, *P=0.09*). Result for DENV-4 was not shown as the sample size was inadequate for statistic analyses (n=7). An illustration of influence of IgG response status on NS1 sensitivity in DENV-1, DENV-2, and DENV-3 is shown in **Fig.5.9**.

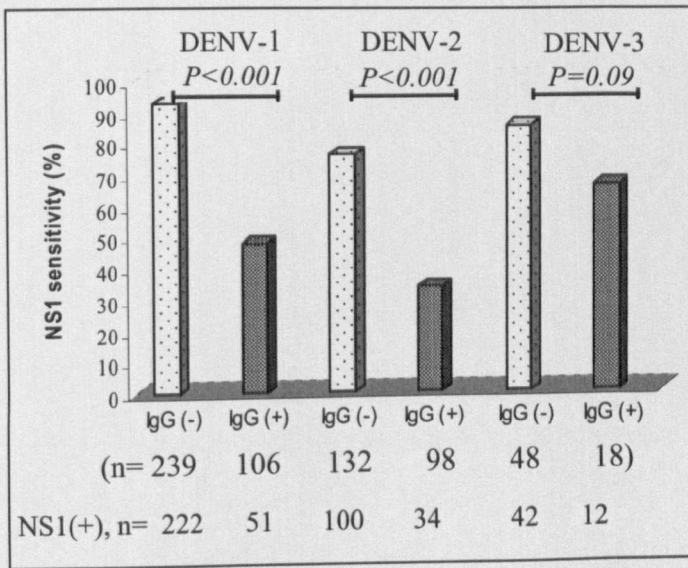


Fig.5.9. NS1 sensitivity by DENV-IgG status in individual serotypes. Shown is NS1 sensitivity by DENV-IgG status in test sample across individual infecting serotypes. NS1 sensitivity in IgG (-) samples is consistently higher than in IgG (+) in 3 DENV-1, -2 and DENV-3. (n: Number of samples in each IgG group)

5.4.6. Influence of infecting DENV serotype on sensitivity of Platelia NS1

The DENV serotype was characterized in 88.5% (648/732) of acute dengue patients. Detection rate of NS1 in DENV-3 or DENV-1 infected patients was significantly higher than in DENV-2 infected patients (**Fig.5.10.A**, Fisher's exact). NS1 sensitivity in DENV-4 was also non-statistically lower than DENV-1 or DENV-3 (*P=0.1*, **Fig.5.10.A**, Fisher's exact). One possible explanation for this difference was the immune status in these individuals. To investigate this assumption, we analysed proportion of secondary and primary infection in individual serotypes. The proportion of secondary infection in patients infected with DENV-2 (88%) was statistically higher than either DENV-1 (65%) or DENV-3 (74%), [**Fig.5.10 B**, Fisher's exact]. There was also a statistically greater proportion of DENV-2 infected patients having measurable IgG in the test samples than either DENV-1 or DENV-3 (**Fig.5.10.C**, Fisher's exact). Proportion of secondary cases in DENV-4 was also higher than DENV-1 or DENV-3, though this was not statistically significant [**Fig.5.10 B,** (*P=0.1* and *P=0.06,* Fisher's exact)]. Moreover, the percentage of patients infected with DENV-4 having measurable reactive IgG was significantly greater than DENV-1 or DENV-3 (*P=0.001*, **Fig.5.10.C**, Fisher's exact). This lower NS1 sensitivity in DENV-2 and DENV-4 was associated with viraemia levels, as viraemia levels in DENV-2 or DENV-4 was lower than DENV-1 or DENV-3, though this was not statistically significant. [log10viraemia, median (SD): DENV-2: 6.48 (1.79), DENV-4: 6.13 (1.75), DENV-1: 6.80 (1.86), and DENV-3: 6.68 (1.69), *P=0.2*, Kruskal–Wallis test].

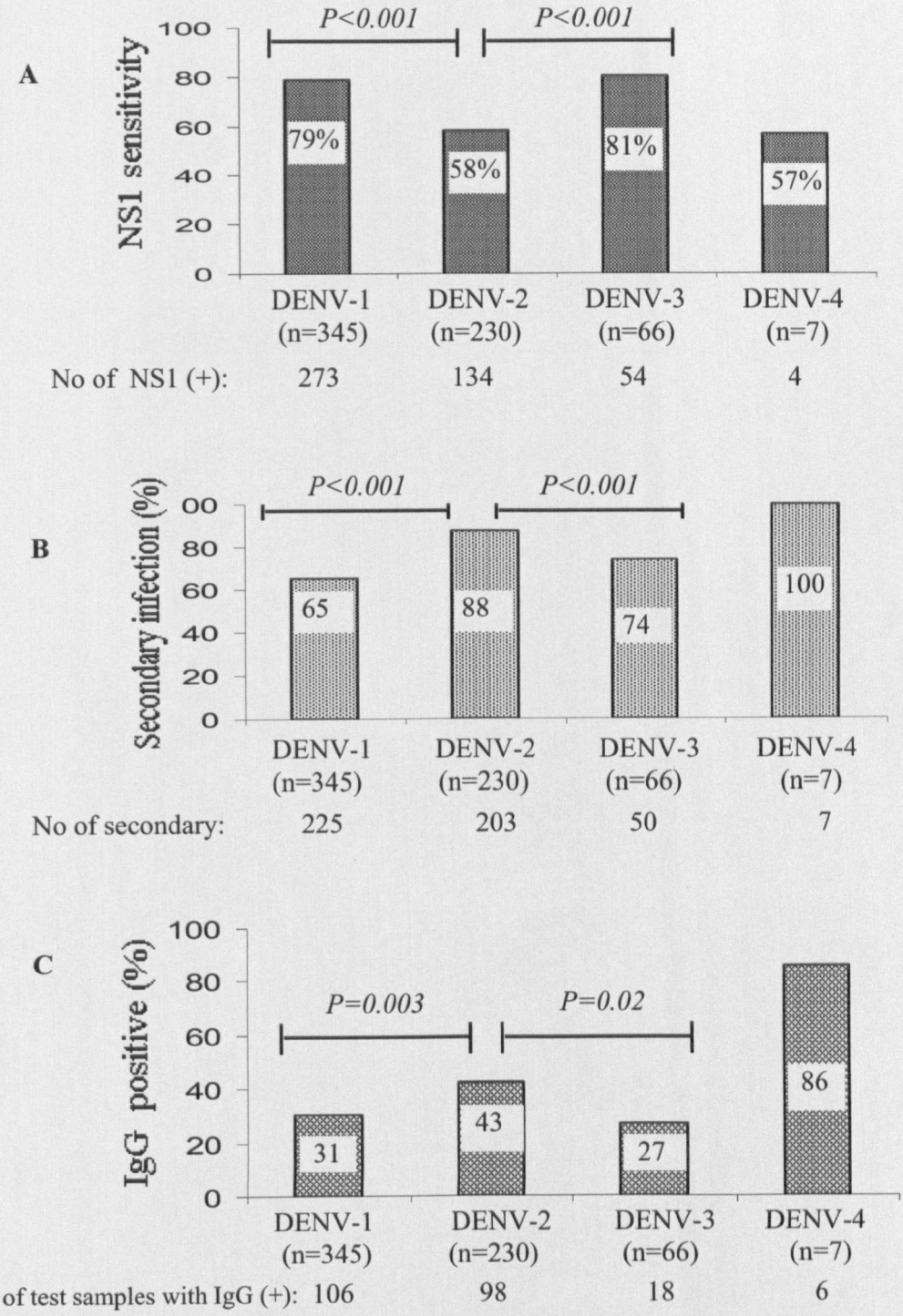


Fig.5.10. NS1 sensitivity in relation to immune response status. Shown in (**A**) is NS1 sensitivity in test samples at the enrollment in individual infecting serotypes. NS1 detection rate in DENV-2 is significantly lower than in DENV-1 or DENV-3. Proportion of secondary cases (**B**) or patients with DENV reactive-IgG measurable (**C**) in DENV-2 infected patients is significantly greater than DENV-1 or DENV-3. NS1 detection rate in DENV-4 (57%) is also lower than DENV-1 or DNEV-3, though this is not statistically significant.

However, low NS1 sensitivity in DENV-2 or DENV-4 was not affected by the difference in the duration of illness at the time of sampling, as mean illness day in DENV-2 infected patients was significantly shorter than either DENV-1 or DENV-3 [DENV-2 illness day; mean (SD), 3.28 (0.88) versus 3.50 (0.96), *P=0.008* or 3.52 (0.91), *P=0.03*]; and illness day in DENV-4 infected patients was non-significantly longer than either DENV-1 or DENV-3 [3.71 (1.25) versus 3.50 (0.96), *P=0.7* or 3.52 (0.91), *P=0.8*]. Thus, reduced sensitivity in DENV-2 or DENV-4 infected patients was probably due to an increased proportion of patients having DENV-reactive IgG in their test samples (indicative of secondary infection). Furthermore, there would be other factor impacting on lower NS1 sensitivity in DENV-2. To test this hypothesis, we analyzed Platelia NS1 sensitivity relative to DENV serotype in patient samples that satisfied 2 conditions: primary infection and negative for DENV-reactive IgG. This further analysis revealed that the detection rate for NS1 in DENV-2 (n=20) was significantly lower than either DENV-1 (n=99) or DENV-3 (n=14) [DENV-2 NS1 sensitivity (95%CI): 56.5% (34.5-76.8) versus DENV-1: 92.6% (86.0-96.7) or DENV-3: 73.3% (44.8-92.2), *P<0.0001*, Pearson test]. No primary case belonged to DENV-4. In brief, besides immune response status, probably the mAb used for capture and detecting NS1 had lower affinity to DENV-2 NS1 which contributes to lower NS1 sensitivity in DENV-2.

5.4.7. Relative associations with NS1 antigenaemia in test sample

Since Platelia NS1 sensitivity was associated with many factors in univariate analysis; IgG status in test sample, viraemia level, infecting serotype, and number of day of illness; we then further investigated the influence of these variables on the NS1 sensitivity via logistic regression analyses (expressed with odds ratio - OR). Shown in **Table 5.5** are positive and negative associations with NS1 antigenaemia. The absence of DENV reactive IgG from test sample was strongly positively associated with NS1 sensitivity [IgG (-): OR (95% CI), 9.97 (6.05-16.39), *P<0.001* versus IgG (+): 0.1 (0.07-0.15), *P<0.001*], whereas IgM showed no

influence on NS1 sensitivity (OR IgM (-) (95% CI) versus IgM (+), 1.08 (0.79-1.49), *P=0.6* versus 0.92 (0.67-1.26), *P=0.6*). To a lesser extent, primary infection and higher viraemia levels were also positively associated with NS1 sensitivity. In contrast, secondary infection and duration of illness prior to test sample collected were negatively associated with NS1 sensitivity. In relation to infecting serotypes, DENV-2 [OR (95% CI), 1.86 (1.12-3.08), *P=0.01*] showed less positive association with NS1 sensitivity than DENV-1 and DENV-3 [OR (95% CI), 5.05 (3.05-8.36), *P<0.001* and 5.43 (2.58-11.44), *P<0.001*; respectively]. DENV-4 was not included in the logistic regression as the sample size of DENV-4 infected patients was small (n=7).

Positive and negative associations with NS1 antigenaemia			
Variables	Odds Ratio	95% CI	*P value*
IgG (-)	9.97	6.05 - 16.39	
IgG (+)	0.1	0.07 - 0.15	*<0.001*
IgM (-)	1.08	0.79 - 1.49	
IgM (+)	0.92	0.67 - 1.26	0.6
Primary infection	3.33	2.09 - 5.32	
Secondary infection	0.29	0.18 - 0.48	*<0.001*
Viraemia level	2	1.68 - 2.17	*<0.001*
PCR (+)	5.65	3.09 - 10.34	*0.001*
DENV-1	5.05	3.05 – 8.36	*<0.001*
DENV-2	1.86	1.12 – 3.08	*0.01*
DENV-3	5.43	2.58 - 11.44	*<0.001*
Number of illness days	0.77	0.65 - 0.91	0.002

Table 5.5 Positive and negative associations with NS1 antigenaemia. Shown are factors with associations with NS1 sensitivity in test sample. Absence of IgG from test sample, host primary infection and viraemia are factors associated with increased NS1 sensitivity.

5.5. Discussion

In this study, we report the assessment of diagnostic accuracy of Platelia NS1 assay conducted in children and adults admitted to HCMC hospitals from August 2006 to March 2007. This assessment includes sensitivity and specificity spanning across different serotypes, host infection status; viraemia levels, and duration of illness. The results from this study show that NS1 detection by means of Platelia NS1 assay provides a highly specific (100%) and satisfactorily sensitive approach to dengue diagnosis using a single specimen.

In univariate and multivariate analysis, we demonstrated NS1 sensitivity was associated with viraemia level detected by TaqMan RT-PCR. Patients who were NS1 negative had significantly lower median viraemia levels than NS1 positive patients with the same duration of illness history. In particular, patients who presented within ≤72hrs and who were NS1 positive had viraemia levels much greater than in those who were NS1 negative. These data suggests that NS1 antigenaemia is the reflection of the viraemia levels.

The assessment of NS1 sensitivity using the Platelia NS1 assay relative to day of illness has been conducted in both children and adults in Thailand and showed no association between the sensitivity and patient's duration of illness for Platelia NS1 [26]. Unlike this previous study, our results show that NS1 detection rate decreases with increased duration of illness. This probably because NS1 decays over the infection time, leading to decreased NS1 level in the blood stream. Another possible explanation is that increased Igs in the blood stream over acute phase may reduce free plasma NS1 due to Igs-sequestered NS1. In fact, Libraty et al. have shown plasma sNS1 concentration peaking at day 3 to day 4 of illness then declining to undetectable at day 7 to 8 in both DF and DHF secondary sera [23].

In agreement with previous studies [26, 29], the results of this study demonstrate that the Platelia NS1 assay is less sensitive in secondary dengue cases. This is also consistent with our finding of reduced NS1 sensitivity in test sample with a measurable level of anti-DENV IgG

antibodies. Furthermore, as reported in previous studies [21, 29, 140], unlike DENV-IgG antibodies, detection of NS1 antigen was not influenced by the presence of DENV-IgM antibodies in the test sample. Study of the profiles of NS1-specific antibody in primary and secondary patient's sera has revealed a higher level of NS1 specific-antibody in secondary sera than primary sera [156]; thus, a possible explanation for reduced NS1 sensitivity in secondary immune response is that plasma NS1 is sequestered in immune complexes and that target epitopes are not accessible to either the plate-bound or probe mAb in the NS1 ELISA. Indeed, efforts to dissociate immune complexes can enhance the sensitivity of the Platelia NS1 assay [26, 140].

Studies of the sensitivity and specificity of the Platelia ELISA have been previously conducted in different settings [24, 26-30, 157], some of these assessments (French Guiana [28, 30]; Malaysia [27]) have reported a relatively higher sensitivity for Platelia NS1 (88% and 93%) than the results reported in this study. Given that the sensitivity was affected by infection status, it is possible that the lower NS1 sensitivity for Platelia reported here was partly due to high proportion of secondary infection in this hospitalized-based study (76% secondary dengue cases in the study population), concomitant with greater proportion of samples with measurable DENV-reactive IgG. Our study is more consistent with the reported sensitivity of Platelia NS1 (63%) from Thailand where most cases were secondary dengue [26].

The Platelia NS1 assay showed higher sensitivity for DENV-1 or DENV-3 than DENV-2. It is possible that the higher proportion of secondary cases with DENV-2 is the cause of this result. However, this cannot be the only reason for low Platelia NS1 sensitivity in our DENV-2 study population. Our further assessment of Platelia NS1 sensitivity in the test sample from cases with primary infection and negative for DENV-IgG in the test sample revealed lower rates of NS1 detection for DENV-2. An inherent lower sensitivity for DENV-2 NS1 was supported by the logistic regression analysis in which the odds of being NS1 positive was lowest even after

including other factors such as day of illness, viraemia and serological status in the test sample. A possible explanation for lower sensitivity in DENV-2 infections could be lower avidity of the mAbs in the assay for the target epitope(s) in DENV-2 NS1. This serotype-related difference in sensitivity may reduce the utility of this assay in investigations of DENV-2 endemic or epidemic areas.

Another study conducted in Puerto Rico has shown lower NS1 sensitivity for DENV-4 for Platelia NS1 [29]. This is consistent with our finding of low Platelia -NS1 sensitivity for DENV-4, although the number of samples was inadequate for statistic analyses, because DENV-4 was present at a very low frequency in this study.

Similar to previous studies [21, 140], we demonstrated that NS1 can be detected in patients whose blood sample is negative by RT-PCR. The most likely explanation for this is that bloodstream NS1 clearance kinetics is different to those of the RNA genome.

In summary, our study confirms that as no single diagnostic assay is adequately sensitive and specific enough to diagnose all acute cases of dengue at every time point during the acute illness. DENV-NS1 antigen detection based on ELISA principle provides an alternative tool to diagnosis for acute dengue. The ease of this assay and relatively modest cost (~ $5 per sample) make it feasible to perform in most routine hospital laboratories in southern Vietnam. NS1 detection would be a diagnostic choice for improving dengue diagnosis in the first few days of illness before DENV IgM is measurable. Importantly, our results suggest very high specificity for the Platelia NS1 assay. Though results from this study reveal a high specificity for the Platelia assay; other brands of NS1 assay also need to be evaluated. Such evaluations of NS1 sensitivity and specificity should be conducted on a wide range of patients in different settings in order to recognize the strengths and weaknesses of these tests.

Chapter 6

6. Assessment of the utility of NS1-LFRT versus Platelia NS1 ELISA for diagnosis of acute dengue virus infection

6.1. Introduction

Timely, sensitive, and specific diagnosis of DENV infection can assist in patient management. Prompt diagnosis of index cases can also facilitate vector control activities in the community so as to mitigate further transmission. The availability of commercial ELISA assays to detect the DENV NS1 protein in acute plasma provides an additional dengue diagnostic tool to the existing approaches of PCR, serology and, less frequently, virus isolation [25-30, 157]. The assessment of NS1 assays as a diagnostic tool across a wide range of patient populations and viral serotypes is an important part of the process of identifying where these assays may fit into existing dengue diagnostic algorithms.

In dengue epidemic areas, a convenient, rapid, and reliable diagnosis for acute dengue will bring a quick and appropriate point-of-care decision. RT-PCR, with a required-stringent workflow, is not widely accessible. The NS1-antigen ELISA has shown to be suitable for early diagnosis with considerably high sensitivity, (83.2% [29]; 93.4% [27]) and specificity (100% [27, 28]), and could be considered as first-line testing for acute dengue diagnosis in a single serum sample [27, 28] but it can only be carried out in well-equipped healthcare services. In a primary healthcare center with minimal laboratory apparatus, an accurate, rapid and simple diagnosis is an important strategy for acute dengue diagnostic. Rapid tests provide an alternative in such a setting. The most significant advantage of this system is ease of application and they are the least time-consuming.

The sensitivity and specificity of NS1-LFRT assay have been previously assessed in several settings [30, 158]. These studies have shown this approach is an improvement for a rapid and accurate diagnosis for acute dengue, and could be used in routine clinical practice. However, further evaluation of this assay in different settings and different ethnic profiles is needed. In this context, the purpose of this study was two-fold. First, to compare the sensitivity and specificity of a lateral flow rapid test (NS1-LFRT) versus the Platelia ELISA, in the context

of different viral serotypes, viral burdens, day of illness, and clinical presentations in Vietnamese patients. Second, to assess the specificity of these NS1 assays in patients with other confirmed infections.

6.2. Materials and Methods

6.2.1. Patient enrolment

A series of prospective clinical research studies on dengue are in progress at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam. Patients greater than 2 years of age admitted to one of the intensive care units (adult or paediatric) or to one of the general wards with a clinical suspicion of dengue as their primary diagnosis are eligible for enrolment following written informed consent by the patient or guardian. In this study, day of onset of illness was defined as day 1 and was self-reported by the patient or patient's parents/guardian. All studies have received approval from the Ethics Committee of the HTD and from the Oxford Tropical Research Ethics Committee. All patients in these studies are assessed daily by a study physician and have serial haematocrit and platelet estimations performed, as well as appropriate sampling for diagnostic serology and virology. Other investigations and clinical management are at the discretion of the attending physicians. After discharge each patient is classified using the current WHO criteria for DF, DHF and DSS. Patients with history of DENV infection later than 6 days or patients with dual acute infections of dengue or lack of written informed consent were excluded from the study.

Plasma samples from patients with another confirmed diagnosis (malaria, enteric fever, JE or leptospirosis) were obtained from stored specimens collected as part of other prospective studies at HTD between 2001-2008. The diagnosis of Plasmodium falciparum malaria was made by blood smear. Enteric fever was diagnosed by blood culture of S. Typhi or S. paratyphi. Leptospirosis was diagnosed by positive serology (microscopic agglutination test). Japanese encephalitis was diagnosed by capture IgM and IgG ELISA assays.

6.2.2. Study procedures

To confirm dengue infection, plasma from all suspect dengue cases were sampled at admission and tested with quantitative serotypic TaqMan RT-PCR. Paired specimens from enrollment and convalescence phase were tested for serologic MAC and GAC ELISA. To evaluate the sensitivity and specificity of NS1-LFRT, all admission samples were blindly tested for dengue NS1 antigen by NS1-LFRT and Platelia ELISA according to the manufacturer's instructions. Both NS1 tests were performed with freshly collected acute plasma samples from each patient. Samples that were defined as equivocal in the NS1 Platelia ELISA assay were repeated. If they were still equivocal they were regarded as being negative. Samples that were defined as equivocal in the NS1-LFRT were considered as being negative. Details of NS1-LFRT assay are described in Chapter 2. Shown in **Fig.6.1** is an example of a NS1 LFRT result. Each assay strip was independently assessed twice.

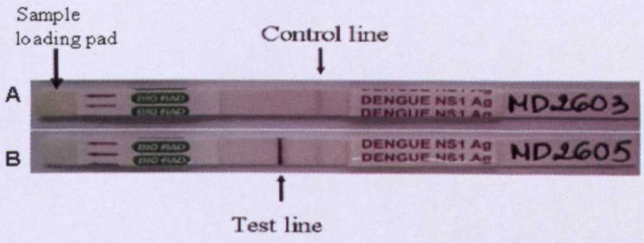


Fig.6.1. An example of NS1-LFRT read. **(A):** negative result, with only Control Line. **(B):** positive result, with Control Line and Test Line.

6.2.3. Dengue diagnostics and result analysis

Diagnostic results for dengue were as described in Chapter 5. Sensitivity, specificity, PPV, and NPV analyses were as described in Chapter 2. The interpretation of primary and secondary serological responses was also as described in Chapter 5.

6.3. Results

6.3.1. Characteristics of the study population

From November 2007 to January 2008, we prospectively tested 138 consecutive acute plasma samples from all children and adults enrolled in these studies. The mean duration of illness prior to the test plasma sample being collected was 3 days (range; 1-6). Patients with a breadth

of disease severities were represented in the study, including patients with DSS. There were 117 patients diagnosed with "acute dengue", and 8 cases diagnosed as "recent dengue" according to the laboratory reference algorithm. Of the "recent dengue" cases, 1/8 had DHF and 7/8 had established DSS. Since the clinical picture was so clear in these patients, and was supported by highly suggestive serology (IgM positive in MAC ELISA in acute and early convalescent plasma samples and IgG titre rising, but not 4-fold rising), we included these patients in the "acute dengue" category, for a total of 125 acute dengue patients. Of 125 acute dengue cases, 11 cases were diagnosed by PCR alone, 14 were diagnosed be serology alone, and 100 were diagnosed by both PCR and serology. There were 13 patients in whom there was no evidence of acute or recent dengue. The baseline characteristics of the study population are shown in **Table 6.1**.

Sample characteristics	Acute dengue (n=125)								OFI
	Clinical severity				Serological status			Overall	
	DF	DHF I	DHF II	DHF III	Primary	Secondary	Indeter-minate		
Number of cases	49 (39.2%)	16 (12.8%)	40 (32.0%)	20 (16.0%)	24 (19%)	93 (74%)	8 (6%)	125	13
Male/Female	1:2.1	1.7:1	1:1.2	1.5:1	2:1	1:1		1:1.2	1:1.2
Median age in year (range)	20 (7-41)	15 (10-42)	14 (6-40)	11 (4-31)	13 (6-39)	17 (4-42)		16 (4-42)	12
Mean day of illness (range)	2.8 (1-5)	3.1 (2-5)	3.5 (1-6)	4.6 (3-6)	3.3 (1-5)	3.6 (1-6)		3 (1-6)	3
DENV-1	29	8	21	5	14	44	5	63	
DENV-2	5	3	8	4	3	17		20	
DENV-3	11	5	6	3	6	16	3	25	
DENV-4	2	0	1	0		3		3	
Serotype not defined	2		4	8	1	13		14	

Table 6.1 Summary of key clinical, viral and demographic information on the study population
OFI: Other febrile illness

6.3.2. Infecting serotype prevalence

DENV serotype was characterized in 111 samples, of which 63 samples were DENV-1, 20 samples were DENV-2, 25 samples were DENV-3, and 3 samples were DENV-4. Fourteen samples were not defined for infecting serotype. A frequency of individual DENV serotype is shown in **Fig.6.2**.

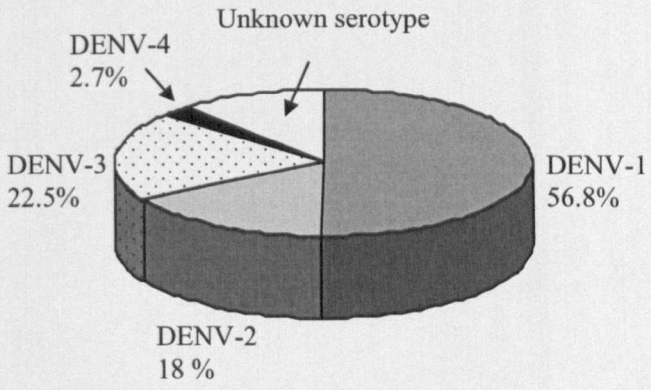


Fig.6.2. Frequency of infecting serotype in the study population

6.3.3. NS1 result description

Platelia NS1 antigen-capture ELISA and NS1-LFRT were completed on 138 patient samples at admission. One-hundred and four samples were positive for Platelia NS1-ELISA, 91 samples were positive for NS1-LFRT. Samples that were equivocal in the Platelia NS1 assays on first testing (n=2) were re-tested; one sample was negative whilst the other remained equivocal and thereafter was regarded as negative. During the study, there were no examples of discordance in the interpretation of any NS1-LFRT. None of plasma samples that had no laboratory confirmation for acute dengue infection (n = 13) reacted with NS1 antigen in both tests. Results of NS1 assay in both assays in 138 samples are summarised in **Table.6.2**.

Diagnosis / NS1 status		Acute Dengue infection					OFI	Total
		DENV-1	DENV-2	DENV-3	DENV-4	Unknown serotype		
ELISA-NS1	NS1 (+)	62	11	24	3	4	0	104
	NS1 (-)	1	9	1	0	10	13	34
LFRT-NS1	NS1 (+)	56	7	22	3	3	0	91
	NS1 (-)	7	13	3	0	11	13	47

Table 6.2 NS1 results in 138 study samples from NS1-LFRT and Platelia NS1 assays. 104 samples are positive with Platelia NS1, 91 samples are positive with NS1-LFRT. None of patient specimens in OFI (n=13) is NS1 reactive in both NS1 assays.

6.3.4. Overall sensitivity of NS1-LFRT versus NS1-ELISA

Overall, the NS1-ELISA assay was more sensitive than the NS1-LFRT test for the diagnosis of acute dengue relative to the reference algorithm (83% versus 73%, *P=0.047*; **Table 6.3**). Against RT-PCR, the sensitivity of the NS1-ELISA was also greater than the NS1-LFRT (90% versus 79%, *P=0.025*; **Table 6.3**). There were 4 and 3 patients who were positive in the NS1 ELISA assay and NS1-LFRT respectively who were negative in the RT-PCR.

NS1 sensitivity against reference algorithm								
NS1 assays	Total analysis	Acute dengue	NS1 positive	Sensitivity % (95% CI)	Specificity % (95% CI)[a]	PPV% (95% CI)[a]	NPV(%) (95% CI)	P value
Platelia™ NS1	138	125	104	83.2 (75.5 - 89.3)	100 (86.7-100.0)	100 (97.2-100.0)	38.2 (22.2- 56.4)	*0.047*
LFRT-NS1	138	125	91	72.8 (64.1 - 80.3)	100 (91.6 - 100)	100 (96.7 - 100.0)	27.6 (15.6 - 42.6)	
NS1 sensitivity against RT-PCR								
Platelia™ NS1	138	111	100	90.1 (83.0 - 95.0)	100 (76.2-100.0)	100 (97.0-100.0)	71.1 (54.1- 84.6)	*0.025*
LFRT-NS1	138	111	88	79.3 (71.0 - 86.3)	100 (87.8 - 100)	100 (96.7 - 100.0)	54.0 (39.3 - 68.1)	

[a] one-sided, 95% Confidence Interval

Table 6.3 Diagnostic accuracy for each NS1 assay against the reference algorithm and RT-PCR.

6.3.5. Sensitivity of NS1 tests by day of illness

The sensitivity of both NS1 ELISA and LFRT tests was influenced by the patient's duration of illness prior to study entry (**Fig.6.3**). Thus, both the ELISA and NS1-LFRT were significantly more sensitive in test sample collected within 3 days of illness onset versus those collected at later times, *P=0.002* and *0.01*, (**Table 6.4**). The reduced sensitivity of NS1 assays in patients presented after 72hrs was associated with viraemia levels in test sample [Viraemia level in patients presented within 72 hrs, log10cDNA (SD); 8.5 (1.3) versus viraemia level after 72 hrs, 6.9 (1.2), *P<0.0001*, Mann-Whitney, (**Fig.6.4**)].

Sample group	NS1 (+)	NS1-ELISA sensitivity (95%CI)	*P* value[a]	NS1-LFRT sensitivity (95%CI)	NS1 (+)	*P* value[a]
< =3 day (n=75)	69	92.0 (83.4 - 97.0)	*0.002*	81.3 (70.6 - 89.4)	61	*0.01*
> 3 day (n=50)	35	70.0 (55.4 - 82.1)		60.0 (45.2 - 73.5)	30	

[a]: Fisher's exact test

Table 6.4 Sensitivity of NS1 assays in plasma samples collected within 3 days of illness onset versus those collected at a later time.

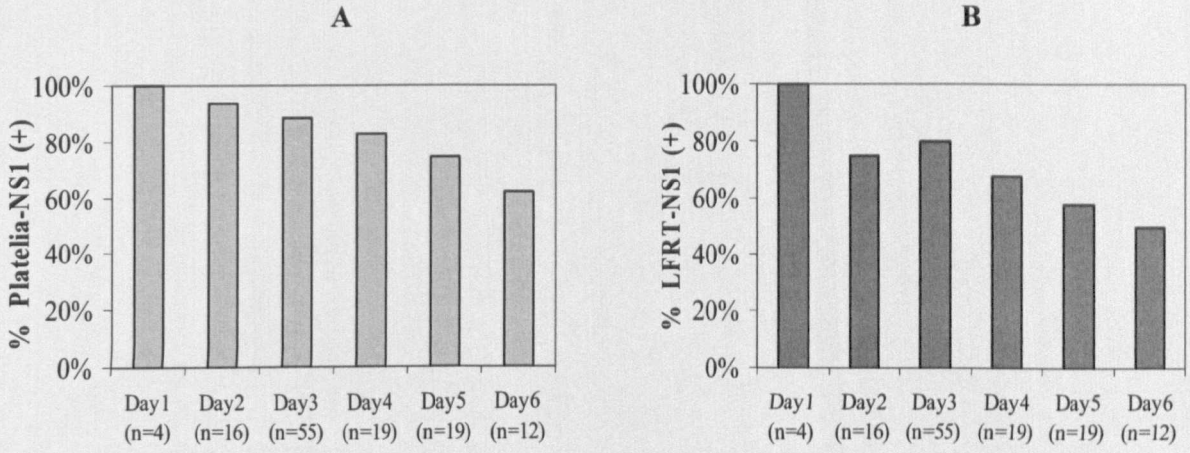


Fig.6.3. Relationship between day of illness and NS1 sensitivity. **(A)** Shown is percentage of positive Platelia ELISA and **(B)** LFRT assays by day of illness in patients with confirmed dengue (n=125).

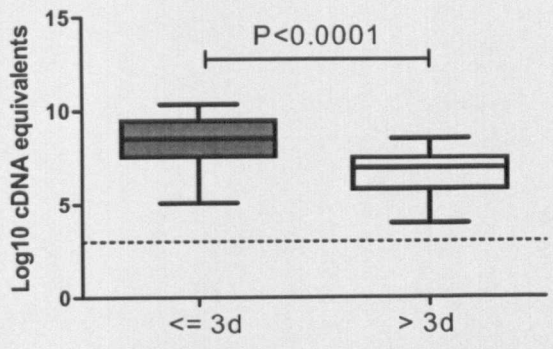


Fig.6.4. Viraemia level in samples collected within 72hrs of illness onset and at later times. Shown is median (interquartile and range) viraemia level in patient samples collected within 72 hrs and at later time. The limit of detection of the assay is shown with a dashed line.

6.3.6. NS1 sensitivity in primary or secondary infection

In general, NS1 detection was higher in patients with primary dengue (n=24) than secondary dengue (n=93) (**Table 6.5**). This difference was statistically significant for the NS1-LFRT (*P=0.01*) and of borderline significance for the ELISA (*P=0.07*). The difference in sensitivity between primary and secondary dengue was not significantly associated with the illness day at the time of testing [mean illness day (SD) of primary dengue versus secondary dengue: 3.3 (1.0) versus 3.7 (1.3), *P=0.2*)]. Reduced NS1 sensitivity in secondary infection was also not associated with viraemia levels between primary and secondary dengue cases [median log10 viraemia (SD), primary versus secondary: 8.3 (1.4) versus 7.7 (1.5), *P*=0.2].

Sample group	NS1 status	Platelia % (95% CI)	P value[a]	NS1-LFRT % (95% CI)	P value[a]
Primary infection (n=24)	NS1 (+)	23	*0.07*	22	*0.01*
	NS1 (-)	1		2	
	NS1 sensitivity	95.8 (78.9 - 99.9)		91.7 (73.0 - 98.9)	
Secondary infection (n=93)	NS1 (+)	73		61	
	NS1 (-)	20		32	
	NS1 sensitivity	78.5 (68.8 - 86.3)		65.6 (55.0 - 75.1)	

[a] Fisher's exact

Table 6.5 Sensitivity of NS1 assays in patients with primary and secondary serological profiles. Sensitivity in primary is greater than in secondary cases in both test kits, but the significant difference is only in NS1-LFRT (*P=0.01*).

As shown in Chapter 5, NS1 sensitivity has reverse association with DENVs-reactive IgG. To assess the consistency of this occurrence, we analysed NS1 detection sensitivity in the context of DENV-reactive IgG and IgM antibody in the test samples in both assays. Consistent with our report in Chapter 5, the presence of measurable DENV-reactive IgG in the test samples was associated with a significant reduction (*P<0.001*) in NS1 sensitivity in both assays (**Table 6.6**). DENV-reactive IgM was also associated with a reduction in NS1-LFRT sensitivity and was statistically significant in the ELISA assay (*P=0.03*) (**Table 6.7**).

NS1 assay / IgG status[a]	Total	NS1-Platelia		NS1-LFRT		Sensitivity % (95% CI)	
		Positive	Negative	Positive	Negative	NS1-Platelia	NS1-LFRT
IgG positive	38	23	15	18	20	60.5 (43.4 - 76.0)	47.4 (31.0 - 64.2)
IgG negative	87	81	6	73	14	93.1 (85.6 - 97.4)	83.9 (74.5 - 90.9)
P value						*P < 0.001*	*P < 0.001*

[a]: IgG in test sample

Table 6.6 Sensitivity of each NS1 assay in the presence or absence of measurable DENV-reactive IgG in test samples.

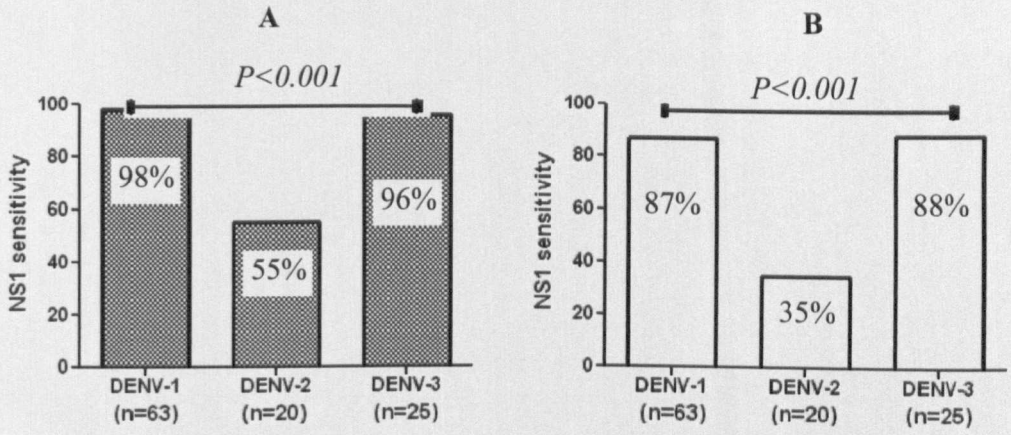
NS1 assays / IgM status[a]	Total	NS1-Platelia		NS1-LFRT		Sensitivity % (95% CI)	
		Positive	Negative	Positive	Negative	NS1-Platelia	NS1-LFRT
IgM positive	35	25	10	23	12	71.4 (53.7 - 85.3)	65.7 (47.8 - 80.9)
IgM negative	90	79	11	68	22	87.7 (79.2 - 93.7)	75.5 (65.4 - 84.0)
P value						*P=0.03*	*P=0.26*

[a]: IgM in test sample

Table 6.7 Sensitivity of each NS1 assay in the presence or absence of measurable DENV-reactive IgM in test samples.

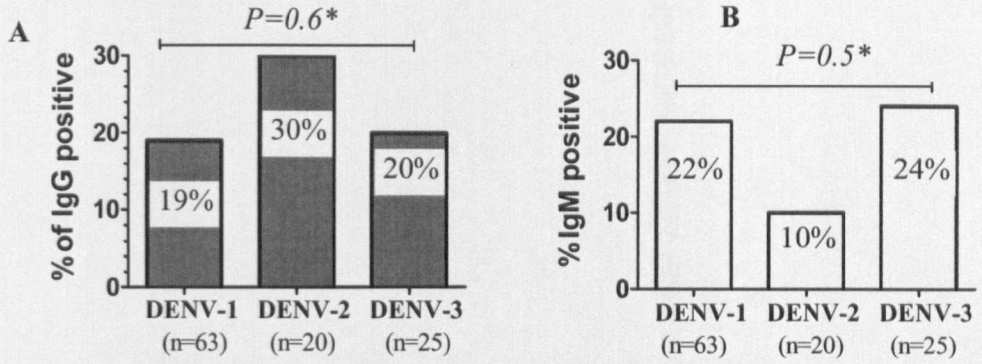
6.3.7. NS1 sensitivity in relation to viral serotype

In line with the results from previous Chapter, NS1 sensitivity in DENV-2 was lower than DENV-1 or DENV-3 in both NS1-Platelia (**Fig.6.5.A**) and LFRT-NS1 (**Fig.6.5.B**). The reduced sensitivity in DENV-2 was non-statistically associated with the serological response in these individuals. Secondary dengue in DENV-2 infected patients was greater than either DENV-1 or DENV-3 (85% versus 75% and 76%, *P=0.6*, Pearson chi square test). A relatively greater proportion of DENV-2 infected patients had measurable DENV reactive IgG, (**Fig.6.6.A,** Pearson chi square test), rather than IgM (**Fig.6.6.B,** Pearson chi square test) in the test sample.



* Pearson chi square

Fig.6.5. Sensitivity of NS1 detection in Platelia ELISA **(A)** and LFRT **(B)** in the enrolment samples according to the infecting serotype identified by real-time RT-PCR. NS1 detection in DENV-2 infected patients was significantly lower [55% **(A)** and 35% **(B)**] than for DENV-1 [98% **(A)** and 87% **(B)**] or DENV-3 [96% **(A)** and 88% **(B)**] in both Platelia ELISA [*P<0.001,* **(A)**] and LFRT [*P<0.001*, **(B)**]. Results for DENV-4 not shown as the sample size was small (n=3).



* Pearson chi square test

Fig.6.6. DENV reactive IgG and IgM status by serotypes. Proportion of patients with detectable DENV-reactive IgG **(A)** or IgM **(B)** antibodies (measured by capture ELISAs) in the test sample was also related to the infecting serotype. Test sample from DENV-2 infected patients were more likely to have measurable levels of DENV-reactive IgG but not IgM, though this was not statistically significant.

Again, the reduced sensitivity of NS1 detection in DENV-2 infected patients was non-statistically associated with lower viraemia level in the test sample from DENV-2 infected patients [DENV-2 median log10cDNA (SD); 7.4 (1.7) versus DENV-1; 7.9 (1.5) or DENV-3; 7.8 (1.5), *P=0.3*, Kruskal-Wallis test]. The mean duration of illness at the time of sampling showed no association with lower NS1 sensitivity in DENV-2 also (DENV-1: mean 3.3 days; DENV-2: mean 3.4 days; DENV-3: mean 3.3 days, *P=0.7*, Kruskal–Wallis test). In brief, NS1 detection was a robust diagnostic test in DENV-1 and DENV-3 infections, but was less sensitive in DENV-2 infections in part because test sample from these patients were generally more likely to have a concomitant DENV-IgG response, suggestive of secondary infection.

6.3.8. NS1 sensitivity in relation to clinical profile

In general, NS1 sensitivity in DF patients was higher than in DHF for both NS1-ELISA and LFRT [(92% versus 78%, *P=0.049*) and (80% versus 68%, *P=0.22*, respectively)]. Lower NS1 sensitivity in DHF was associated with greater illness days and lower viraemia levels in DHF patients relative to DF (**Table.6.8**). Reduced NS1 sensitivity in DHF was also associated

with measurable DENV-IgG in test sample [IgG (+) in DHF versus DF: 39% versus 18%, *P=0.009*, Fisher's exact].

NS1 assay and other variables	DF (n=49)	DHF (n=76)	*P value*
Platelia sensitivity % (95%CI)	91.8 (80.4 - 97.7)	77.6 (66.6 - 86.4)	*0.049* [a]
LFRT sensitivity % (95%CI)	79.6 (65.7 - 89.8)	68.4 (56.7 - 78.6)	*0.22* [a]
Mean day of illness (SD)	2.87 (0.12)	3.97 (0.15)	*<0.001* [b]
Median viraemia (SD)	8.13 (0.22)	7.53 (0.20)	*0.04* [b]

[a] Fisher's exact test [b]: Mann-Whitney test

Table 6.8. Sensitivity of each NS1 assay in DF and DHF patients. NS1 sensitivity in DHF patients who presented later and had lower viraemia than DF patients is lower than NS1 sensitivity in DF patients for both NS1-ELISA and LFRT (92% and 80% versus 78% and 68%, respectively).

6.3.9. NS1 specificity in patients with other confirmed diagnoses

Since the number of patients with no evidence of acute dengue was small (n=13) in this study (**Table.6.1**), further effort was made to assess the specificity of dengue NS1 assays in patients with other infectious diseases whose transmission geographically overlaps with dengue. To this end, frozen acute (within 10 days of illness onset) plasma samples from patients with culture confirmed enteric fever (S. *typhi*, n=25 and *paratyphi*, n=25), smear positive *P. falciparum* malaria (n=52), serological-proven Japanese encephalitis (n=11) and *Leptospirosis* (n=12) were tested in parallel by both NS1-ELISA and NS1-LFRT. In all cases, NS1 tests were negative in these samples.

6.4. Discussion

An accurate, early and convenient laboratory diagnosis for acute dengue is still problematic in most dengue endemic areas, particularly where laboratories are poorly equipped and have limited resources. Approaches currently used for laboratory dengue diagnosis such as DENV-antibody detection (IgM/IgG ELISA), or less frequently, viral isolation and viral nucleic acid amplification assays require skilled staffs and well-equipped laboratories. The dengue immunochromatographic test for detection of dengue specific IgM/IgG, despite its advantage for obtaining results within 7 minutes [159], has similar limitations to serology by ELISA, since antibody detection is not sensitive in the very early stages of disease. It strictly requires paired specimens for definitive laboratory determination and lacks specificity (IgG) because of cross-reactivity with other flaviviruses. In this study, we compared the accuracy of NS1-LFRT assay and NS1-ELISA, performed in parallel on the same day, in a group of paediatric and adult patients encompassing a broad range of dengue disease severities and considered the results in the context of viral burden and humoral immune response. A number of issues have been raised by this study.

In general, sensitivity of NS1-LFRT was consistently lower than NS1-ELISA when matched in the first 3 days of illness (81% versus 92%) or after 3 illness days (60% versus 70%). The NS1 antigenaemia test marketed as Dengue NS1 Ag STRIP (LFRT) has proven to be a promising first-line test in the field when only a single specimen is available [30]. Results from our study showed that NS1-LFRT sensitivity (73%) was consistently lower than in previous reports (81.5%, French Guiana [30], 90.4%, Malaysia [158]). The observed lower sensitivity was probably due to higher ratio of secondary infections in the study population (79%).

In agreement with other studies [27, 30, 158] and our previous report for NS1-ELISA, we observed that NS1-ELISA and LFRT were less sensitive in secondary dengue cases. This is

also consistent with our finding of substantially decreased sensitivity in test sample with a measurable level of anti-DENV IgG. Previous studies have reported contradictory findings on the influence of DENV-reactive IgM on NS1 detection [21, 22, 24, 27, 29, 140, 160]. In our study, NS1 sensitivity was also affected by the presence of measurable IgM and this was more clearly observed in Platelia ELISA (P=0.03) than in LFRT (P=0.26). If the NS1 sensitivity is truly affected by both DENV-measurable IgM and IgG, immune-complex dissociation must be considered to increase the utility of the test, regardless of whether test samples are collected from primary or secondary infection patients.

In line with previous study [157], detection rate of positive NS1 was higher in DF patients rather than in DHF for both LFRT and ELISA. This could result from lower viraemia levels likely related to being present late in DHF patients.

Overall, though the NS1-LFRT assay was modestly less sensitive than the Platelia NS1-ELISA in this study (73% versus 83%) but importantly, retained high specificity (100%). The attraction of the NS1-LFRT relative to the NS1 ELISA is its ease of use and speed (15 minutes versus 2 hrs), though this comes at a greater cost per test (~$10 versus $5). An obvious setting in which to use this assay format is in primary health care clinics for testing of febrile patients presenting early in their illness. In our hospital-based setting, the sensitivity of the NS1-LFRT was 81% in patients admitted within 3 days of illness onset which is acceptable for a diagnostic test. The ongoing development of specific anti-viral drugs for dengue [46, 47] makes the availability of accurate rapid tests, such as the NS1-LFRT even more important since diagnosing patients quickly and early will provide the greatest window of opportunity for an anti-viral drug to be delivered with clinical benefit.

A weakness of the current study is that relatively few of the patients in the prospectively assessed patient population were negative for dengue. We compensated for this by including a large number of patients with known alternative diagnoses. The strengths of the current study,

and point of difference from published studies, are that we included patients with severe clinical presentations and investigated the relationship between NS1 positivity, viraemia levels, illness history and Ig responses. The finding that viraemia levels are (in previous Chapter also), on average, higher in NS1-positive patients is a novel finding in the context of these commercial assays. The significance of this observation is tied to the widely accepted view that high viraemia levels are associated with disease severity [36, 161]. Thus, NS1 detection may be biased towards detecting those patients who, generally, have the highest viraemias and with relatively higher risks of developing complications during their illness. Future studies should measure the prognostic value of early NS1 measurements for predicting patients at risk of developing severe complications, e.g. DSS.

Chapter 7

7. Conclusions and future directions

Dengue is a globally important public health problem. There is no vaccine or anti-viral drug available for dengue at the moment. Vector control programs are complicated and not always sustainable [162] and thus a vaccine would be the most effective control strategy. Dengue viruses, like other RNA viruses, possess an RNA-dependent RNA polymerase that lacks proofreading activities leading to high mutation rates and genetic diversity. Thus, in moving towards designing vaccines or developing anti-viral drugs against dengue, an understanding of DENV genomic diversity is of crucial importance. Moreover, knowing how the changes in serotype or lineage prevalence relate to dengue epidemics would be useful for better dengue control. In addition, an accurate and timely diagnosis to differentiate dengue infection from other viral infections in the first few days of illness is also an important practical requirement because it is helpful for clinical management, disease surveillance and research activities. Against this background, this study was carried out to; **1)** investigate molecular epidemiology of the 3 most prevalent DENV serotypes over the last 10 years in Southern Vietnam (HCMC and surrounding provinces); **2)** examine molecular epidemiology of DENV-1 and DENV-2 in a Mekong delta province during a dengue outbreak in 2007; and **3)** assess the utility of two commercial NS1 antigen detection assays in the contexts of viraemia, infecting serotype, host immune response and duration of fever. This section will discuss what implications for vaccine/drug targets can be drawn from results of this study; how our results could contribute to dengue prevention and a control strategy; and how results from the evaluation of NS1 commercial test kits could contribute to improving this diagnostic assay. Finally, additional future research directions will be proposed.

7.1. Co-circulation of multiple DENV lineages and the emergence of DENV-2 Asian 1: implication for the current dengue vaccine/drug effort

The most challenging issue for dengue vaccine developers is making a safe vaccine that elicits broad and durable protective immune responses against all four serotypes of DENV. The

results of this study highlight the extent of sequence diversity within individual serotypes of DENV (notably, DENV-1 and DENV-2) and thus the importance of providing broad coverage against all of the discrete virus lineages within each serotype. For example, we demonstrate that 3 different DENV-2 genotypes have been circulating in HCMC; American/Asian, Asian 1 and Cosmopolitan genotypes. Differences in amino acid sequence between the DENV-2 genotypes were low for the complete CDS (2.6%); however, divergence varied from gene to gene, for example, 5.2% for NS2A; 4.2% for Capsid; 3.9% for NS1 and M protein. Whilst the amino acid differences in the E proteins of viruses from these different lineages were small (2.4%), it is plausible that even subtle changes could provide for immune escape from vaccine-elicited neutralising antibodies. We also showed that DENV-2 Asian 1 replaced the American/Asian genotype in southern Vietnam. The DENV-2 Asian 1 lineage is probably the most prevalent lineage of DENV-2 in SE Asia, suggesting vaccine developers must at least ensure that their vaccines can elicit protective immunity against this lineage. Similarly, drug developers should be using members from this lineage as reference DENV-2 viruses. Furthermore, the Asian 1 genotype could potentially become widely distributed geographically in many DENV endemic areas and particularly the Americas where currently Am/As DENV-2 viruses are prevalent (shown in **Fig.3.7**).

The large genetic diversity in DENV-1 genotype I viruses (6 distinct lineages with sequence divergence ranging from 0.1-1.17 %) suggests southern Vietnam has sufficiently high transmission levels to support substantial genetic (but not amino acid) diversity in this single genotype of DENV-1 viruses. The concept of virus reservoirs has been suggested for influenza, with tropical regions of Asia being nominated as reservoirs for new influenza subtypes or lineages [163]. Conceivably Vietnam and other countries in SE Asia act as geographic reservoirs that cultivate multiple lineages of DENV of which the fittest find new niches for transmission in other neighbouring countries or globally. However, overriding

evolutionary mechanism acting on DENV is purifying selection, as exemplified by the limited amino acid diversity in the DENV-1 genotype I viruses sampled in this study.

7.2. Signs to indicate a dengue epidemic and potential precautionary measures

Results from this study showed no evidence for the temporal coincidence between DENV-2 lineage replacement and serotype change as in other previously reported studies [118, 127]. However, the DENV-2 lineage replacement was associated with increased DENV-2 incidence occurring in 2003, 2004 and 2005 (**Fig.3.12**). Previous studies have shown lineage shifts are either coincidental with an increase in severe cases or precede a dengue outbreak [130, 133]. For example, DENV-1 lineage replacement in Myanmar (1998-2000) resulted in an outbreak in 2001 [130]. In our research, results from DENV epidemiology investigation have shown that multiple DENV serotypes co-circulate in Southern Vietnam and serotype shifts have continually occurred. Notably, the conclusion drawn from a dengue surveillance program in Southern Vietnam over the last 12 years showed that DENV serotype shifts are temporally associated with increased dengue incidence in years during which the serotype change occurred (for example 1998, 2004 and 2007). Taken together, our results suggest that genetic diversity is temporally linked to increased disease incidence. As a consequence, monitoring shifts in lineages or serotypes could provide a warning of potential outbreaks of dengue.

7.3. NS1 antigen as a candidate for early dengue diagnosis

Our evaluation of commercial NS1 assay kits (Platelia ELISA and NS1-LFRT) has consistently shown that NS1-positivity is higher in patients with high viraemia levels than in those who have lower viraemia levels. For both NS1 tests, the NPVs have a wide range of values [20-88%, depending on the day of illness (Platelia ELISA)]. It is critical that laboratory staff and clinicians understand that a negative result for NS1 does not exclude dengue as a diagnosis. Clearly rapid point of care tests could be very useful for diagnosis of dengue in outpatient or primary care level facilities. Similarly, they could be useful for intensive care

facilities where the differential diagnosis of a patient with shock could include sepsis or other syndromes. As drugs for dengue advance down development pathways, rapid tests that can diagnose dengue early will be valuable for identifying those patients who might benefit from an anti-viral therapy.

Future NS1 test possibilities could include serotype specific NS1 tests that would be useful for research purposes. It might also be possible to configure semi-quantitative NS1 rapid tests that could be evaluated for their diagnostic and prognostic accuracy. Data from my colleague, Miss Than Ha Quyen suggests that early NS1 levels in plasma could be useful as a prognostic marker of DSS.

It would be a useful aid to rational development of antibody tools that have equal sensitivity and specificity for the DENV serotypes. Our sequencing results in Chapters 3 and 4 have contributed to NS1 test developers as it suggests there is limited amino acid diversity in NS1 sequences from the same serotype.

7.4. Future directions

The most interesting questions emanating from this thesis and for future research are:

1. Why do Asian 1 DENV-2 viruses have a fitness advantage over American/Asian genotype viruses in southern Vietnam?
2. Are there measurable biological phenotypic differences amongst the different DENV-1 lineages we have detected here, and what will happen to the various lineages in the DENV-1 population during the next shifts in dominant serotype?
3. Do the current dengue vaccines that are in development, many of which are based on viruses that circulated >20 years ago, elicit antibodies capable of neutralising the currently circulating viruses from southern Viet Nam?
4. Can NS1 be used as a prognostic marker of DSS?

The first question, on the fitness of Asian 1 DENV-2 viruses, could be addressed by virus-host studies in *A. aegypti* mosquitoes. One reasonable hypothesis is that Asian 1 viruses have a fitness advantage over American/Asian viruses in the mosquito vector by having a shorter extrinsic incubation period or a lower infectious dose. An alternative possibility is that Asian - 1 viruses have a fitness advantage because there are more susceptible hosts in the population. This could be explored by measuring neutralising antibody titres to both Asian 1 and American/Asian viruses in plasma from healthy community cohort samples collected between 2001-2005 in southern Vietnam by the OUCRU.

There may not be a measurable clinical phenotype to each of the DENV-1 or DENV-2 lineages we have measured here. If there was, it would be fascinating as it would suggest much more subtle relationships between virus sequences and "virulence" than previously appreciated for dengue. One way of looking at this is by a careful investigation of clinical and virological findings in the patients in whom we have recovered a genome sequence. Of note, as of end-July 2009, our group has sequenced 590 DENV-1 genomes from patients with a breadth of disease severities in whom the viraemia levels in daily specimens are currently being determined. With such numbers, it might be possible to link a genotype to a virological or clinical phenotype. These ongoing studies will also allow us to see how the DENV-1 population structure changes as the prevalence of DENV-1 inevitably decreases to be replaced by a different serotype.

It is very important that dengue vaccines offer immunity to contemporary viruses that are in circulation, and that vaccine development keeps pace with ongoing changes in viral diversity. Protective value of vaccines under development could be assessed by measuring titres of neutralising Ab against the viruses we have sampled here. Although neutralising antibody is not a confirmed surrogate of immunity, it would be re-assuring to see that these vaccines elicit antibodies capable of neutralising representative contemporary strains.

The possibility of using NS1 to predict clinical outcomes has been suggested but not confirmed [23]. An obvious next step for NS1 assays is to make them quantitative and evaluate them in very large prospective cohort studies. A simple diagnostic and prognostic marker for severe dengue would be a very valuable aid to clinicians in dengue endemic countries such as Vietnam.

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9. Appendices

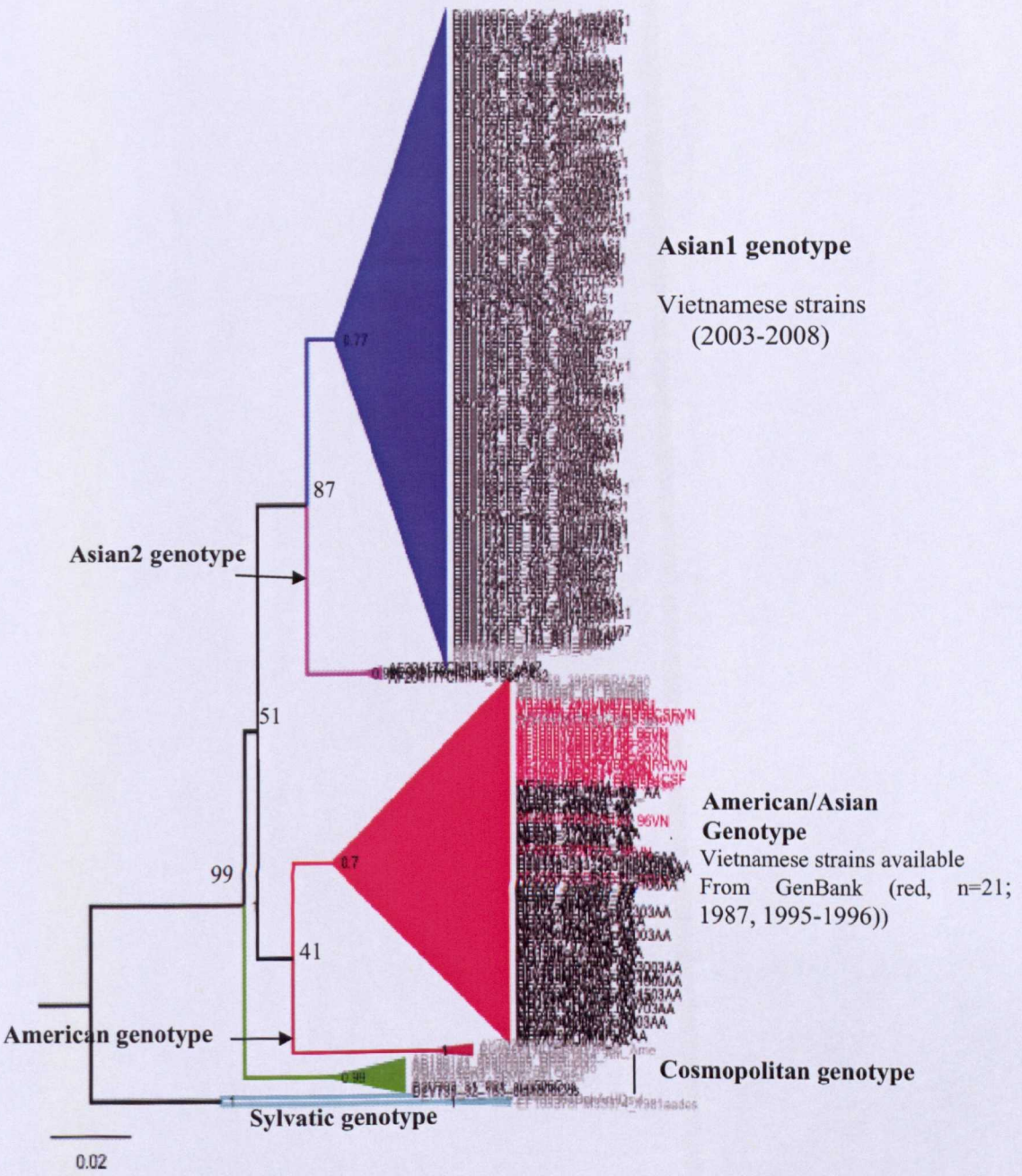

Fig.3.9. NJ phylogenetic tree of 239 DENV-2 E/NS1 sequences (240 nt) including 83 strains available from GenBank (21 are Vietnamese strains highlighted in red) and 156 Vietnamese strains generated from this study. All Vietnamese strains sampled in 1987, 1995 and 1996 fall in American/ Asian genotype.

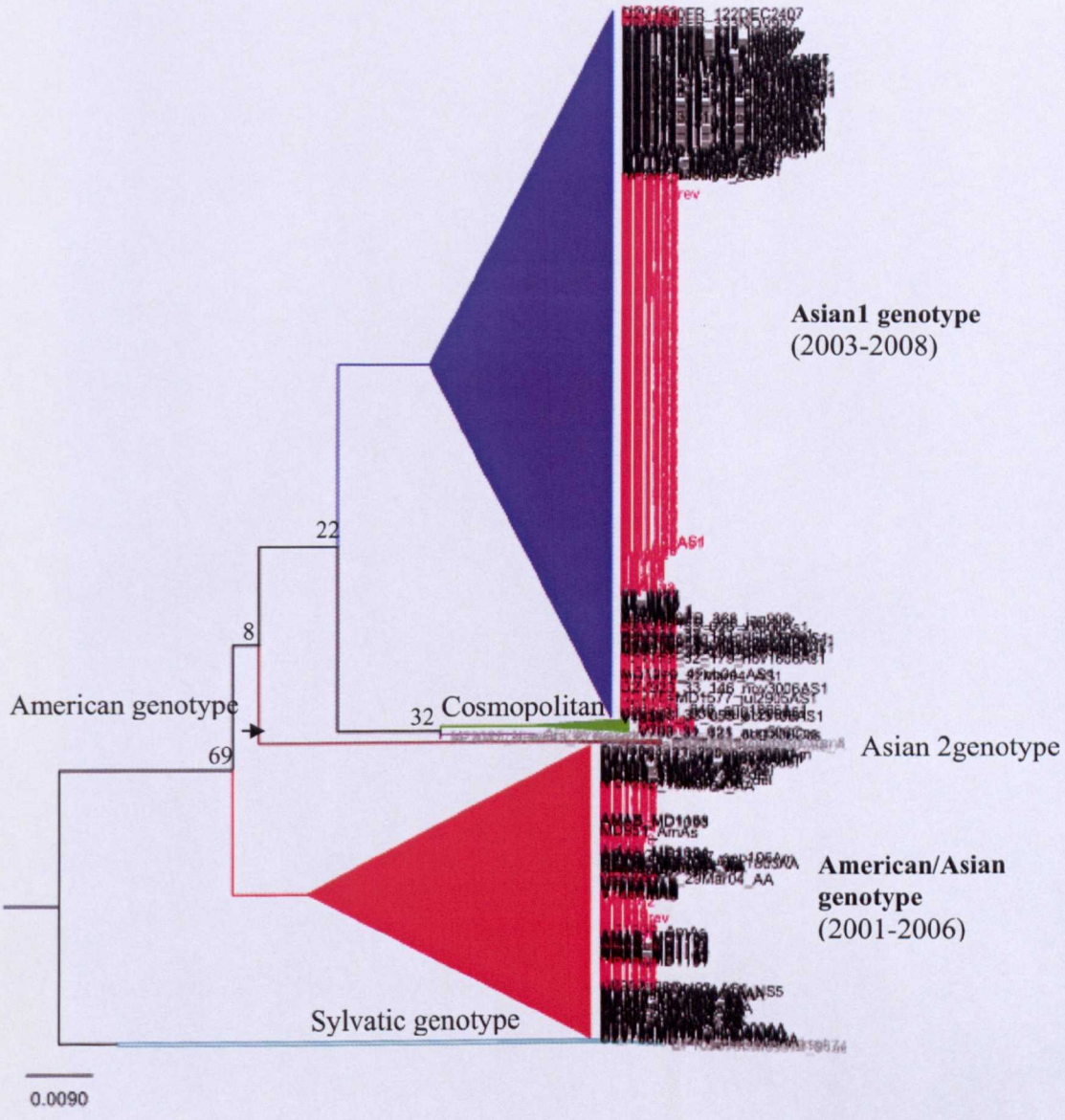

Fig.3.10. NJ phylogenetic tree of 502 DENV-2 NS5 sequences (78nt) including 11 reference sequences from GenBank, 156 sequences derived from whole genome sequences (38 from Dong Thap province) and 335 attempted sequences. The American/Asian genotype contains sequences sampled from 2001-2008 (n=130), Asian 1 genotype contains sequences sampled from 2003-2008 (n=354), and Cosmopolitan comprises sequences sampled in 2006, 2007, and 2008 (n=7). Bootstrap values are shown at main nodes.

List 1: Full genome sequences available from GenBank used in this study

No	Country	Accession No	Year	Serotype
1	Malaysia	EF457905	1972	DENV-1
2	Cote d'voire	AF298807	N/A	DENV-1
3	Brunei	EU179860	2005	DENV-1
4	Singapore	AY762084	N/A	DENV-1
5	Thailand	AY732474	1990	DENV-1
6	Thailand	AY732476	1990	DENV-1
7	Myanmar	AY713473	1971	DENV-1
8	Myanmar	AY722801	1976	DENV-1
9	Myanmar	AY722802	1996	DENV-1
10	Myanmar	AY722803	1998	DENV-1
11	Singapore	EU081258	2005	DENV-1
12	Argentina	AY277665	N/A	DENV-1
13	Brazil	AF311957	N/A	DENV-1
14	Brazil	AF311958	N/A	DENV-1
15	Brazil	AF513110	N/A	DENV-1
16	Brazil	AF311956	N/A	DENV-1
17	Brazil	FJ384655	N/A	DENV-1
18	Brazil	AF226685	1990	DENV-1
19	Nicaragua	EU482615	2005	DENV-1
20	Nicaragua	FJ432720	2005	DENV-1
21	Nicaragua	FJ024485	2005	DENV-1
22	Nicaragua	EU482617	2005	DENV-1
23	Nicaragua	EU596502	2005	DENV-1
24	Nicaragua	FJ024478	2005	DENV-1
25	Nicaragua	FJ432721	2005	DENV-1
26	Nicaragua	EU482619	2005	DENV-1
27	Nicaragua	EU482618	2005	DENV-1
28	Nicaragua	EU596503	2005	DENV-1
29	Nicaragua	FJ024480	2005	DENV-1
30	Nicaragua	EU596504	2005	DENV-1
31	Nicaragua	FJ024481	2005	DENV-1
32	Nicaragua	FJ024479	2006	DENV-1
33	Nicaragua	EU482616	2005	DENV-1
34	Nicaragua	FJ024423	2005	DENV-1
35	Nicaragua	FJ024484	2005	DENV-1
36	Nicaragua	FJ024482	2005	DENV-1
37	Nicaragua	FJ024483	2005	DENV-1
38	Nicaragua	EU596501	2004	DENV-1
39	Puerto Rico	EU482591	2006	DENV-1
40	Venezuela	EU482610	2007	DENV-1
41	Venezuela	EU482611	2007	DENV-1
42	Venezuela	EU482609	2007	DENV-1

43	French Guiana	AF226686	N/A	DENV-1
44	French Guiana	AF226686	N/A	DENV-1
45	French Guiana	EF122231	N/A	DENV-1
46	French Guiana	EF122232	N/A	DENV-1
47	Puerto Rico	FJ410186	1992	DENV-1
48	Puerto Rico	FJ410188	1996	DENV-1
49	Puerto Rico	FJ478457	1996	DENV-1
50	Puerto Rico	FJ410184	1993	DENV-1
51	Puerto Rico	EU482567	1998	DENV-1
52	Puerto Rico	FJ410183	1993	DENV-1
53	Puerto Rico	FJ410181	1995	DENV-1
54	Puerto Rico	FJ410185	1993	DENV-1
55	Puerto Rico	FJ410190	1987	DENV-1
56	Puerto Rico	FJ410175	1994	DENV-1
57	Puerto Rico	FJ410180	1995	DENV-1
58	Puerto Rico	FJ410174	1995	DENV-1
59	Puerto Rico	FJ410182	1996	DENV-1
60	Puerto Rico	FJ410189	1996	DENV-1
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65	Puerto Rico	FJ410187	1992	DENV-1
66	Puerto Rico	EU482592	1998	DENV-1
67	Puerto Rico	FJ390378	1998	DENV-1
68	Puerto Rico	FJ390380	1998	DENV-1
69	Puerto Rico	FJ410173	1998	DENV-1
70	Argentina	AY277664	N/A	DENV-1
71	Argentina	AY277666	N/A	DENV-1
72	Brunei	EU179861	2006	DENV-1
73	Hawaii	DQ672564	2001	DENV-1
74	Nauru Island	U88535	1974	DENV-1
75	Nauru Island	U88536	N/A	DENV-1
76	Nauru Island	U88537	N/A	DENV-1
77	China	EF025110	N/A	DENV-1
78	Indonesia	AB189121	1998	DENV-1
79	China	EF032590	N/A	DENV-1
80	Japan	AB195673	N/A	DENV-1
81	French Polynesia	DQ672556	N/A	DENV-1
82	French Polynesia	DQ672557	N/A	DENV-1
83	French Polynesia	DQ672559	N/A	DENV-1
84	French Polynesia	DQ672560	N/A	DENV-1
85	Hawaii	DQ672561	2001	DENV-1
86	Hawaii	DQ672562	2001	DENV-1

87	Hawaii	DQ672563	2001	DENV-1
88	French Polynesia	DQ672558	N/A	DENV-1
89	Indonesia	AB189120	1998	DENV-1
90	Indonesia	AB074761	1988	DENV-1
91	Japan	AB204803	N/A	DENV-1
92	Cambodia	AF309641	N/A	DENV-1
93	China	DQ193572	N/A	DENV-1
94	Singapore	EU081277	2005	DENV-1
95	Singapore	EU081226	2005	DENV-1
96	Singapore	EU081252	2005	DENV-1
97	Singapore	EU081269	2005	DENV-1
98	Singapore	EU081270	2005	DENV-1
99	Singapore	EU081274	2005	DENV-1
100	Singapore	EU081267	2005	DENV-1
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102	Singapore	EU081227	2005	DENV-1
103	Singapore	EU081228	2005	DENV-1
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105	Singapore	EU081229	2005	DENV-1
106	Singapore	EU081231	2005	DENV-1
107	Singapore	EU081233	2005	DENV-1
108	Singapore	EU081236	2005	DENV-1
109	Singapore	EU081240	2005	DENV-1
110	Singapore	EU081250	2005	DENV-1
111	Singapore	EU081251	2005	DENV-1
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116	Singapore	EU081243	2005	DENV-1
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130	Singapore	EU081266	2005	DENV-1
131	Singapore	EU081232	2005	DENV-1
132	Singapore	EU081241	2005	DENV-1
133	Singapore	EU081248	2005	DENV-1

134	Singapore	EU081249	2005	DENV-1
135	Singapore	EU081247	2005	DENV-1
136	Singapore	EU081263	2005	DENV-1
137	Singapore	EU081230	2005	DENV-1
138	Singapore	EU081238	2005	DENV-1
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149	Singapore	EU081276	2005	DENV-1
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152	Myanmar	AY726549	2001	DENV-1
153	Myanmar	AY726551	2001	DENV-1
154	Myanmar	AY726553	2002	DENV-1
155	Myanmar	AY726554	1998	DENV-1
156	Myanmar	AY713476	2001	DENV-1
157	China	AY835999	N/A	DENV-1
158	Thailand	AY732479	2001	DENV-1
159	Thailand	AY732482	2001	DENV-1
160	China	EU280167	N/A	DENV-1
161	Singapore	M87512	1990	DENV-1
162	Thailand	AY732475	1994	DENV-1
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164	China.GZ/80	AF350498	1980	DENV-1
165	Djibouti	AF298808	1998	DENV-1
166	Thailand	AY732477	1991	DENV-1
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168	Myanmar	AY713474	2001	DENV-1
169	Myanmar	AY726552	2002	DENV-1
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171	Myanmar	AY726550	2001	DENV-1
172	Myanmar	AY726555	1998	DENV-1
173	Thailand	AY732481	1982	DENV-1
174	Thailand	AY732483	1981	DENV-1
175	Hawaii	EU848545	1944	DENV-1
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179	Ivory Coast	EF105383	1980	DENV-2
180	Burkina Faso	EF105382	1980	DENV-2
181	Burkina Faso	EF105386	1980	DENV-2

182	Guinea	EF105378	1981	DENV-2
183	Senegal	EF105390	1999	DENV-2
184	Senegal	EF105389	1999	DENV-2
185	Senegal	EF457904	1991	DENV-2
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195	China	AF119661	1985	DENV-2
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197	Colombia	EU854294	2005	DENV-2
198	Colombia	FJ024473	2005	DENV-2
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217	Nicaragua	EU482633	2006	DENV-2
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224	Nicaragua	EU482629	2007	DENV-2
225	Nicaragua	EU482639	2006	DENV-2
226	Nicaragua	EU482760	2005	DENV-2
227	Nicaragua	EU482688	2006	DENV-2
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244	Nicaragua	EU482752	2005	DENV-2
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263	Nicaragua	EU660406	2007	DENV-2
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272	Nicaragua	EU660404	2007	DENV-2
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276	Nicaragua	EU569702	2007	DENV-2
277	Nicaragua	FJ373300	2007	DENV-2

278	Nicaragua	EU596496	2007	DENV-2
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280	Nicaragua	EU660405	2007	DENV-2
281	Nicaragua	EU482684	2006	DENV-2
282	Nicaragua	EU482685	2006	DENV-2
283	Nicaragua	EU482686	2006	DENV-2
284	Nicaragua	EU482772	2006	DENV-2
285	Nicaragua	EU482773	2006	DENV-2
286	Nicaragua	EU482694	2007	DENV-2
287	Nicaragua	EU482620	2006	DENV-2
288	Nicaragua	EU482683	2006	DENV-2
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317	Puerto Rico	EU482726	2006	DENV-2
318	Puerto Rico	EU482731	2005	DENV-2
319	Puerto Rico	EU596491	2007	DENV-2
320	Puerto Rico	EU687217	2005	DENV-2
321	Puerto Rico	EU677145	1999	DENV-2
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323	Puerto Rico	EU482546	1998	DENV-2
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325	Puerto Rico	EU677142	1999	DENV-2
326	Puerto Rico	EU482545	1998	DENV-2
327	Puerto Rico	EU482734	1998	DENV-2
328	Puerto Rico	EU482735	1998	DENV-2
329	Puerto Rico	EU482544	2006	DENV-2
330	Puerto Rico	EU482548	2006	DENV-2
331	Puerto Rico	EU482549	2006	DENV-2
332	Puerto Rico	EU482719	2006	DENV-2
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334	Puerto Rico	EU596488	2007	DENV-2
335	Puerto Rico	EU482725	2006	DENV-2
336	Puerto Rico	EU726770	2005	DENV-2
337	Puerto Rico	EU482551	2006	DENV-2
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339	Puerto Rico	EU596489	2007	DENV-2
340	Puerto Rico	EU529693	2004	DENV-2
341	Puerto Rico	EU687213	2004	DENV-2
342	Puerto Rico	EU482552	2006	DENV-2
343	Puerto Rico	EU781135	2005	DENV-2
344	Puerto Rico	EU482724	2005	DENV-2
345	Puerto Rico	EU687235	2003	DENV-2
346	Puerto Rico	EU482553	2006	DENV-2
347	Puerto Rico	EU687215	2004	DENV-2
348	Puerto Rico	EU482556	2005	DENV-2
349	Puerto Rico	EU687214	2004	DENV-2
350	Puerto Rico	EU687244	2004	DENV-2
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357	Puerto Rico	EU687238	2003	DENV-2
358	Puerto Rico	EU687241	2003	DENV-2
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363	Puerto Rico	EU687240	2003	DENV-2
364	Puerto Rico	EU687230	2001	DENV-2
365	Puerto Rico	EU687228	2001	DENV-2
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367	Puerto Rico	EU687231	2001	DENV-2
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369	Puerto Rico	EU687227	2001	DENV-2
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371	Puerto Rico	EU687224	2000	DENV-2
372	Puerto Rico	EU687222	2000	DENV-2
373	Puerto Rico	EU687223	2000	DENV-2
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377	Puerto Rico	EU482732	1998	DENV-2
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384	Puerto Rico	EU482565	1998	DENV-2
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386	Puerto Rico	EU569717	1997	DENV-2
387	Puerto Rico	EU569703	1996	DENV-2
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391	Puerto Rico	EU482738	1998	DENV-2
392	Puerto Rico	EU482729	1999	DENV-2
393	Puerto Rico	EU569718	1997	DENV-2
394	Puerto Rico	EU569719	1997	DENV-2
395	Puerto Rico	EU482728	1998	DENV-2
396	Puerto Rico	EU482733	1998	DENV-2
397	Puerto Rico	EU482739	1994	DENV-2
398	Puerto Rico	EU529700	1994	DENV-2
399	Puerto Rico	EU569706	1995	DENV-2
400	Puerto Rico	EU482550	1998	DENV-2
401	Puerto Rico	EU482554	1998	DENV-2
402	Puerto Rico	EU569716	1997	DENV-2
403	Puerto Rico	EU596486	1996	DENV-2
404	Puerto Rico	EU569709	1996	DENV-2
405	Puerto Rico	EU677141	1996	DENV-2
406	Puerto Rico	EU569712	1998	DENV-2
407	Puerto Rico	EU677144	1999	DENV-2
408	Puerto Rico	EU569713	1997	DENV-2
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457	Puerto Rico	EU529701	1991	DENV-2
458	New Guinea	M29095/M19727	1944	DENV-2
459	Thailand	AF022434	1993	DENV-2
460	Thailand	DQ181798	1999	DENV-2
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498	Brunei	EU179857	2005	DENV-2
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500	China	EU359009	N/A	DENV-2
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508	Bangladesh	AY496874	2002	DENV-3
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536	Thailand	AY676353	1987	DENV-3
537	Singapore	AY766104	1995	DENV-3
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542	East Timor	AB214880	2005	DENV-3
543	East Timor	AB214881	2005	DENV-3
544	East Timor	AB214882	2005	DENV-3
545	Indonesia	AB189125	1998	DENV-3
546	Indonesia	AY858042	2004	DENV-3
547	Indonesia	AY858048	2004	DENV-3
548	Indonesia	AY858047	2004	DENV-3
549	Singapore	EU081221	2005	DENV-3
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560	Indonesia	AY858043	2004	DENV-3
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562	Indonesia	AY858038	1988	DENV-3

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567	French Polynesia	AY744683	1992	DENV-3
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