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THESIS

A dissertation submitted to the graduate faculty
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by

Veasna DUONG

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DENGUE IN CAMBODIA:

EPIDEMIOLOGY, MOLECULAR EVOLUTION, CLINICAL PRESENTATION, LABORATORY DIAGNOSTIC AND MARKERS OF SEVERITY

LA DENGUE AU CAMBODGE:

EPIDEMIOLOGIE, EPIDEMIOLOGIE MOLECULAIRE, PRESENTATIONS CLINIQUES, DIAGNOSTIC DE LABORATOIRE ET MARQUEURS DE LA SEVERITE

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under supervision of Dr Philippe BUCHY and Dr Thierry Dupressoir

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DENGUE IN CAMBODIA: EPIDEMIOLOGY, MOLECULAR EVOLUTION, CLINICAL PRESENTATION, LABORATORY DIAGNOSTIC AND MARKERS OF SEVERITY

SUMMARY

Dengue infection, caused by one of the four serotypes of dengue virus (DENV), is a major cause of morbidity and mortality in the world with an estimated 50-100 million cases annually. In Cambodia, dengue is hyperendemic and all four serotypes are circulating. The active hospital-based surveillance has been established in 2000 and provided important insights in the understanding of the epidemiological profile and of the DENV evolution. The dynamic of evolution of DENV is characterized by complex patterns of lineage turnovers within each serotype, with lineages increasing and decreasing in frequency through time. Dengue manifests in various clinical forms - from asymptomatic to severe form with shock syndrome - and is sometimes difficult to differentiate from other febrile diseases. We have evaluated the performance of a recent diagnostic tool (NS1 antigen detection) - developed to identify dengue at a very early stage on the infection - depending on various clinical and biological patterns. Additionally, genome-wide expression analysis has characterized a large amount of gene signatures specific to dengue shock syndrome which could be used as prognostic markers as well as potential targets for drug design.

Keywords: Cambodia, dengue, epidemiology, evolution dynamic and severity.

LA DENGUE AU CAMBODGE: EPIDEMIOLOGIE, EPIDEMIOLOGIE MOLECULAIRE, PRESENTATIONS CLINIQUES, DIAGNOSTIC DE LABORATOIRE ET MARQUEURS DE LA SEVERITE

RÉSUMÉ

La dengue, maladie transmise par des moustiques, est causée par l'un des 4 sérotypes du virus de la dengue (DENV). Le Cambodge est un pays d'hyperendémicité de la dengue où les 4 sérotypes en co-circulent. Le programme national de lutte contre la dengue a été établi en 2000 et a fourni des données nécessaires dans la compréhension du profile épidémiologique et de l'évolution du DENV. La dynamique d'évolution virale est caractérisée par des évènements de disparition et d'émergence de lignées au sein de chaque sérotype en fonction du temps. La dengue se présente sous différentes formes cliniques allant d'une forme asymptomatique aux formes sévères accompagnée d'un syndrome de choc (DSS) et le diagnostic différentiel est souvent compliqué. Dans des contextes cliniques différents et avec des profils biologiques variables, nous avons évalué un test de diagnostic précoce de détection de l'antigène NS1 da la dengue. De plus, nous avons identifié de nombreux profils d'expression biologique spécifiques aux cas de DSS qui pourraient être utilisés comme d'éventuelles cibles pour le développement de nouveaux traitements ou comme des marqueurs de pronostic.

Mots clés : Cambodge, dengue, épidémiologie, dynamique d'évolution et sévérité.

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LIST OF ABBREVIATIONS

ADE:	Antibody-dependent enhancement
BSP:	Bayesian skyline plot
DENV:	Dengue virus
DF:	Dengue fever
DHF:	Dengue hemorrhage fever
DSS:	Dengue shock syndrome
C:	Capsid protein
E:	Envelope protein
HI:	Hemagglutination Inhibition assay
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IPC:	Institut Pasteur in Cambodia
M:	Membrane protein
MAC-ELISA:	M antibody-capture Enzyme Linked Immunosorbent Assay
NDCP:	National Dengue Program
NS:	Nonstructural protein
prM:	Precursor membrane protein
RNA:	Ribonucleic Acid
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
WHO:	World Health Organization

LIST OF FIGURES AND TABLES

SUMMARY

Dengue infection, caused by fours serotype of dengue virus (DENV), is one of major causes of morbidity and mortality among mosquito-borne diseases. Globally, 2.5 billion people are at risk of infection with an estimated 50 million dengue cases annually among whom approximately 500,000 cases suffer from life threatening forms (DHF/DSS). The geographic distribution of area affected by dengue keeps increasing since the last 50 years with more than 69 countries reporting dengue activity to WHO. With global warming and many other factors, dengue has pushed its boundary toward more temperate regions. With an increase in number of severe forms and no specific drugs and preventive vaccine available, dengue is one of the major public health threats.

Cambodia is one of Southeast Asia countries where dengue is highly endemic with seasonal epidemic occurring during rainy season. The National Dengue Control Program (NDCP) has been conducting dengue surveillance since 1980 and all four DENV serotypes are found in circulation. Dengue poses a considerable public health and economic burden due to high annual incidence.

This thesis manuscript reports results obtained by multidisciplinary and integrative approaches aiming to better understand dengue disease in Cambodia by combining epidemiology, DENV evolution studies, and evaluation of recent diagnostic tools in various clinical presentations of the infection which could potentially be used as predictive markers of disease severity.

The epidemiology pattern of dengue in Cambodia is marked by a shifting cycle of predominance in prevalence of DENV-2 and DENV-3 every 3-4 years and by large epidemic occurring every 8-9 years and coinciding with a change of predominant serotype. The annual incidence ranged from 0.7 to 3 per 1000 inhabitants and children aged less than 6 year are the most affected group. Dengue vector control using temephos and media was shown to have only limited impact on dengue incidence.

Bioinformatics tools were used to describe the evolution dynamics of DENV at the molecular level. We have revealed that DENV genotypes circulating in Cambodia are similar to those observed in neighboring countries - in particular Thailand and Vietnam - and probably have a Thai origin. Our analysis demonstrated phenomenon of lineages extinction and emergence in all 4 DENV serotypes and also suggested that the evolutionary process was probably shaped by at least 3 events including introduction of new virus genotype/lineage, stochastic event caused by flood and drought and herd cross-protective immunity.

Our clinical study in Southeast Asia (SEA) and Latin Americas (LA) has demonstrated that dengue symptoms were poorly specific and often not able to distinguish dengue from other undifferentiated fever etiologies. Among patients with dengue-like symptoms, one third of patients in SEA and two third in LA were not dengue cases. Among dengue cases (215), 13% were severe dengue cases and all of them were originating from SEA. Furthermore, we also identified high proportions of dengue infections among household members of dengue index cases. Among these patients, 74.4% experienced unapparent infection with higher incidence in SEA than in LA.

In Cambodia, we found that the NS1 Antigen capture kit evaluated had an overall moderate sensitivity (57.7%) using a broad range of well characterized specimens. However, this detection rate varied greatly in relation to the various forms of dengue infection with highest sensitivity in patients sampled during the first 3 days after onset of fever, with primary infection, with DENV-1 infection, with high level of viraemia, with DF rather than DHF/DSS and with symptomatic presentation (compared to asymptomatic individuals). Additionally, we observed a significant increase of sensitivity if the NS1 antigen capture test was used in combination with an anti-DENV IgM capture ELISA assay. We also demonstrated that mild disease severity was observed in patients with level of viraemia >5 log10 cDNA equivalents/mL or in high level of NS1 antigen at day 4-8 after onset of fever or with DENV-1 infection.

In order to have a better understanding on factors associated with dengue disease severity, a genome-wide expression profiles study was carried out. Over 2950 gene signatures differentiating DSS from DF/DHF patients were identified. Briefly, the gene signature expression showed that DF and DHF grades I/II were not two separate disease phenotypes and this result supports the new WHO 2009 dengue cases classification which considers dengue as a dynamic disease. Additionally, we described that a significant number of gene expression profiles in DSS children blood cells were up or down-regulated such as a decreased abundance of transcripts related to T and NK lymphocyte responses, an increased abundance of anti-inflammatory and repair/remodeling transcripts. We have also observed that pro-inflammatory gene patterns (innate immunity, inflammation and host lipid metabolism) were up-regulated in the samples obtained from DSS children. Our results suggested that some cellular and biological markers identified should be considered as putative therapeutic targets or biomarkers of progression to DSS.

GENERAL INTRODUCTION

Dengue is a mosquito-borne disease cause by DENV belonging to *Flavivirus* genus in *Flaviviridae* family and transmitted by mosquitoes (Calisher and Gould, 2003). *Aedes aegypti* is the principal vector of dengue and adapted extremely well to the urban environment. *Aedes albopictus* is considered as secondary vector but is believed to have a high potential in spreading DENV and other mosquito-borne viruses due to resistance of the eggs to subfreezing temperatures (Hawley et al., 1987) and also because of its more efficient vertical transmission (Rosen et al., 1983).

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. While most patients recover following a self-limiting, non-severe clinical course, a small proportion (~1%) progress to severe disease, mostly characterized by plasma leakage with or without hemorrhage (Duong et al., 2009; WHO, 2009). Dengue remains a major public health problem in tropical and sub-tropical countries despite lots of efforts to control the mosquito vector (Guzman et al., 2010a). Since the 1950s, the incidence of DHF/DSS has increased over 500-fold, with more than 100 countries affected by outbreaks of dengue (Kyle and Harris, 2008).

DENV consists of four distinct but antigenically related serotypes. Nucleic acid sequencing progress has allowed classification of four DENV serotypes into several distinct genotypes using phylogenetic analysis (Rico-Hesse, 1990; Vasilakis and Weaver, 2008). New bioinformatics tools have been developed and have allowed scientists to gain more in-depth understanding of the history and evolution of DENV. It has been demonstrated that the endemic DENVs derived from sylvatic monkey virus (Wang et al., 2000) and the origin of DENV was approximately about 1000 years ago (Twiddy et al., 2003). Large scale epidemiological data have shown that the evolution pattern of dengue follows a cyclical fluctuation of individual serotype and that the dominant serotype is sequentially replaced over time (Huy et al., 2010b; Vu et al., 2010; Zhang et al., 2005). In Cambodia, the evolution pattern of DENV is characterized by larger epidemics at intervals of about 3–4 years (Huy et al., 2010b), with DENV-2 and DENV-3 being predominant alternatively (Chapter 2). With the introduction of comparative gene sequence analysis and mathematical models, it has been possible to dissect the genetic structure of DENV populations and to attempt at disclosing the processes influencing the DENV evolution. Through the analysis of long term epidemiological data, it has become apparent that DENV lineages or clades frequently arise,

persist for a period of time and disappear (Holmes and Twiddy, 2003). Hypothesis (Zhang et al., 2005) has been raised trying to explain this extinction and replacement phenomenon in evolutionary process of DENV: (1) DENV lineages or clades differ in **fitness** (i.e. producing higher viraemia or possessing mutations that allow them to evade cross-protective immunity) that can outcompete another; this superiority in fitness has been demonstrated in DENV-2 in Vietnam (Vu et al., 2010), (2) replacement event are due entirely to **stochastic** factors such as large-scale epidemiological processes that are independent of viral genotype such as random population bottleneck of mosquitoes population caused by severe flood and continuous drought, (3) clade replacement is dependent of the complex of **herd immunity profile** (cross-immunity protection) to DENV in circulation. Antibodies against infecting DENV serotype is known to cross-react with other 3 serotypes. While providing long lasting protection against the current infecting serotype, this cross-immunity temporarily protects against infection caused by other serotypes for 1-2 months (Sabin, 1952). These phenomena may interact together in shaping the ecology and evolution of DENV.

In clinical settings, medical doctors are facing two major drawbacks in dengue disease management as there are not yet specific antiviral drugs or vaccine: (1) the need of reliable, feasible, less time consuming and low cost tests for early diagnosis and (2) lack of tools and reliable markers that can predict a risk of subsequent evolution to severe dengue disease. Early diagnosis in dengue infection is desirable in order to improve the clinical management of dengue infection and reduce the unnecessary use of antibiotics, hospitalization of patients with milder disease in countries with limited resources and with high incidence of dengue as Cambodia. Commonly used serological methods are easy to perform and inexpensive (WHO/TDR, 2009) but fail to provide results within a reasonable time. They are also sometimes difficult to interpret due to cross-reactivity with other flaviviruses (Gubler, 2002) (WHO, 2009). Virus isolation and RT-PCR are time-consuming, expensive and fastidious, and require specialized laboratory equipment and experienced staff. One of the most promising methods is the detection of the NS1 antigen (Alcon et al., 2002; Kao et al., 2005; Lapphra et al., 2008; Libraty et al., 2002; Schilling et al., 2004). The amount of secreted NS1 in the serum of individuals infected with DENV can be detected as early as first day of fever and has been shown to directly correlate with viraemia and the pathogenesis of dengue infection (Hang et al., 2009; Libraty et al., 2002; Vaughn et al., 2000; Wang et al., 2006; Young et al., 2000). Given all these advantages, NS1-based ELISAs and immunochromatographic

tests may be important diagnostic tools for those acute samples in which IgM is not detectable and for which PCR is not available.

Biological markers of dengue severity have been studied and several factors have been implicated such as virus virulence, autoimmunity, host genetic predisposition, antibody-dependent enhancement, and cross-reactive T-cell response as well as soluble factors (cytokine storm, complement, etc.) (Martina et al., 2009). Identification and implementation of these factors into tools that can provide early severity prognostic would be crucial for clinician to closely monitor and provide the potentially severe patients an appropriate management.

In Cambodia, dengue is considered endemic with annual incidence ranging from 13.4 - 57.8/1,000 person-seasons using active surveillance (Vong et al., 2011). The case-fatality rate was 1-2% over the past 5 years (Huy et al., 2010a). Cambodia has poor health and economic indicators (ADB, 2009) with an estimated population was 14.6 million in 2008 (NIS, 2009). The DENV was first detected in Cambodia in 1963 (Chastel, 1963) and became highly endemics with an epidemic seasonality during rainy season from May to November. The NDCP has been established and dengue infections have been monitored through passive surveillance since 1980. Dengue cases are clinically diagnosed based on standardized procedure. This passive hospital based surveillance tends to report only the most severe cases that require hospitalization. This national surveillance was enhanced in 2001 with active hospital based surveillance in 5 sentinel sites (national hospitals) spread across the country. This enhanced surveillance program includes laboratory diagnosis (virological and serological testing) for randomly selected samples from patients suspected of dengue infection. The burden of dengue disease was estimated between US$36 - $75 over 2006-2008 (Beaute and Vong, 2010). Patients sustain the highest share of costs by paying an average of 78% of total costs and 63% of direct medical costs.

The data used to generate results in this thesis were from the NDCP and DENFRAME project which are consortia of multidisciplinary research groups (13 institutions) aiming at the development of new diagnostic tools and therapeutic approaches (www.denframe.org) to improve the management of dengue disease. The two studies have provided precious data to approach the problematic mentioned above. The aims were (1) to describe the surveillance data from NDCP since 1980 to 2008 (patients' demography, incidence, fatality rate, transmission pattern) and to better understand the epidemiological pattern of circulating DENV (seasonality, cycle, serotypes in circulation); (2) to study evolution dynamic of each DENV serotype at the molecular level using virological data of NDCP and IPC from 1998 to 2010. In this objective, we investigated factors

involved in the evolutionary process and traced back the origin and ancestor of DENV in Cambodia; (3) to describe,using data from DENFRAME project, the various forms of dengue disease from asymptomatic cases to severe dengue infection and to compare clinical and biological data from asymptomatic or symptomatic dengue-infected patients; (4) to study the performance of dengue NS1 antigen-capture assay in relation with various clinical and virological factors, to assess the potential association of the level of NS1 antigenemia (using simple semi-quantitative estimation) and that of viraemia with dengue disease severity using well characterized sera from hospitalized patients and to evaluate the test in asymptomatically dengue-infected individuals; (5) to identify the critical mechanisms involved in the vascular leakage, that represent targets for future therapeutic strategies and to identify cellular markers that could be used as predictive factors of evolution towards DSS in dengue-infected patients.

The outline will be displayed in form of chapters with results presented as a form of published, submitted or in preparation articles. The articles included in this thesis are listed below:

1. **FIRST PART: Literature review in epidemiological trend in Southeast Asia**
   - **Duong V**, Vong S, Buchy P. [Dengue and other arboviral diseases in South-East Asia]. Med Trop (Mars). **2009** Aug;69(4):339-44. French.

2. **CHAPTER 1 :**
   - Huy R, Buchy P, Conan A, Ngan C, Ong S, Ali R, **Duong V**, Yit S, Ung S, Te V, Chroeung N, Pheaktra NC, Uok V, Vong S. National dengue surveillance in Cambodia 1980-2008: epidemiological and virological trends and the impact of vector control. Bull World Health Organ. **2010** Sep 1;88(9):650-7. Epub 2010 Apr 7.

3. **CHAPTER 2:**
   - **Duong, V.,** Simmons, C., Gavotte, L., Viari, A., Ong, S., Chantha, N., Lennon, N.J., Birren, B.W., Vong, S., Farrar, J.J., Henn, M.R., Deubel, V., Frutos, R., Buchy, P., **2011**. Genetic diversity and lineage dynamic of dengue virus serotype 1 (DENV-1) in Cambodia. Infect Genet Evol. 2011 Jul 2. [Epub ahead of print]
   - **Veasna Duong**, Eddie Holmes, NganChantha, RekolHuy, Matthew R. Henn, Niall J. Lennon, Bruce W. Birren, Sreyrath Lay, Roger Frutos, Cameron P. Simmons, Philippe Buchy. Molecular evolution of dengue virus in Cambodia from 1998 to 2010. (**in preparation**)
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4. CHAPTER 3:

- Philippe Dussart, Laurence Baril, Laure Petit, Lydie Beniguel, Luong Chan Quang, Sowath Ly, Raimunda do Socorro Azevedo, Jean-Baptiste Meynard, Sirenda Vong, Loic Chartier, Aba Diop, Ong Sivuth, **Veasna Duong**, Cao Minh Thang, Michael Jacobs, Anavaj Sakuntabhai, Marcio R Texeira Nunes, Vu Ti Que Huong, Philippe Buchy, Pedro Fernando C Vasconcelos.Study of dengue cases and their household members: a familial cluster analysis – the multinational DENFRAME project. PLoS Negl Trop Dis (in press)

5. CHAPTER 4:

- **Duong V**, Ly S, Lorn Try P, Tuiskunen A, Ong S, et al. (**2011**) Clinical and Virological Factors Influencing the Performance of a NS1 Antigen-Capture Assay and Potential Use as a Marker of Dengue Disease Severity. PLoS Negl Trop Dis 5(7): e1244. doi:10.1371/journal.pntd.0001244

6. CHAPTER 5:

- Devignot S, Sapet C, **Duong V**, Bergon A, Rihet P, Ong S, Lorn PT, Chroeung N, Ngeav S, Tolou HJ, Buchy P, Couissinier-Paris P. Genome-wide expression profiling deciphers host responses altered during dengue shock syndrome and reveals the role of innate immunity in severe dengue. PLoS One. **2010** Jul 20;5(7):e11671.

PART I

LITERATURE REVIEW

I.1 Flavivirus

I.1.1 Classification

According to the International Committee on Taxonomy of Viruses, flaviviruses constitute one of three genera within the virus family *Flaviviridae* (Calisher and Gould, 2003). The other two are the genera *Pestivirus* (from Latin pestis = ‘‘plague’’) and *Hepacivirus* (from Greek hepatos = ‘‘liver’’). Although pestiviruses and hepaciviruses have genome replication strategies similar to that of flaviviruses, they are antigenically distinct from the flaviviruses and are not transmitted by mosquitoes. They may represent lineages that diverged early in the evolution of the family (Figure 1).

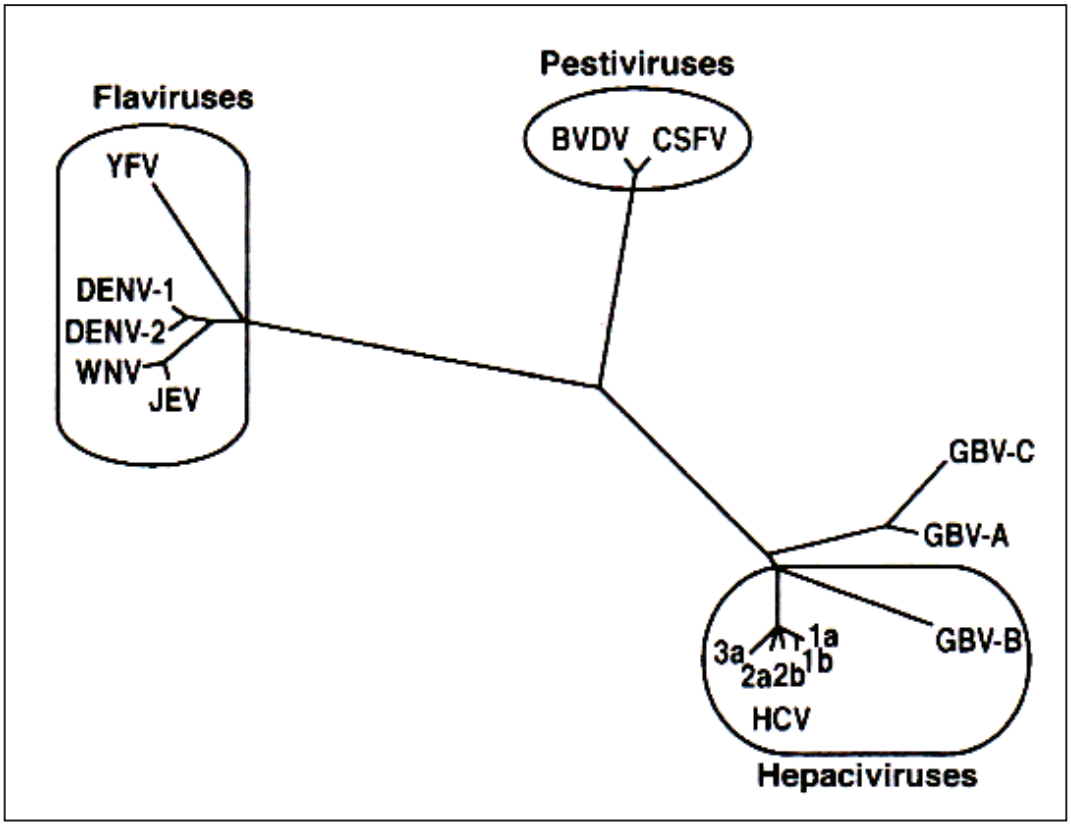


Figure 1: Phylogenetic tree showing the 3 genera in the *Flaviviridae* family.

The family name is due to the flavivirus prototype, yellow fever virus (YFV), which was the first identified human virus. The name is derived from the Latin word “flavus”, meaning yellow, evoking the jaundice caused by YFV (Vasilakis and Weaver, 2008). The flaviviruses are primarily transmitted by arthropods and consist of more than 73 viruses including DENV, Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and YFV (Gould et al., 2003). Although viruses in the genus *Flavivirus* share complex antigenic interrelationships, they can be

divided into four phylogenetic/ecological groups with regard to their vector association: two mosquito-borne groups, a tick-borne group, and non-vectored viruses (Vasilakis and Weaver, 2008) (Figure 2).

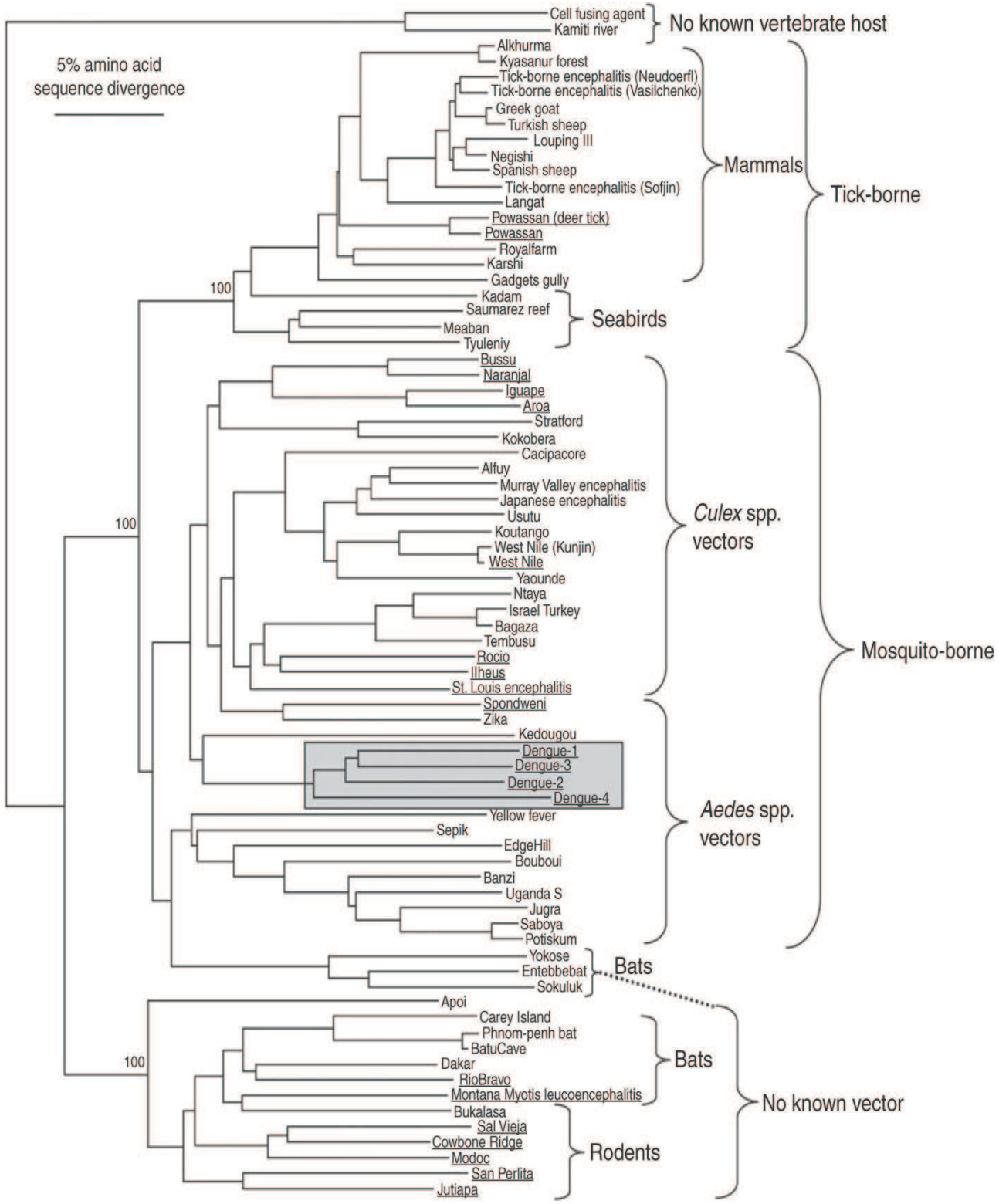


Figure 2: Phylogenetic tree of the flaviviruses derived from partial NS5 sequences from the GenBank library. Subtypes are written in parentheses after virus names. New World viruses are printed in bold and underlined (Vasilakis and Weaver, 2008).

I.1.2 Genome structure

Flaviviruses virion are 50 nm in diameter, spherical, and enveloped (Calisher and Gould, 2003) (Figure 3). The genome is a positive sense single stranded RNA of about 10.8 kb in size. The genome lacks a 3' C-terminal poly (A) tract and contains a 5' N-terminal type I cap (Wengler and Gross, 1978) (Figure 4A). As for all positive-stranded RNA viruses, flavivirus genomic RNA is infectious (Peleg, 1969). It is the only viral mRNA found in infected cells (Calisher and Gould, 2003). It contains a single long open reading frame flanked by 5' N- and 3' C-terminal noncoding regions that form specific secondary stem–loop structures required for genome replication (Lindenbach and Rice, 2003). In flaviviruses, translation initiation is cap–dependent (Calisher and Gould, 2003).

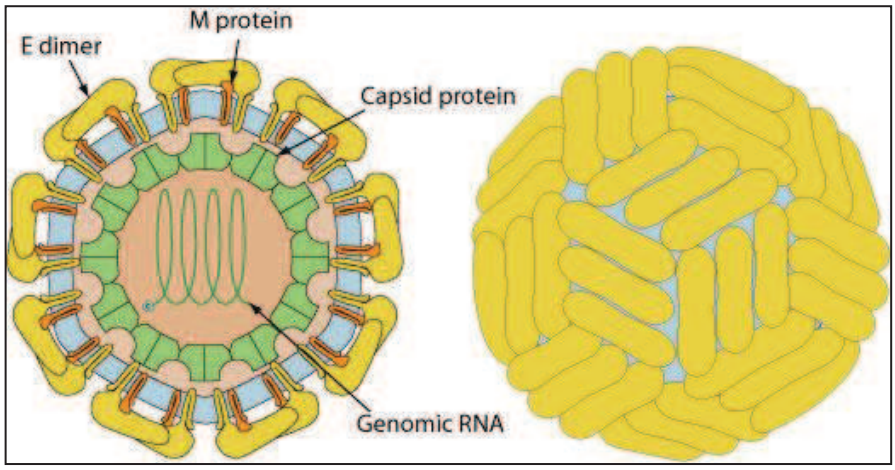
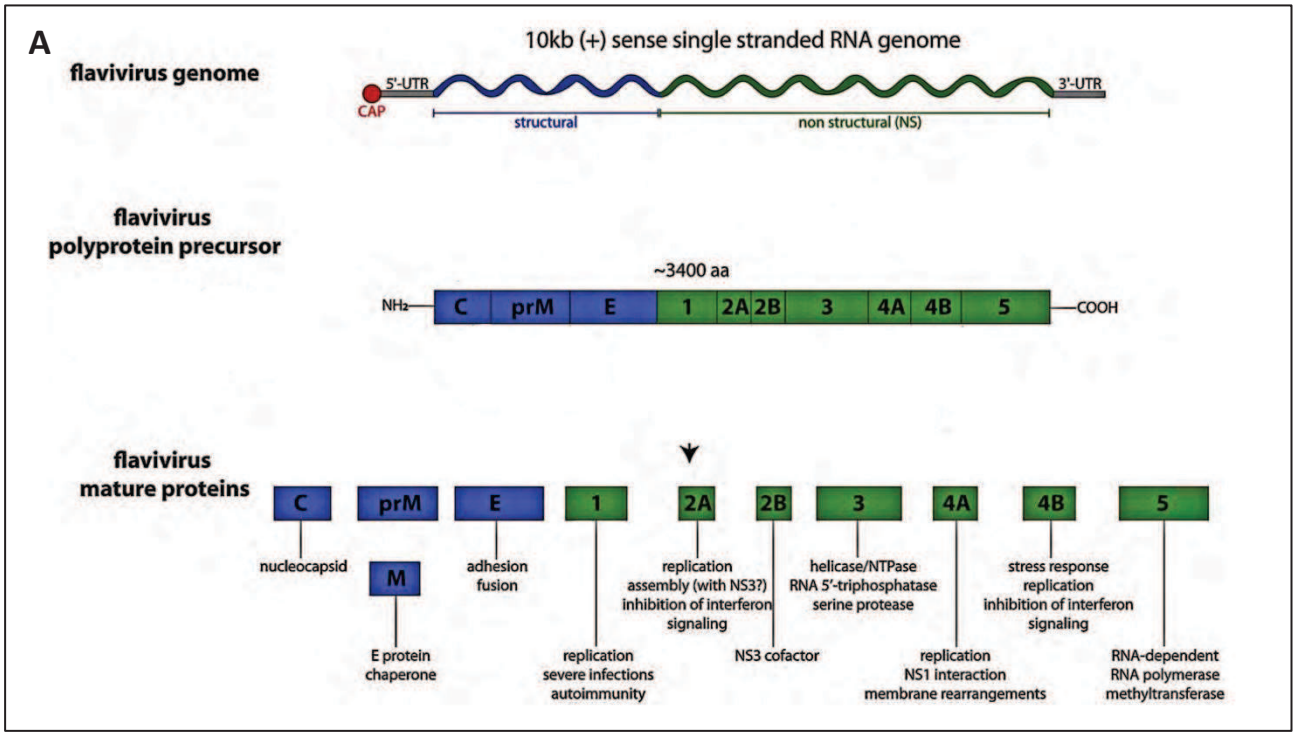


Figure 3: Schematic representations of flavivirus virion.

The flavivirus genome has one open reading frame encoding a single polyprotein at the origin of structural and non-structural proteins (Cleaves et al., 1981) (Figure 4A). The N-terminal end of the genome encodes three structural proteins — capsid (C), membrane (M, which is expressed as prM, the precursor to M) and envelope (E) — that constitute the virus particle. The **capsid protein** consists of ~120 amino acids (a.a.) and is involved with packaging of the viral genome and forming the nucleocapsid (NC) core. **prM** (~165 a.a. and E (~495 a.a.) are glycoproteins, each of which contains two transmembrane helix (Figure 4B). Before it is cleaved during particle maturation to yield the "pr" peptide and the M protein (~75 a.a.), the prM protein might function as a chaperone for the E protein folding and assembly. The **E protein**, the major surface protein of the viral particle, contains one or several cellular receptor-binding site(s) and a fusion peptide mediating virus–cell membrane fusion (Mukhopadhyay et al., 2005).

Seven non-structural (NS) proteins (NS1–NS2A–NS2B-NS3–NS4A–NS4B–NS5) that are essential for viral replication are encoded by the remainder of the genome (Figure 4A). NS3 (70 kDa; 618 a.a.) and NS5 (104 kDa; ~900 a.a.) are the best characterized non-structural proteins, with multiple enzyme activities that are required for viral replication. **NS3** protein has three distinct activities: serine protease together with the cofactor **NS2B** (~130 a.a.), required for polyprotein processing (Aleshin et al., 2007; Assenberg et al., 2009); helicase/NTPase activity, required for unwinding of the double-stranded replicative form of RNA; and RNA triphosphatase, needed for capping newly synthesized viral RNA (Bollati et al., 2009). **NS5** is the largest and most conserved flaviviral protein, with greater than 75% sequence identity across all DENV serotypes (Davidson, 2009). It contains two distinct enzymatic activities, separated by an interdomain region: an S-adenosyl methyltransferase and an RNA-dependent RNA polymerase (RdRp) (Mazzon et al., 2009). **NS1** protein (46 kDa; ~352 a.a.) is required for flavivirus replication and is presumably involved in negative-strand synthesis by an unknown mechanism (Pastorino et al., 2010). **NS2A** (22 kDa; ~218 a.a.) is a small hydrophobic transmembrane protein that is involved in generation of virus-induced membranes during virus assembly (Leung et al., 2008). **NS4A** (16 kDa; ~150 a.a.) is an integral membrane protein which induces membrane rearrangements to form the viral replication complex (Miller et al., 2007). **NS4B** protein (27 kDa; ~248 a.a.) inhibits the type I interferon response of host cells (Puig-Basagoiti et al., 2007), and may modulate viral replication via its interaction with NS3 protein (Umareddy et al., 2006).



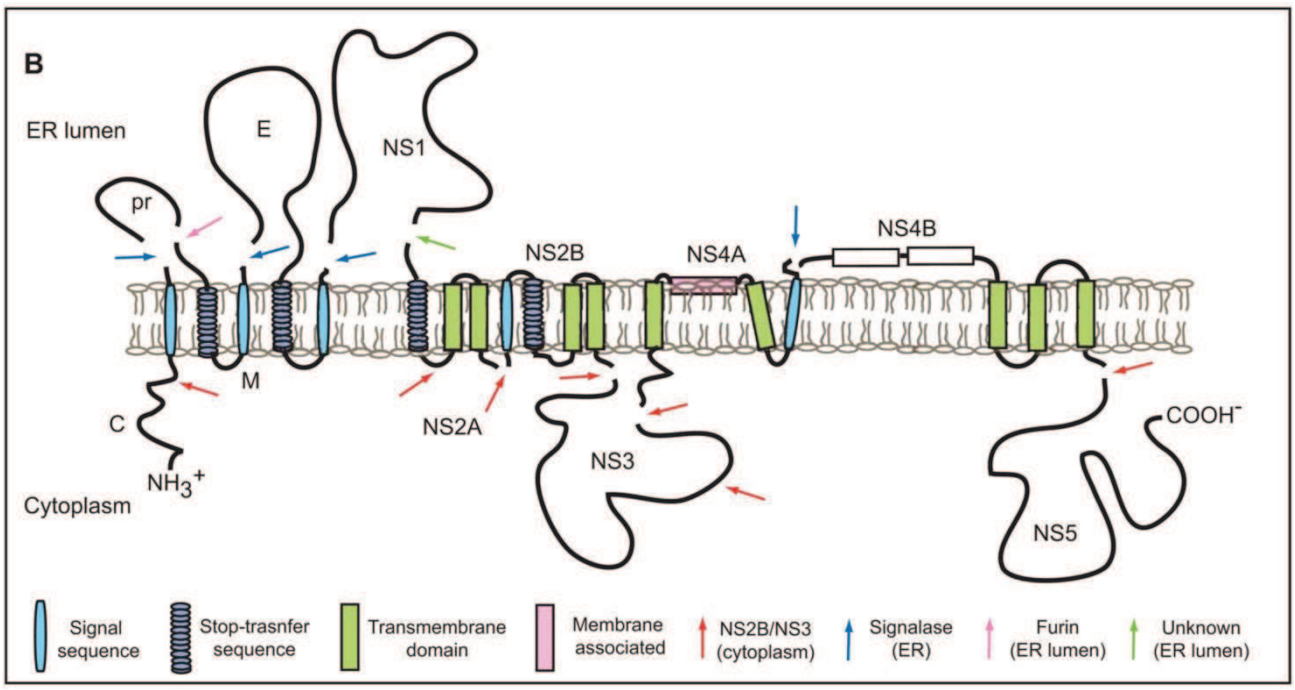


Figure 4: Flavivirus genome and polyprotein organization.
(A) The single open reading frame is depicted with the structural and non-structural protein coding regions (colored in blue and green respectively), the 5'-CAP and the 5' and 3' untranslated region (UTR). The single open reading frame encodes an immature polyprotein precursor that is co- and post-translationally cleaved into three structural proteins (in blue) and seven non-structural proteins (in green) (Pastorino et al., 2010). (B) The detail of cleavage sites for cellular proteases, NS2B/NS3 and unknown protease are indicated (Perera and Kuhn, 2008).

I.1.3 Flavivirus replication cycle

Flaviviruses enter host cells by receptor-mediated endocytosis (Lozach et al., 2005) (Figure 5). Two types of cell receptors appear to be involved in facilitating entry of DENV into human target cells, depending on the cell (Huerta et al., 2008). The first type corresponds to receptors of low affinity and specificity, including aminoglycan-type adhesion molecules such as heparan sulfate that are expressed in many cell types (Germi et al., 2002). The second type corresponds to lectin-type receptors such as DC-SIGN expressed in some antigen-presenting cells such as immature dendritic cells (DC) (Lozach et al., 2005).

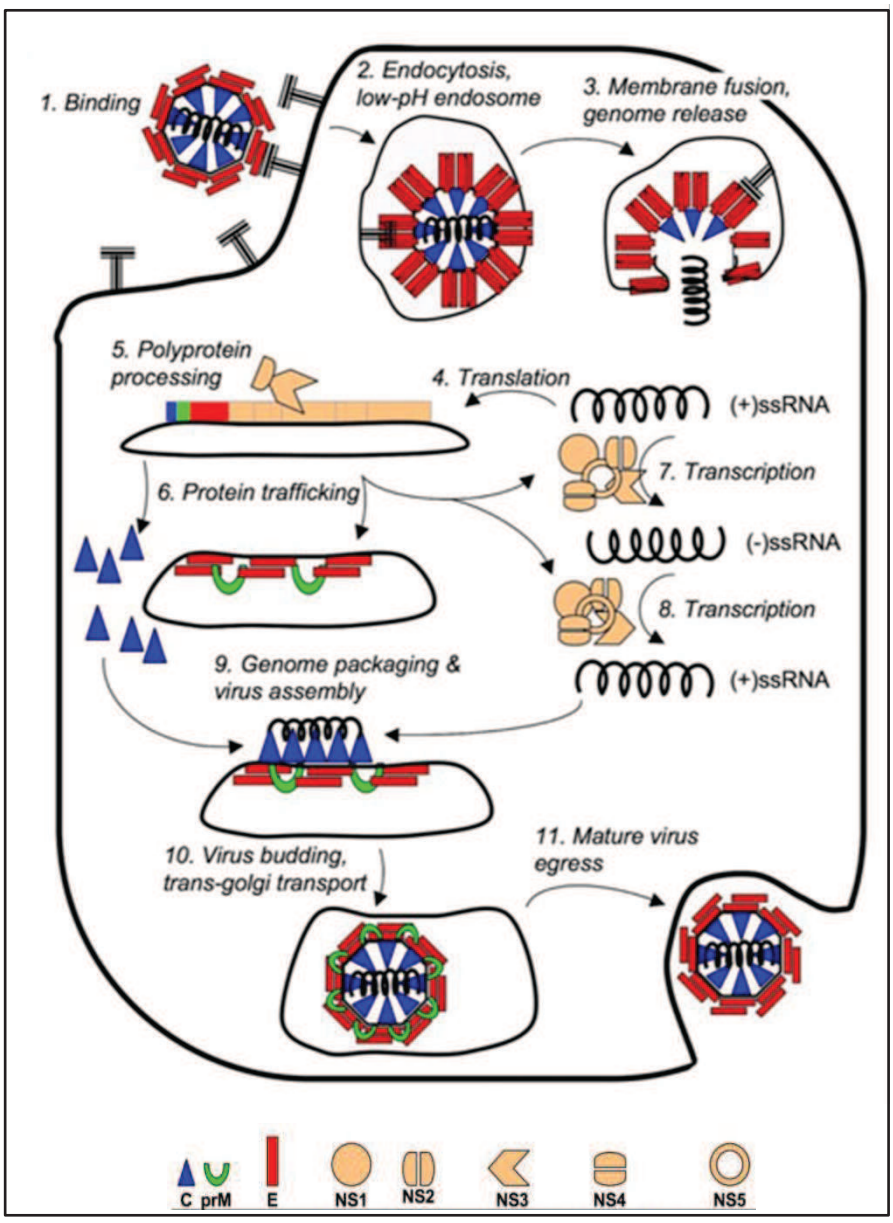


Figure 5: Schematic representations of a flavivirus life cycle (Tomlinson et al., 2009).

The acidic environment of the endosome triggers an irreversible trimerization of the E protein that results in fusion of the viral and endosomal membranes. After fusion has occurred, the NC is released into the cytoplasm (Rey et al., 1995), the capsid protein and RNA dissociate. The positive-sense RNA is translated into a single polyprotein that is co- and post-translationally cleaved by viral and host proteases and transcribed into negative-sense RNA. The cleavage sites and topology of the polyprotein at the ER membrane are illustrated in Figure 4 B.

Genome replication occurs on intracellular membranes and is synthesized using the complementary negative-sense genomic RNA as template. The newly synthesized viral RNA is extruded in the intermembranary space of Golgi-derived vesicles packets , from which it exits into

the cytoplasm by an unknown mechanism (Uchil and Satchidanandam, 2003). Assembly of virus particles occurs in the lumen of the Rough Endoplasmic Reticulum (RER). The first step in this process is the coating of the newly synthesized viral RNA with the C protein (Khromykh and Westaway, 1996; Perera and Kuhn, 2008). Next, E and PrM proteins hetero-dimerize and wrap the NC, forming an immature virus particle that buds from the lumen of the RER into the Golgi complex (Mackenzie and Westaway, 2001). Maturation of virus particles occurs in the trans-Golgi network, where prM is cleaved to M by furin at low pH condition, along with conformational rearrangements of E protein (Mukhopadhyay et al., 2005). Mature virions are subsequently released by exocytosis. (Pastorino et al., 2010)

I.2 Dengue virus

I.2.1 Classification

DENV consists of four distinct but antigenically related serotypes (DENV-1, -2, -3, and -4) in DENV antigenic complex (Calisher et al., 1989). As with other viruses, the evidence for strain differences among DENVs was first detected serologically using antibodies made by inoculating laboratory animals (Sabin, 1952). Later, nucleic acid sequencing allowed for the classification of DENV into genetically distinct groups or genotypes within each serotype (Rico-Hesse, 1990). Rico-Hesse defined these 'genotypes' as clusters of DENV viruses having nucleotide sequence divergence not greater than 6% within a given genome region (i.e. E/NS1 junction), which was based on the clustering of strains for which associations could be inferred on epidemiological grounds (Rico-Hesse, 1990).

Various phylogenetic analyses based on partial E/NS1 or complete E nucleotide sequences (Vasilakis and Weaver, 2008; Weaver and Vasilakis, 2009) indicated that **DENV-1** are grouped in five genotypes: (1) **genotype I**, representing strains from Southeast Asia, China, and East Africa; (2) **genotype II**, representing strains from Thailand collected in the 1950s and 1960s; (3) **genotype III**, representing the sylvatic strain collected in Malaysia; (4) **genotype IV**, representing strains from the West Pacific islands and Australia; and (5) **genotype V**, representing all strains collected in the Americas, strains from West Africa, and a limited number of strains collected from Asia (Goncalvez et al., 2002; Rico-Hesse, 1990).

Similar phylogenetic analyses based on E nucleotide sequences indicated that **DENV-2** comprises five genotypes: (1) the Asian genotype, consisting of **Asian genotype 1** representing strains from Malaysia, Cambodia, Vietnam and Thailand, and **Asian genotype 2** representing strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines; (2) the **cosmopolitan genotype**, representing strains of wide geographic distribution including Australia, East and West Africa, the Pacific and Indian ocean islands, the Indian subcontinent and the Middle East; (3) the **American genotype**, representing strains from Latin America and older strains collected from the Caribbean, the Indian subcontinent and Pacific Islands in the 1950s and 1960s; (4) the **Southeast Asian/American genotype**, representing strains from Thailand, Cambodia and Vietnam and strains collected in the Americas over the last 30 years; and (5) the **sylvatic genotype**, representing strains collected from humans, forest mosquitoes, or sentinel monkeys in West Africa and Southeast Asia (Lewis et al., 1993; Rico-Hesse, 1990; Twiddy et al., 2002a).

DENV-3 isolates were grouped based on prM/E nucleotide (Lanciotti et al., 1994) and, later, on complete genome sequences (Chao et al., 2005) into four genotypes: (1) **genotype I**, representing strains from Indonesia, Malaysia, the Philippines and recent isolates from the South Pacific islands; (2) **genotype II**, representing strains from Thailand, Cambodia, Vietnam and Bangladesh; (3) **genotype III**, representing strains from Sri Lanka, India, Africa and Samoa; however, the complete genome phylogenetic analysis includes the 1962 strain from Thailand within this genotype (Chao et al., 2005); and (4) **genotype IV**, representing strains from Puerto Rico, Latin and central America and a 1965 Tahiti strain. **Sylvatic** strains of DENV-3 have not been isolated, but are believed to exist in Malaysia, based on the seroconversion of sentinel monkeys (Rudnick, 1986).

Lastly, initial analyses revealed that **DENV-4** strains exhibited greater sequence conservation than the other DENV serotypes (92%) and 96–100% conservation in E protein amino acids. Current DENV-4 phylogenies complete E gene sequences delineate four genotypes based on the E gene (AbuBakar et al., 2002; Foster et al., 2003; Lanciotti et al., 1997) or complete genome sequences (Klungthong et al., 2004): (1) **genotype I**, representing strains from Thailand, Cambodia, the Philippines, Sri Lanka, and Japan (strains were imported into Japan from Southeast Asia); (2) **genotype II**, representing strains from Indonesia, Malaysia, Tahiti, the Caribbean and the Americas. Subsequent analysis with additional strains revealed putative evidence of intra-serotypic recombination among DENV-4 from independent lineages (most likely Indonesia 1976 and Malaysia 1969), which may have contributed to the emergence of a distinct genotype, representing all Malaysian strains (AbuBakar et al., 2002). Genotype II has become well established in the Caribbean since its introduction in the area in the early 1980s from Southeast Asia (Bennett et al., 2003; Foster et al., 2003); (3) **genotype III**, representing recently sampled Thai strains that are distinct from other Thai strains (Klungthong et al., 2004); and (4) **genotype IV**, representing the sylvatic strains of DENV-4 from Malaysia.

I.2.2 Genetic evolution

RNA viruses show great genetic variability, due to the intrinsically high mutation rate associated with RNA-dependent RNA polymerase (Drake and Holland, 1999), their rapid rates of replication, and their huge population sizes. In DENV, this genetic variability most obviously manifests in the existence of four antigenically distinct serotypes. With the introduction of comparative gene sequence analysis, it has been possible to dissect the genetic structure of DENV populations and to reveal the processes governing viral evolution (Rico-Hesse, 2003).

Genotypes frequently harbor viruses sampled from very different geographical locations as, for instance, the DENV-2 "Cosmopolitan" genotype which is covering most of viruses isolated in tropical countries (Twiddy et al., 2003). This wide range of distribution is an important indicator of how far infected hosts and vectors can spread the virus and leads to questions whether the difference in geographical distribution shown by dengue genotypes has any sort of selective basis and whether some genotypes have more "epidemic potential" than others (Gubler et al., 1981).

By measuring rates of synonymous (dS) and nonsynonymous (dN) substitutions, Twiddy et al. (Twiddy et al., 2002a) found sporadic **positive selection** in the E gene sequences of DENV-3 and DENV-4, in some genotypes of DENV-2, but not in DENV-1. Since the E protein is the major antigenic determinant of DENV, it was not surprising that the majority of these selected sites were located in or near B- or T-cell epitopes, suggesting that the selection pressure was related to immune evasion.

On the other hand, dN/dS values in DENV were usually very low (<0.2) indicating that the vast majority of nonsynonymous mutations that arise in DENV are slightly deleterious (see below), fall onto the external branches of molecular phylogenetic trees, and are eventually removed from the population by **purifying selection** (Zhang et al., 2005). The importance of transient deleterious mutations has been increasingly recognized in DENV evolution and supports a "slightly deleterious" model of molecular evolution in which most mutations lower fitness and which can be fixed by genetic drift only in relatively small populations (Zhang et al., 2005). Another aspect observed in Twiddy et al. is a similar and generally very low dN/dS ratios in the human and sylvatic strains of DENV-2 indicating there was no major change in selection pressure, or host-specific adaptations in the E gene, as the virus changed transmission cycles (Twiddy et al., 2003). If this observation remains true when a larger sample of monkey-associated strains are analyzed, the implication is that sylvatic viruses will have little trouble spreading in human populations should the opportunity arises nowadays (Holmes and Twiddy, 2003).

The widely distributed Cosmopolitan genotype appears to be subject to stronger positive selection pressure than other DENV-2 genotypes, which may correlate with its dispersal ability (Twiddy et al., 2002a). Furthermore, viruses in the Cosmopolitan and American genotypes have characteristic mutations at amino acid E-390 in the envelope gene, which was identified as a key virulence determinant in experimental studies (Sanchez and Ruiz, 1996). Therefore, these studies tentatively imply that viral genotypes might differ in **fitness**. Another possible signature for the action of

natural selection in DENV is strain extinction and replacement. For example, in Thailand there was seemingly a turnover of DENV-2 strains between 1980 and 1987 (Sittisombut et al., 1997) and of DENV-3 strains in the 1990s (Wittke et al., 2002). Such strain replacement would be expected if the viruses in question differ in fitness, although this has not been formally demonstrated. A recent study in Vietnam has revealed that the lineage replacement in DENV-2 (from Asian/American to Asian 1) observed in Vietnam was highly likely to be linked to an underlying difference in fitness (Vu et al., 2010). They have shown that the patients infected with Asian 1 had higher level of viraemia relative to Asian/American viruses.

Additionally, this pattern of extinction and replacement could also be explained by entirely **stochastic processes**, such as population bottlenecks following a decline in mosquito numbers during inter-epidemic years or large scale natural catastrophe such as flooding or continuous draught (Holmes and Twiddy, 2003). Large-scale variation in vector population sizes would also mean that **genetic drift or bottleneck** plays a major role in the evolution of DENV, so that the fate of a virus in a population will not always reflect its fitness. Whatever the mechanism, it is likely that strain extinction, perhaps even involving entire genotypes, has been a regular occurrence in viral evolution, especially at times when the numbers of susceptible hosts or mosquitoes were low (Holmes and Twiddy, 2003).

Further explanation of clade replacement or seasonal fluctuation of DENV is the composite "**herd**" **immunologic profile** (antibody and T-cell priming) to each of the four serotypes. Serotype and severity-specific data collected between 1973 and 1999 in Thailand showed that each serotype displays a somewhat different pattern of oscillation across this time period, and that together the four serotypes exhibit rather complex dynamics (Zhang et al., 2005). Mathematical models have been designed to test the theory that the interaction between the periodicity of alternating epidemics due to different serotypes and host immunity can explain the patterns seen in Bangkok (Adams and Boots, 2006; Adams et al., 2006; Wearing and Rohani, 2006). One model describes a scenario in which temporary **cross-immunity protection** between serotypes and seasonal fluctuations in vector populations explains serotype dynamics in Bangkok (Wearing and Rohani, 2006), and suggests that Antibody-dependent Enhancement (ADE) and differences in viral virulence are less important in shaping patterns of transmission (although it does not exclude both playing a key role in disease). Another model postulates that moderate cross-immunity alone can explain the oscillations and periodicity of individual serotypes (Adams et al., 2006), and that clade

replacement events seen within each serotype are also associated with serotype-specific periodicity in combination with cross-reactive protection (Adams et al., 2006; Zhang et al., 2005).

Finally, it has been shown that dengue genotypes are not fixed entities as there is now evidence that **recombination** can occur among them (Holmes et al., 1999; Tolou et al., 2001; Uzcategui et al., 2001; Worobey et al., 1999). Although recombination has been documented within all four serotypes, it has not been observed between them, as expected given their extensive genetic divergence. As in all RNA viruses where recombination has been detected, recombination in dengue most likely occurs through a copy-choice mechanism in which the polymerase switches between parental viral molecules during replication (Lai, 1992). Given the huge numbers of infected hosts and vectors, the fact that *A. aegypti* is known to engage in multiple feeding, and the evidence for mixed infections (Lorono-Pino et al., 1999), it is not surprising that there is evidence for recombination in DENV. However, it is less clear how important recombination has been in shaping DENV evolution in the long-term. The relatively low level of recombination observed to date suggests that it is more a sporadic occurrence rather than a selectively determined trait that increases viral fitness.

I.3 Vectors

I.3.1 Aedes aegypti and Aedes albopictus

The principal vector of DENV is the *A. aegypti* mosquito, an anthropophilic species that has adapted extremely well to the urban environment and is found both indoors and outdoors in close proximity to human dwellings (Gubler, 1997). *A. aegypti* is believed to have originated in the jungles of Africa, where the ancestral form, *A. aegypti* formosus, uses tree holes as larval development habitats (Tabachnick and Powell, 1979) and was most likely spread throughout the rest of the world via slave and trading ships during the seventeenth to nineteenth centuries (Gubler, 1997; Smith, 1956). Now a fully domesticated mosquito, *A. aegypti* is an efficient vector of DENV because of its preference for laying its eggs in artificial containers, biting humans, and remaining indoors, where it has access to its favorite host (Gubler, 1997, 1998). Of note, a majority of *Aedes* circulating in Cambodia is *A. aegypti* (Huy et al., 2010b).

A. albopictus is a secondary vector of DENV in SEA, the Western Pacific, and increasingly in Central and South America (Gratz, 2004), but it has also been documented as the sole vector during certain dengue epidemics (Ali et al., 2003; Effler et al., 2005). Prior to 1979, this species was found only in Asia and in the Western Pacific, but it has spread to much of the rest of the world in recent decades (Gratz, 2004; Smith, 1956). The range of *A. albopictus* stretches farther north than that of *A. aegypti*, and its eggs are somewhat resistant to subfreezing temperatures (Hawley et al., 1987), raising the possibility that *A. albopictus* could mediate a re-emergence of dengue in the United States or Europe. For example, *A. albopictus* can survive the winters in northern Italy (Romi, 1995) and was recently implicated in an outbreak of Chikungunya virus in Italy (Watson, 2007) and indigenous dengue infection in France (La Ruche et al., 2010).

I.3.2 Transmission cycles

Although most human infections today are caused by DENV strains (endemic cycles) that rely only on humans as reservoir and amplification hosts, and principally on *Aedes* spp. as vectors. The ancestral forms of DENV are believed to be viruses that circulate in forest habitats, presumably among nonhuman primates (NHPs), transmitted by arboreal mosquitoes (Vasilakis and Weaver, 2008) (Figure 6). These DENV sylvatic cycles have been demonstrated in Asia and in Africa.

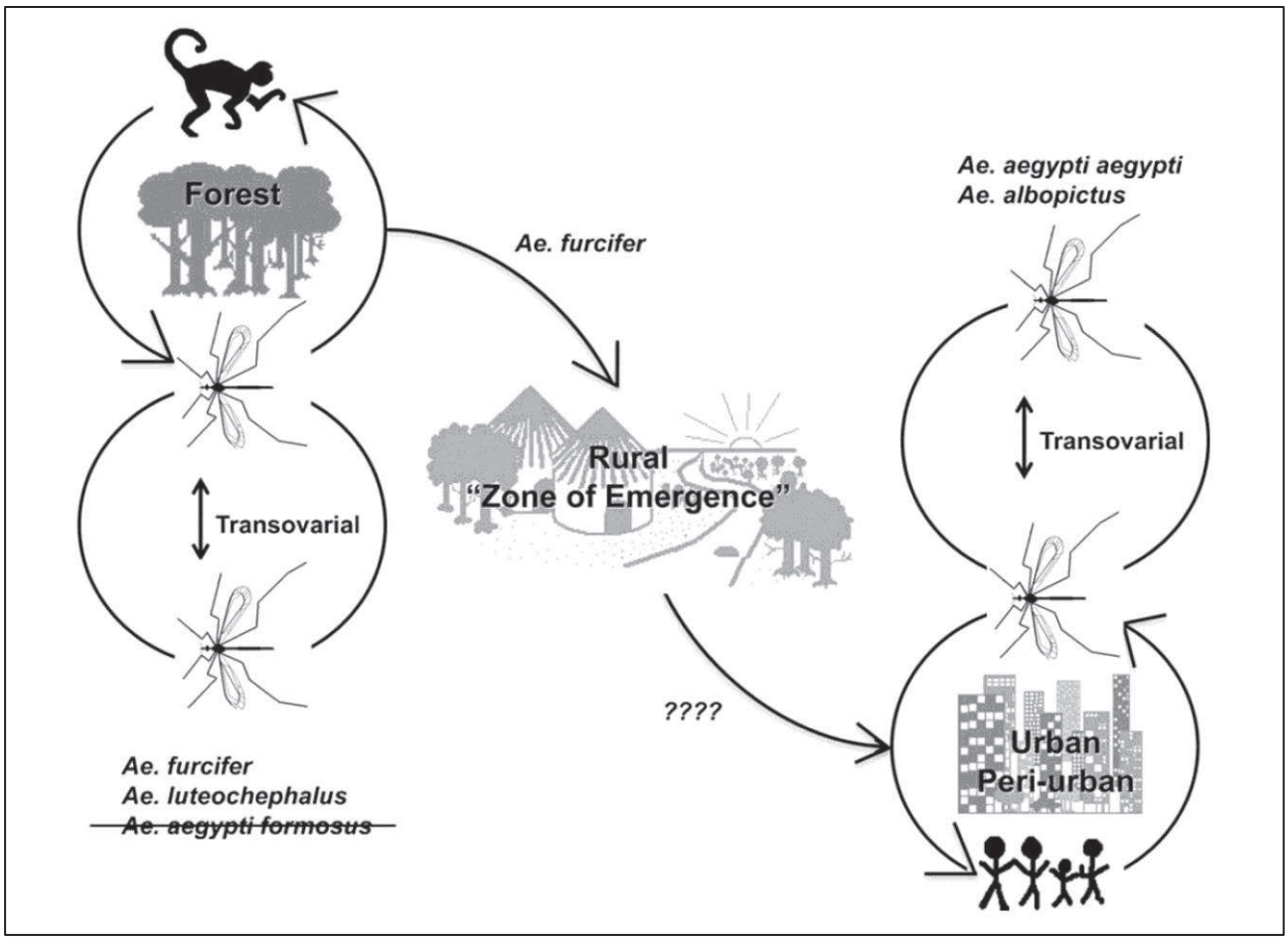


Figure 6: Cartoon depicting the hypothetical evolutionary history of endemic/epidemic DENV emergence from zoonotic transmission cycles (Weaver and Vasilakis, 2009).

I.3.2.1 Sylvatic DENV cycles

Transmission cycle of sylvatic DENV most likely involves NHPs as reservoir hosts and several arboreal canopy-dwelling *Aedes* spp. mosquitoes (Figure 6). These DENV sylvatic cycles have been demonstrated in Asia, where serological evidence as well as virus isolation suggest transmission of sylvatic strains of DENV-1, -2, and -4 among Macaca and Presbytis monkeys vectored by *Aedes niveus* (Peiris et al., 1993; Rudnick, 1965).

In West Africa, only sylvatic DENV-2 has been shown to circulate regularly between Erythrocebus patas monkeys and various sylvatic *Aede*s sp., including *A. taylori*, *A. furcifer*, *A. vitattus*, and *A. luteocephalus* (Diallo et al., 2003; Rodhain, 1991) and was isolated from 5 humans in eastern Senegal (Saluzzo et al., 1986; Zeller et al., 1992). All sylvatic isolates are genetically distinct from all endemic isolates, and are isolated evolutionarily (Rico-Hesse, 1990; Wang et al., 2000).

I.3.2.2 Endemic/epidemic DENV cycles

Endemic DENV circulates among humans, which serve as both reservoir and amplification hosts, by the peridomestic *A. aegypti* and other *Aedes* spp. (i.e. *A. albopictus*) serving as secondary vectors. The efficiency of the endemic cycle is now completely independent both evolutionarily and ecologically from the ancestral, sylvatic cycles. The viruses are maintained in an *A. aegypti*-human-*A. aegypti* cycle with periodic epidemics (Figure 6).

The adult mosquitoes prefer to stay indoors, do not travel great distances by themselves (usually less than 200 m), and prefer to feed on humans during daylight hours (Ordonez-Gonzalez et al., 2001). There are two peaks of biting activity: early morning for 2 to 3 h after daybreak and in the afternoon for several hours before dark (Gubler, 1998). However, these mosquitoes will feed all day indoors and on overcast days. The female mosquitoes are very nervous feeders, disrupting the feeding process at the slightest movement, and tend to feed multiple times. Because of this behavior, *A. aegypti* females will often feed on several persons during a single blood meal and, if infective, may transmit DENV to multiple persons in a short time (Gubler and Rosen, 1976; Platt et al., 1997). After a person is bitten by an infective mosquito, the virus undergoes an incubation period of 3 to 14 days (average, 4 to 7 days), after which the person may experience acute onset of fever accompanied by a variety of nonspecific signs and symptoms and DENV may circulate in the peripheral blood for as long as 10 days (Gubler et al., 1981). If other *A. aegypti* mosquitoes bite the patient during this febrile viraemic stage, those mosquitoes may become infected and subsequently transmit the virus to other uninfected persons, after an extrinsic incubation period of 4 to 12 days (Gubler and Rosen, 1976) (Figure 7).

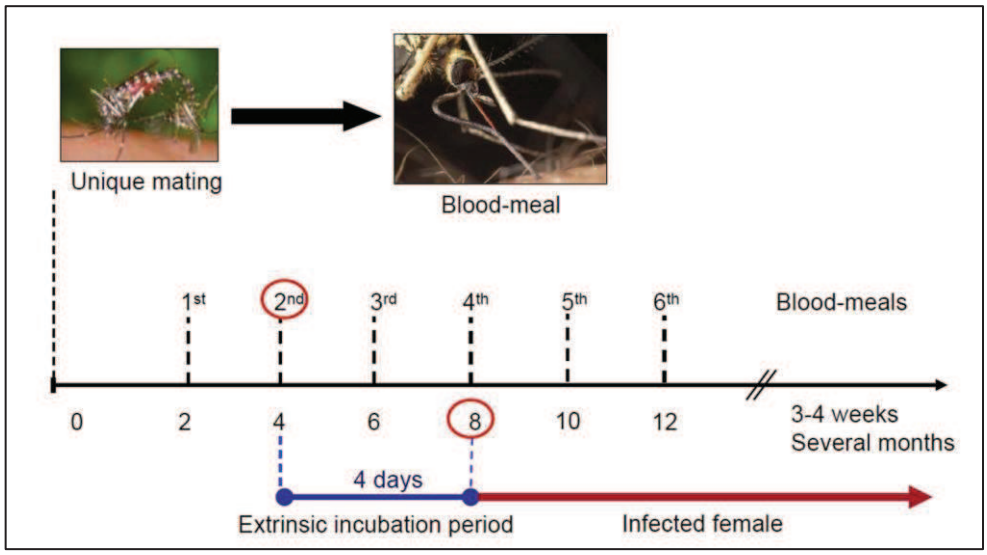


Figure 7: Mosquitoes blood meal and transmission.

I.3.3 Maintenance of DENV and vertical transmission of DENV in mosquitoes

In most endemic countries, dengue displays a seasonal pattern related to temperature and rainfall (Halstead, 2008). This has raised the question of how the virus overwinters, or persists during dry or cold seasons. One possibility is that a population of infected mosquitoes could survive throughout the interim and introduce the virus during the next season. Once infected, *Aedes* mosquitoes remain infected with DENV for life, and the longest lifespan recorded to date is 174 days, although a more typical survival rate is 1–2 weeks (Gubler, 1997). A second possibility is passage of the virus to the next generation of mosquitoes via survival in an infected egg. Recent studies have demonstrated that vertical transmission is possible both in the laboratory and in the wild (Gunther et al., 2007). Some evidence shows that *A. albopictus* mosquitoes are more efficient at vertical transmission than *A. aegypti*, which would make them a candidate for maintaining DENV during interepidemic periods (Rosen et al., 1983). Thus, vertical transmission of DENV in mosquitoes is possible, whether or not the mechanism is truly transovarial or mediated by infection of the mature egg at the time of oviposition (Gubler, 1997). Finally, given the high number of asymptomatic cases of dengue (Balmaseda et al., 2006; Burke et al., 1988; Endy et al., 2002), it is also possible that silent transmission in humans by a reduced number of vectors maintains DENV circulation between epidemic seasons.

I.4 DENV Epidemiology

I.4.1 History and origin of DENV

There is no consensus on when dengue first appeared in human populations, largely because its symptoms are non-specific (influenza-like symptoms) and often not diagnosed. The earliest known clinical descriptions of a dengue-like illnesses are from Chinese encyclopedia written during the Chin Dynasty [Common Era (CE) 265–420], Tang Dynasty (CE 610) and Northern Sung Dynasty (CE 992) (Gubler, 1998). These reports described a disease called 'water poison,' due to its association with water-associated flying insects, and whose clinical description included fever, rash, arthralgia, myalgia and hemorrhagic manifestations. The next reports of a similar illness appear almost seven centuries later, describing an acute illness with prolonged convalescence in the French West Indies and Panama during 1635 and 1699 respectively (Vasilakis and Weaver, 2008). A century later (1779–1788), the first reports of a possible dengue pandemic in Batavia (nowadays Jakarta) (Pepper, 1941), Cairo (Christie, 1881), Philadelphia (Weaver and Vasilakis, 2009), and Cadiz and Seville, Spain (Christie, 1881) were described.

The first serodiagnosis of dengue was done in human volunteers in Fort McKinley in the Philippines and it was concluded that the etiologic agent of dengue was filterable (Ashburn and Craig, 1907). The role of *A. aegypti* in transmission of DENV was confirmed in 1926 by the extensive and well-controlled experiments of Siler (Siler et al., 1926), followed by the incrimination of *A.* (Stegomyia) *albopictus* in 1931 (Simmons et al., 1931).

But it was not until World War II that Japanese scientists isolated DENV-1 (Mochizuki strain) in Nagasaki in 1943, as well as other DENV-1 strains from affected patients in Japan (Hotta, 1952). Later, Sabin isolated both DENV-1 (Hawaii strain) and DENV-2 (New Guinea C strain) from U.S. soldiers in 1944 (Sabin and Schlesinger, 1945). The first well documented outbreak of dengue haemorrhagic fever (DHF) took place in Manila in 1953/54 (Quintos et al., 1954), and was followed by a larger outbreak in Bangkok in 1958 (Hammon et al., 1960). The viruses isolated from patients during the 1956 Philippines epidemic were members of the DENV-3 (H87 strain) and DENV-4 (H241 strain) (Hammon et al., 1960). Since that time hemorrhagic fever/dengue shock syndrome (DHF/DSS) have become endemic in all countries in Southeast Asia, with a dramatic increase in case numbers and dengue is considered an "emerging" disease. At the same time the geographic range of DHF/DSS has expanded considerably and since the 1950s, the incidence of DHF/DSS has increased over 30 fold (WHO, 2009).

Only recently, molecular phylogenetic analysis has contributed on the evolutionary history of the virus (Gaunt et al., 2001; Kuno et al., 1998; Zanotto et al., 1996) . Although the lack of resolution in the flavivirus tree makes it difficult to accurately reconstruct the origin of DENVs, some inferences can be made. The key findings are that sylvatic transmission cycles involving monkeys have been identified in Asia and West Africa (Rodhain, 1991; Rudnick, 1965; Teoh et al., 2010), and that in DENV-2 and DENV-4, these sylvatic strains fall basal to the human DENV within their respective serotype (this may also be true of DENV-1, although there is little resolution in the phylogeny of this serotype) (Wang et al., 2000) (Figure 8). Although no sylvatic strains have yet been identified in DENV-3, the presence of DENV-3 antibodies in Malaysian monkeys suggests that a sylvatic cycle also exists for this serotype (Rudnick, 1986). Hence, there is strong evidence that dengue was originally a monkey virus and that cross-species transmission to humans has occurred independently in all four serotypes.

Until now, it is still not clear where dengue originated from. Gaunt et al. (Gaunt et al., 2001) suggested an African origin, principally because many of the most divergent mosquito-borne flaviviruses circulate exclusively in Africa and often infect primates, implying that DENV has an origin in Africa. Further, *A. aegypti* is believed to have originated in Africa, although this species is only likely to have been adopted as a vector for human transmission in the relatively recent past. Conversely, ecological and phylogenetic evidence argues for an Asian origin of DENV: (1) greater diversity of sylvatic serotypes (possibly all four) in Southeast Asia, whereas in Africa only circulation of sylvatic DENV-2 has been demonstrated (Cordellier et al., 1983; Rudnick, 1986); (2) phylogenetic analysis demonstrating the deep phylogenetic position of the Asian sylvatic strains (Twiddy et al., 2002a; Wang et al., 2000). Although the high prevalence of dengue in this region also supports this hypothesis, only a small number of African samples (from humans and monkeys) are available for analysis. Nonetheless, conclusive determination of the geographic origin of DENV will require increased sampling of sylvatic strains from both Asia and Africa; currently only seven sylvatic strains of DENV-1, -2, and -4 from Southeast Asia are referenced (Vasilakis and Weaver, 2008).

Insights into the history of DENV can be also obtained by reconstructing a molecular time-scale of its evolution. This was recently achieved by estimating rates of nucleotide substitution using a maximum likelihood method that analyses the amount of evolutionary change that has occurred between viruses sampled at different times (Rambaut, 2000). Employing this method to a large

number of envelope (E) gene sequences revealed that DENV evolution often conforms to a molecular clock, although some lineage-specific rate differences were observed (Twiddy et al., 2003). Using these substitution rates, the origin of DENV (the deepest split in the divergence of the four serotypes) was found to be remarkably recent, at approximately 1000 years ago (Twiddy et al., 2003) (Figure 8), and thus corresponds roughly to the first reports of dengue-like disease. Assuming that DENV evolves at a constant rate, an assumption that may not be completely valid, it was estimated that the endemic DENV-2 genotypes diverged from the sylvatic genotypes no more than 1000 ± 500 years ago, DENV-4 no more than 600 ±300 years ago, and DENV-1 no more than 200 ± 100 years ago (Figure 8) (Wang et al., 2000). Within DENV-2, the African and Malaysian sylvatic lineages diverged from each other about 800 ± 400 years ago, presumably when Asian viruses were introduced into Africa. Slightly more recent dates have been estimated for most recent common ancestors using maximum likelihood methods (Twiddy et al., 2003). They estimated that the dates of cross-species transmission from monkeys to humans only occurred between ~125 (DENV-1), ~320 (DENV-2) and ~200 (DENV-4) years ago, and most of the genetic diversity currently observable within each dengue serotype is estimated to have appeared almost simultaneously and only during the past century.

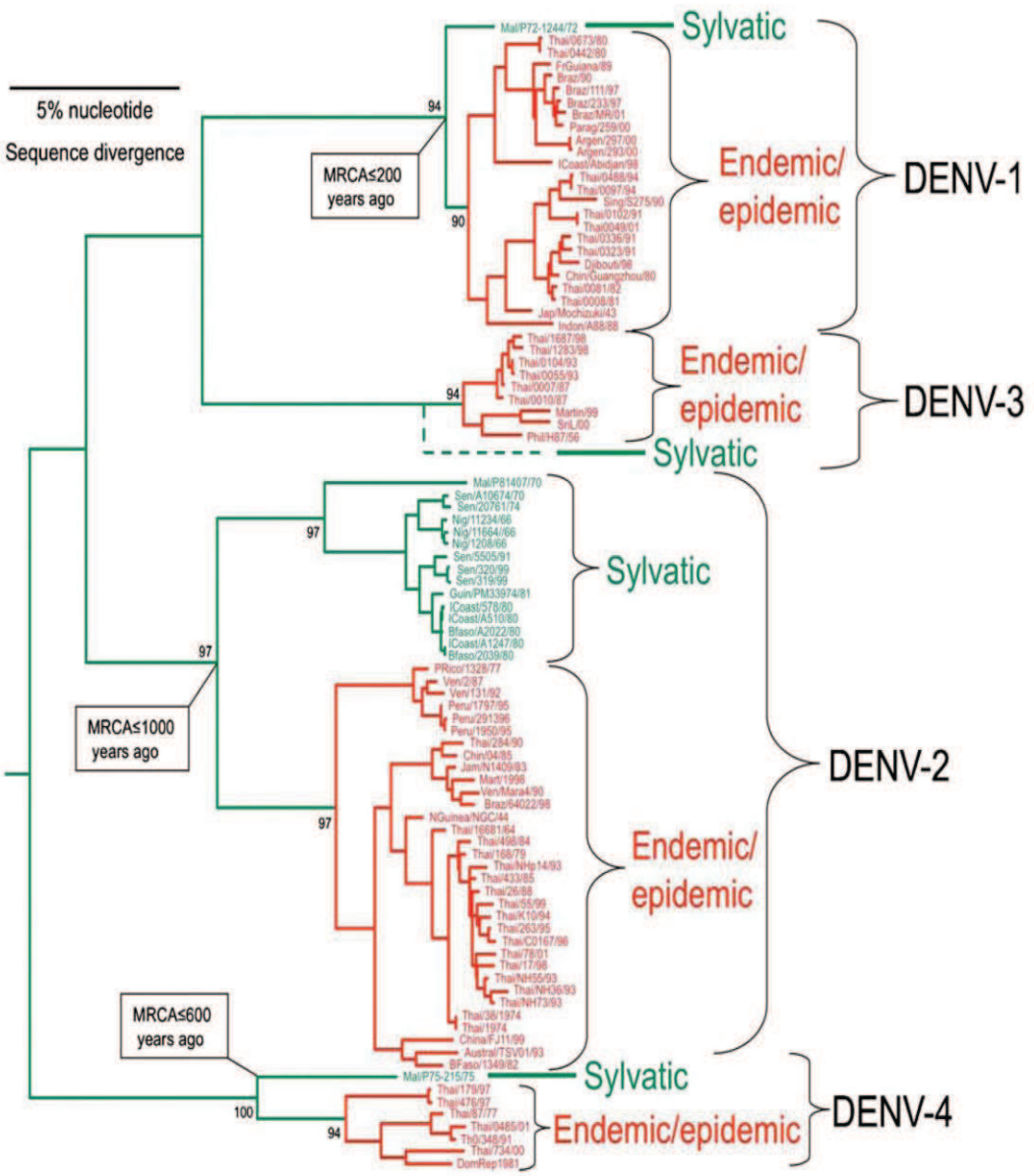


Figure 8: Phylogenetic tree of DENV strains from all 4 serotypes (Weaver and Vasilakis, 2009).

I.4.2 Global burden

Dengue is one of the most rapidly spreading mosquito-borne viral diseases in the world. In the last 50 years, incidence has increased 500-fold with increasing geographic expansion to new countries and from urban to rural settings in tropical and sub-tropical countries in Southeast Asia, the Pacific and the Americas (Figure 9).

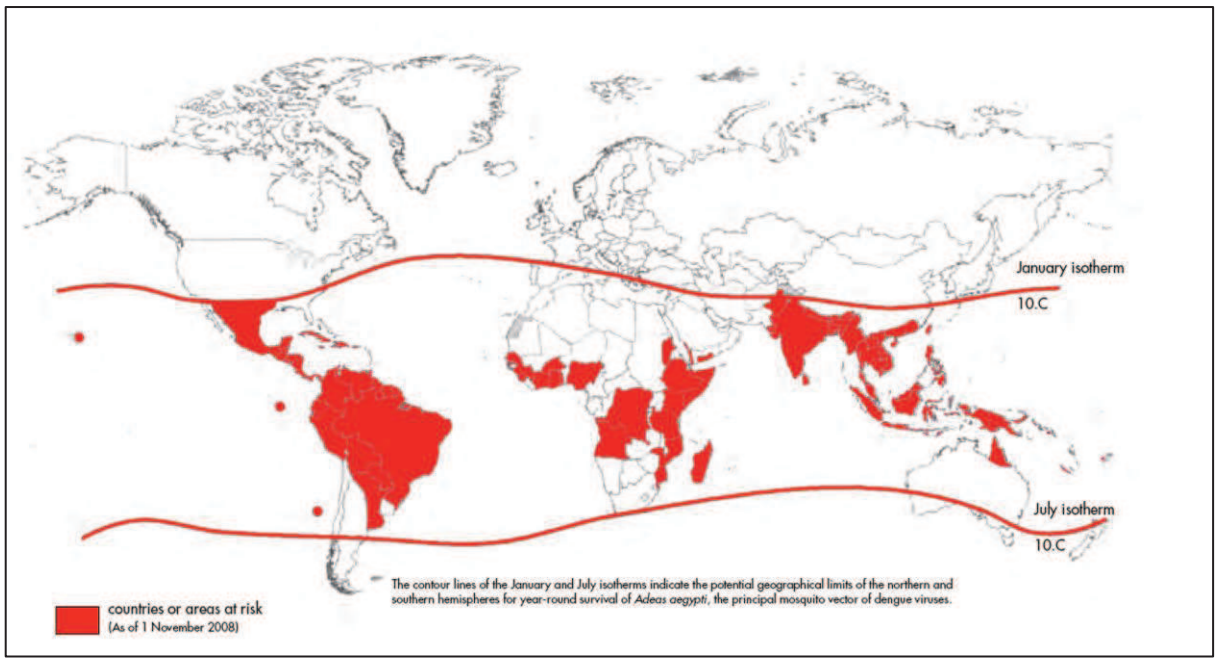


Figure 9: Countries/areas at risk of dengue transmission, 2008 (WHO, 2009).

Worldwide, approximately 2.5 billion people live in dengue endemic countries and an estimated 50 million dengue infections occur annually , including up to 500,000 cases of the life-threatening dengue DHF/DSS, mainly among children, with the case fatality rate exceeding 5% in some area (WHO, 2009). The annual average number of DF/DHF cases reported to the World Health Organization (WHO) has increased dramatically in recent years (Guzman et al., 2010a). For the period 2000–2004, the annual average was 925,896 cases, almost double the figure of 479,848 cases that was reported for the period 1990–1999 (Figure 10). In 2001, a record 69 countries reported dengue activity to WHO (Guzman et al., 2010a).

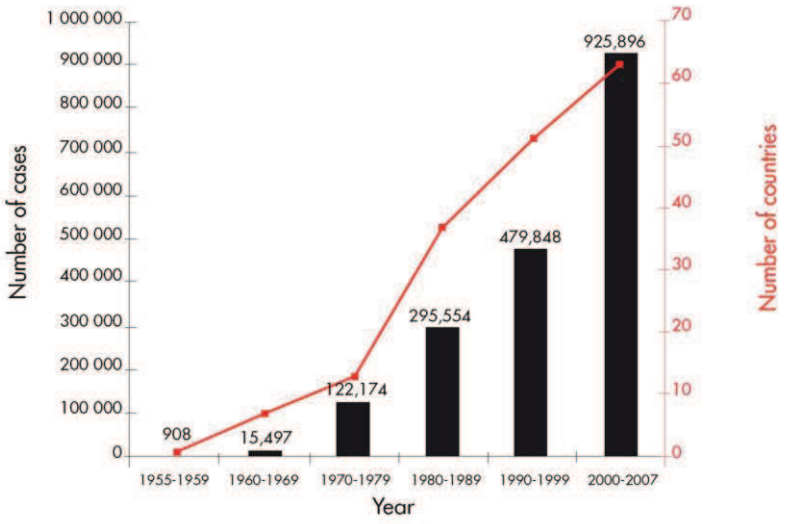


Figure 10: Average annual number of dengue fever (DF) and dengue haemorrhagic fever (DHF) cases reported to WHO, and average of countries reporting dengue, 1955–2007 (WHO, 2009).

All four DENV serotypes are now circulating in Asia, Africa and the Americas, a dramatically different scenario from that which prevailed 20 or 30 years ago (Figure 11). By the 1980s, the American region was experiencing major epidemics of dengue in countries that had been free of the disease for 35 to 130 years (Gubler, 1998). New DENV strains and serotypes were introduced (DENV-1 in 1977, a new strain of DENV-2 in 1981, DENV-4 in 1981, and a new strain of DENV-3 in 1994). Moreover, many countries of the region evolved from nonendemicity or hypoendemicity (one serotype present) to hyperendemicity (multiple serotypes present), and epidemic DHF emerged, much as it had in Southeast Asia 25 years earlier (Gubler, 1998).

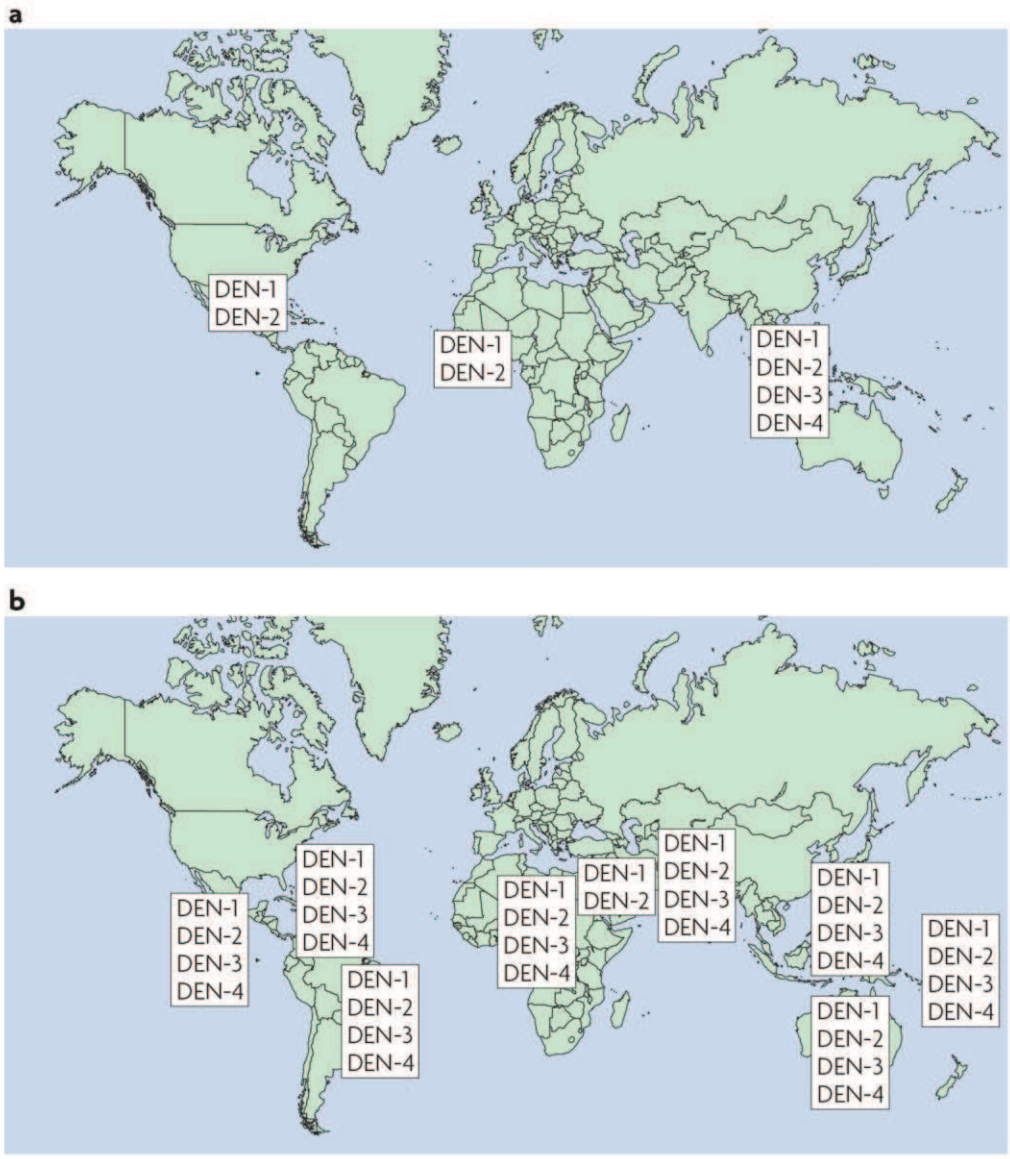


Figure 11: The change in distribution of dengue serotypes. The figure shows the distribution in 1970 (a) and 2004 (b) (Guzman et al., 2010a).

I.4.3 Epidemiological trend in Americas

In the Americas, dengue epidemic during 1960s and 1970s was characterized by the circulation of a single serotype at any given time within a region. The first major epidemic of dengue fever occurred in Cuba in 1977–78 (caused by the DENV-1 serotype). This trend was changed following the introduction of Southeast Asian strain of DENV-2 into Cuba, probably from Vietnam in 1981 (Kouri et al., 1983), followed by an increase in the severity (DHF) during Cuban epidemics in 1981 (Kouri et al., 1989). This DHF epidemic was also the first in the American region and was caused by DENV-2, with secondary dengue infections accounting for 98%–99% of the cases. After this outbreak a very effective and successful control program was launched in Cuba which was free from any dengue viral activity for 16 years (the period 1982–96). In 1989, an epidemic of DHF occurred in Venezuela (Uzcategui et al., 2001), followed by a further epidemic in Cuba in 1997 and both epidemics caused by the DENV-2 serotype. Interestingly, no children were affected during the 1997 DHF epidemic.

During the last two decades the incidence of dengue fever has increased significantly in this region affecting more than 30 Latin American countries. In an analysis of Pan American Health Organization information, the total dengue cases reported increased dramatically from 1,033,417 (16.4/100,000) during the 1980s, to 2,725,405 (35.9/100,000) during the 1990s and 4,759,007 (71.5/100,000) during 2000–2007 (San Martin et al., 2010). Similarly, the number of DHF cases increased from 13,398 during the 1980s, to 58,419 during the 1990s and 111,724 between 2000 and 2007, an 8.3-fold increase. The current epidemiological trend mirrored the events that occurred in Asia, with DHF epidemics occurring every three or four years, with increasing numbers of cases seen within each epidemic (Guzman Tirado et al., 1999). In the USA, from 2001 to 2007, 796 cases of dengue were reported in the United States in the 48 continental states among travelers and immigrants (CDC, 2005, 2007; Ramos et al., 2008).

I.4.4 Epidemiological trend in Africa

There have been few reports of dengue outbreaks among indigenous Africans. Christie in 1881 and Hirsch in 1883 provided the first clinical descriptions of ‘‘dengue’’ in Africa (Fagbami and Fabiyi, 1976). DENV-1 and DENV-2 were isolated at the Virus Research Laboratory at the University of Ibaden, Nigeria, during a fever surveillance program started in 1964 (Carey et al., 1971; Moore et al., 1975). In 1983, the first evidence of autochthonous DENV-4 transmission in Senegal was detected (Saluzzo et al., 1986), and a year later DENV-3 transmission was identified in

Mozambique (Gubler et al., 1986). Although the mosquito vector and all four dengue viral serotypes are present in the African region, to date an epidemic of DHF has not occurred except in rare occasions (Gubler et al., 1986). Since DHF is less frequent among sub-Saharan African population living in areas that experience epidemics of DHF, it is possible that individuals of African origin may have a degree of inherent resistance to the severe form of the disease (Coffey et al., 2009; Sakuntabhai et al., 2005).

I.4.5 Europe, Mediterranean, and the Middle East

Historically, clinical illnesses compatible with dengue were described in Cadiz and Seville, Spain, between 1784 and 1788. Disease occurred throughout the 1800s in the Middle East regions of Suez (1824), the Arabian coast (1835), Yemen (1870–1873), and Israel (1889–1890) (Gubler, 1997). In 1927 and 1928, an extensive dengue outbreak occurred in Greece. Estimates place the total number of infections at greater than one million (Sabin, 1959). Retrospective serologic investigations clearly implicate DENV-1 and possibly DENV-2 as the causative agents (Theiler et al., 1960).

In Europe and the Mediterranean, numerous dengue cases are imported by travelers and expatriates every year. Most infections are acquired in the hyperendemic areas of Southeast Asia or Latin America (Janisch et al., 1997). In 2008, 116 cases were reported, mostly in European travellers; 43% had travelled to Europe from South East Asia, 14% from Latin America, 12% from the Indian subcontinent, 11% from the Caribbean and 4% from Africa, reflecting worldwide dengue activity and travel preferences (Guzman and Isturiz, 2010). Britain (Jacobs et al., 1991), Germany (Eisenhut et al., 1999), the Czech Republic (Chalupa et al., 2001), France (Chippaux and Poveda, 1993), Israel (Schwartz et al., 1996), the Netherlands (Ligtenberg et al., 1991), Norway (Jensenius et al., 1997), Spain (Gascon et al., 1998), and Sweden (Wittesjo et al., 1993) have all reported cases in returning travelers and expatriates. Illnesses resulting from infection usually result in DF; however, cases of DHF have been reported (David et al., 2000; Ligtenberg et al., 1991). Global climatic changes, the importation of tires or other *Aedes* breeding containers, and the subsequent migration of dengue vector populations particularly *A. albopictus* increase the possibility of indigenous dengue transmission in European, Mediterranean, and Middle East regions (Knudsen et al., 1996; Rodhain, 1995; Shope, 1991). Recently, first autochthonous DENV infections have been described in France (La Ruche et al., 2010) and in Croatia (Gjenero-Margan et al., 2011).

I.4.6 Epidemiological trend in the Pacific region

Dengue has been described in the Pacific region for over 100 years. During World War II, DENVs were introduced throughout Southeast Asia, Japan, and the Pacific Islands (Thomas et al., 2003). Since World War II, dengue epidemics have become more and more frequent in the region (Fauran, 1996; Halstead, 1992). Throughout the 1990s, more than 1 million cases of dengue were reported in the western Pacific region. The Philippines, Malaysia, Cook Islands, French Polynesia, New Guinea, and Australia (Russell, 1998) all reported cases early in the decade. In 1998 and 1999, there were 356,554 and 64,066 cases of DF and DHF with 1470 and 112 deaths, respectively (WHO, 1998). DENVs continue to circulate throughout the Pacific region and the incidence of DHF may increase despite many of the islands' geographic isolation, due to the ease of international travel.

I.4.7 Epidemiological trend in Southeast Asia

The first well documented epidemic of DHF was reported in Thailand and in the Philippines in the 1950s. Then, subsequence major outbreaks were reported across Southeast Asia (SEA) with high number of DHF. In 1998, worldwide dengue epidemics accounted for 1.3 million cases of DF and DHF with 3442 deaths and SEA countries alone has reported 218,859 dengue cases and 2075 deaths (Thomas et al., 2003). Since 2000, epidemic of dengue has spread to new areas (i.e. Bhutan, Nepal) and has increased in the already affected area. This explosion of dengue infection was caused mainly by the increase of human activities such as rapid demographic increase, population migration from rural to urban area, anarchic urbanization, transportation, etc. The endemic transmission cycle of dengue in SEA differs from that in Americas and is characterized by transmission activity all year around with peak during rainy season. Although all 4 serotypes are now found in circulation in Americas and in Asia, the severe form of dengue is more than 17 fold higher in Asia. The most affected age group is between 5 to 9 years. However, in Singapore, adult are often affected with moderate clinical symptoms; in Thailand, the incidence seems to move to toward older children and adult in these recent years. Between 2001 and 2008, 1 020 333 cases were reported in Cambodia, Malaysia, the Philippines, and Viet Nam with the high numbers of deaths (WHO, 2009).

This review was summarized in the article below, published in « Médecine Tropicale : revue du Corps de santé colonial» in 2009.

Dengue et autres arboviroses en Asie du Sud-Est

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RÉSUMÉ • En Asie du sud-est, les arbovirus d'intérêt médical sont essentiellement représentés par les *Flavivirus* (dengue, encéphalite japonaise, Kunjin, Zika, etc.) et par les *alphavirus* (Chikungunya, Sindbis, Getah, etc...). Ces virus sont tous transmis par des moustiques. Le virus de la dengue est l'arbovirus le plus fréquent dans la région et pose les problèmes de santé publique les plus importants. L'encéphalite japonaise a une aire de répartition large en Asie et, lorsque l'infection est symptomatique, elle s'accompagne d'une mortalité élevée et de séquelles neurologiques graves. Le Chikungunya est une maladie moins grave mais très douloureuse. Il semble y avoir une nette recrudescence du nombre de cas depuis le début de l'année 2009. Les études épidémiologiques montrent une augmentation constante et parfois exponentielle des cas d'infection par des arbovirus asiatiques. Les facteurs liés à l'extension de ces viroses sont multiples et souvent complexes.

MOTS-CLÉS • Arbovirus. Asie du Sud-Est. épidémiologie. Evolution.

DENGUE AND OTHER ARBOVIRAL DISEASES IN SOUTH-EAST ASIA

ABSTRACT • The most medically significant arboviruses causing human illness in south-east Asia belong to the genera Flavivirus (dengue, Japanese encephalitis, Kunjin, Zika, etc.) and Alphavirus (Chikungunya, Sindbis, Getah, etc.). All of these arboviral diseases are transmitted by mosquitoes. Dengue virus is the most prevalent arbovirus in south-east Asia and constitutes a major public health problem. Japanese encephalitis virus is also widespread in the region and symptomatic infection is associated with high mortality and severe neurological morbidity. Chikungunya virus causes mild but extremely painful illness. The number of Chikungunya cases has been increasing since early 2009. Epidemiological data show a steady, sometimes exponential, increase in the number of arbovirus infections in Asia. The spread of these viral infections can be linked to a number of complex factors.

KEY WORDS • Arboviruses. South-East Asia. Epidemiology. Evolution.

Aspects virologiques et cliniques des principales arboviroses en Asie du Sud-Est

Les arbovirus (pour « arthropod-borne viruses ») regroupent plus de 500 virus appartenant à des familles très distinctes, mais seuls quelques dizaines d'entre eux sont d'intérêt médical. En Asie du Sud-Est, les espèces les plus importantes en pathologie humaine appartiennent aux genres *Flavivirus* et *Alphavirus*.

Le genre *Flavivirus* appartient à la famille des *Flaviviridae.* Les *Flavivirus* sont des virus enveloppés à ARN simple brin, non-segmenté et de polarité positive. Parmi les *Flavivirus* retrouvés en Asie du Sud-Est, la dengue et l'encéphalite japonaise (EJ) posent les problèmes de santé publique les plus préoccupants. D'autres Flavivirus, tels que les virus Kunjin ou Zika, sont beaucoup plus rares et nous ne citerons pas ceux qui ne sont isolés que tout à fait exceptionnellement.

Le virus Kunjin a été pour la première fois décrit en Australie, il y a une cinquantaine d'années. Il appartient au groupe antigénique de l'EJ et est très proche du virus West Nile. Le réservoir principal est constitué par les oiseaux et l'homme n'est qu'un hôte accidentel. Des traces de ce virus ont été retrouvées en Malaisie, à Bornéo et en Papouasie-Nouvelle Guinée lors d'études sérologiques ou entomologiques (1). L'infection chez l'homme est habituellement asymptomatique (2).

Le virus Zika a été initialement découvert en 1947 chez un singe en Ouganda. Il a été à l'origine de cas de fièvres en Indonésie (3) et a pu être isolé chez des moustiques en Malaisie (4).

Le virus de la dengue comprend 4 sérotypes antigéniquement différents (DENV1-4), et chaque sérotype est lui-même divisé en génotypes (Fig. 1). Un génotype est défini par des critères d'analyses phylogénétiques de la séquence du gène codant pour la glycoprotéine d'enveloppe (E).

Le virus de la dengue est transmis par des moustiques du genre A*edes* (*Ae.*) et la principale espèce vectrice est *Ae. aegypti,* moustique anthropophile qui vit à proximité des habitations humaines car ses gîtes larvaires sont essentiellement constitués par des réceptacles artificiels d'eau résultant des activités de l'homme (jarres, pneus usagés, détritus en plastique, etc.). La rapide croissance démographique et l'urbanisation que connaît l'Asie du Sud-Est font partie des facteurs particulièrement propices à l'extension des épidémies de dengue dans cette région. Il existe également un cycle sylvatique de la dengue faisant intervenir des singes et divers moustiques. Ce cycle enzootique a été décrit en Afrique (avec le sérotype DENV-2) et en Asie (avec l'ensemble des 4 sérotypes) où les cas humains sont rares. En effet, si un cas de dengue relativement sévère lié à une souche sylvatique de DENV-2 a été décrit très récemment en Malaisie, le précédent cas découvert en Asie remontait à 1970 (5).

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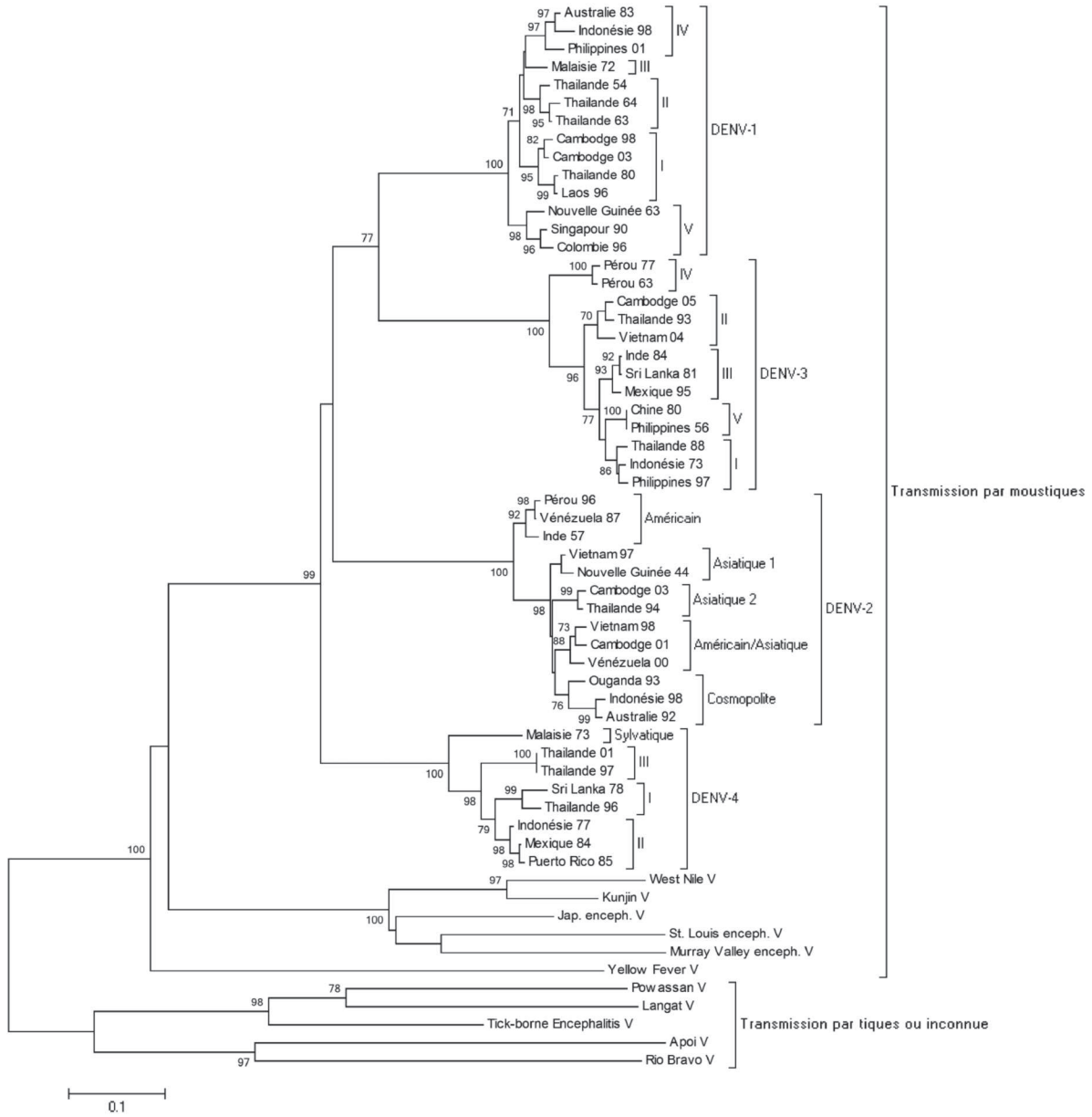

Figure 1. Analyse phylogénétique du virus de la dengue et de quelques autres flavivirus.
Après alignement manuel des séquences nucléotidiques du gène E, l'analyse phylogénétique a été effectuée selon l'algorithme neighbor-joining présent dans le logiciel MEGA (www.medgasoftware.net). Les chiffres sur la droite correspondent aux valeurs des bootstraps (1 000 répétitions). Pour chacun des sérotypes de la dengue, des souches représentatives de chaque sérotype (identifiées par le pays et l'année d'isolement) ont été incluses afin d'identifier les génotypes (en chiffres romains sauf pour les virus DENV-2). Les séquences de ces souches on été obtenues sur GenBank ou générées à partir d'isolats cambodgiens (Buchy P. et Veasna D., données non publiées).

On ne connaît pas avec certitude la date d'apparition de la dengue chez l'homme. Une encyclopédie médicale chinoise datant de l'an 992 décrit les symptômes d'une maladie ressemblant à la dengue (6). L'observation de cas cliniques suspects a également été faite à la fin du 18e siècle et il semble que le virus ait gagné les zones tropicales et subtropicales du globe vers la fin du 19e ou au début du 20e siècle, tandis que les premières formes hémorragiques étaient décrites (7). En 1953-1954 à Manille puis en 1958 à Bangkok, les premières grosses épidémies de formes hémorragiques de dengue sont apparues, touchant majoritairement des enfants (8).

On admet classiquement que la majorité des infections par le virus de la dengue sont asymptomatiques ou pauci-symptomatiques. On distingue 3 formes cliniques de la dengue : la fièvre dengue ou dengue « classique » (DF pour « dengue fever »), la dengue hémorragique (DHF pour « dengue haemorrhagic fever ») et la dengue avec choc (DSS pour « dengue shock syndrome »). La DF est caractérisée par une fièvre élevée d'apparition brutale suivant une période d'incubation de 2 à 7 jours. La fièvre est associée à des céphalées, des douleurs rétro-orbitaires, des myalgies, des arthralgies, une injection conjonctivale, des nausées, des vomissements, une anorexie, un érythème facial. La période fébrile est soit continue (durant 6 à 7 jours), soit bi-phasique avec une phase de défervescence vers le 3e-5e jour au cours de laquelle un rash maculopapulaire ou morbilliforme apparaît sur le tronc et diffuse sur le

corps de manière centripète en épargnant les paumes des mains et les plantes des pieds. La convalescence est souvent longue avec une persistance de l'asthénie et des douleurs. Au moment de la période de défervescence thermique, des hémorragies de la peau et des muqueuses peuvent s'observer : pétéchies, épistaxis, ménorragies, hémorragies digestives, etc. On parle alors de dengue hémorragique. Si cette dénomination est correcte d'un point de vue clinique (encore que des manifestations hémorragiques bénignes peuvent faire partie du tableau de DF), elle ne l'est pas d'un point de vue physiopathologique car la DHF est définie par une fuite plasmatique liée à une augmentation de la perméabilité vasculaire. Lorsque les effusions des séreuses et les hémorragies s'accentuent, le patient peut entrer en état de choc circulatoire (DSS). Les distinctions entre les formes cliniques de la dengue ne sont en réalité pas toujours aussi tranchées et les classifications cliniques du passé sont en cours de révision afin de permettre aux médecins d'administrer le traitement le plus adéquat et d'améliorer les taux de survie dans les formes sévères.

Le virus de l'encéphalite japonaise est surtout transmis par *Culex tritaeniorrhynchus,* un moustique pondant volontiers dans les rizières. Les oiseaux aquatiques (hérons, aigrettes, etc.) constituent le réservoir naturel du virus qui néanmoins passe régulièrement chez le porc (jouant le rôle d'hôte amplificateur), chez certains équidés et bien entendu à l'homme. Les porcs domestiques, nombreux en Asie du Sud-Est et très souvent élevés à proximité si ce n'est directement sous les habitations en zone rurale, constituent un facteur de risque important de transmission du virus à l'homme. Le virus de l'EJ semble être originaire de l'archipel malais et aurait évolué, probablement il y a quelques milliers d'années, en 4 génotypes avant de gagner toute l'Asie. Le 1er cas clinique a été décrit au Japon en 1871. Les formes symptomatiques ne représentent que 0,3 à 0,5 % des infections et se manifestent chez l'homme par une encéphalite fébrile classique survenant environ 1 à 2 semaines après une piqûre infectante (9). La mortalité atteint alors 25 à 50 % et plus de la moitié des survivants souffrent de séquelles neurologiques parfois très invalidantes. Il existe des vaccins efficaces et financièrement abordables, mais ils sont encore peu utilisés dans les pays d'endémie les plus défavorisés. Actuellement les 3 vaccins les plus utilisés sont : le vaccin inactivé et purifié, préparé sur cerveaux de souris en utilisant la souche Nakayama ou Beijing ; le vaccin inactivé produit sur cultures de cellules primaires de reins de hamster ou sur cellules Vero à partir de la souche Beijing P-3 ; le vaccin vivant atténué préparé sur culture cellulaire et utilisant la souche SA 14-14-2. Les inconvénients du vaccin préparé sur cerveau murin sont la nécessité d'administrer plusieurs doses et le coût relativement élevé de chaque dose. L'immunité obtenue après deux administrations varie entre 94 et 100 % chez les enfants de plus d'un an, et pratiquement tous les enfants ou adultes qui bénéficient d'un rappel à un an développent un taux protecteur d'anticorps. On observe des réactions locales (œdème, érythème, etc.) dans environ 20 % des cas et des signes systémiques mineurs (céphalées, fièvre, myalgies, etc.) avec à peu près la même fréquence. L'OMS estime qu'aucune preuve véritable n'existe pour relier l'administration du vaccin préparé sur cerveau de souris à la survenue d'une encéphalomyélite aiguë disséminée. Les vaccins atténués ou inactivés préparés sur cultures cellulaires sont utilisés à grand échelle en Chine où le vaccin atténué tend néanmoins à remplacer progressivement le vaccin inactivé. Le coût de ces vaccins est moins élevé car les doses nécessaires pour obtenir une immunité durable sont plus faibles. Le vaccin vivant atténué est préparé à partir d'une souche neuro-atténuée et génétiquement stable (SA 14-14-2). La récupération d'une neurovirulence est considérée comme hautement improbable. Ce vaccin a une efficacité de 95 % après l'administration de 2 doses. Plusieurs vaccins recombinant ou à ADN sont en cours de développement et le vaccin chimérique JEV-fièvre jaune 17D semble prometteur (10).

Les *Alphavirus* appartiennent à la famille des *Togaviridae* et comptent près d'une trentaine de membres. En Asie du Sud-Est, on rencontre essentiellement le virus Chikungunya (CHIKV), plus rarement les virus Sindbis (SINV) ou Getah (GETV). Comme les flavivirus, les *alphavirus* sont des virus enveloppés à ARN monocaténaire non-segmenté de polarité positive.

Il existe plusieurs génotypes de CHIKV, dont un génotype asiatique. Celui-ci comprend un cluster de souches isolées en Inde (1963-1973) et en Thaïlande (1962-1978), et un autre cluster regroupant des virus découverts aux Philippines (1985), en Indonésie (1985), en Thaïlande (1988 et 1995-1996), et en Malaisie (1988). Des cas suspects avaient aussi été décrits au Myanmar, au Vietnam et au Cambodge. Le Chikungunya est une infection virale transmise, en Asie, essentiellement par *Ae. aegypti* ou *Ae. albopictus.* Le réservoir est humain en période épidémique. Le reste du temps, les singes, les rongeurs, les oiseaux et divers invertébrés constituent les principaux réservoirs.

Après une période d'incubation généralement courte (en moyenne de 2 à 4 jours), l'infection se manifeste par une fièvre d'apparition brutale, des céphalées, des myalgies, un rash maculopapulaire prédominant sur le thorax, et surtout des arthralgies intenses et invalidantes qui lui ont valu son nom qui, en swahili, signifie : « marcher courbé ». On observe parfois des pétéchies et des gingivorragies, essentiellement chez les enfants (11).

Le virus Getah appartient au complexe Semliki Forest (SFV). Il a été isolé pour la première fois de *Culex gelidus* en Malaisie, en 1955, et se retrouve dans de nombreux pays d'Asie du Sud-Est (12-14). Ce virus infecte les chevaux et les porcs. Il a été retrouvé en Malaisie dans des sérums humains et au nord Vietnam chez des moustiques mais ne semble pas être à l'origine de maladie chez l'homme.

Le virus Sindbis a été découvert en Egypte en 1952 chez *Culex univittatus* (15). Sindbis est l'*alphavirus* responsable d'arthrite le plus répandu au monde et on le retrouve pratiquement partout en Asie du Sud-Est. Néanmoins, cette virose est encore mal connue et rarement diagnostiquée. Il existe un cycle enzootique de SINV entre les oiseaux et les moustiques ornithophiles et l'introduction du virus dans la population humaine passe par des *Aedes.* Dans les formes apparentes, le virus est responsable d'une fièvre modérée, accompagnée d'un rash cutané diffus touchant les paumes des mains et les plantes des pieds, et d'arthralgies surtout localisées aux grosses articulations, nécessitant parfois une immobilisation à des fins antalgiques. La guérison survient en environ 2 semaines, mais les arthralgies peuvent persister durant des mois voire des années.

Epidémiologie de la dengue et des autres principales arboviroses

Environ 2,5 milliards de personnes dans le monde vivent dans des zones considérées à risque de transmission de la dengue. On estime que 50 millions d'infections surviennent chaque année, dont 500 000 cas de dengue hémorragique et ceci s'accompagnerait d'au moins 22 000 décès, principalement chez les enfants (16). Les épidémies de dengue et de dengue hémorragique sont apparues comme des problèmes majeurs de santé publique ces dernières décennies, avec l'apparition d'une hyperendémicité dans les zones

urbaines et périurbaines de nombreux pays tropicaux et subtropicaux (17). L'incidence annuelle de la dengue a été multipliée par 30 durant ces 50 dernières années avec une croissance du nombre de cas devenue exponentielle ces 15 dernières années (18). L'Asie du Sud-Est recense environ 70 % des cas mondiaux et la dengue hémorragique est devenue la principale cause d'hospitalisation et de décès chez les enfants dans certains pays asiatiques (19).

Les raisons de cette explosion sont principalement liées à l'augmentation des activités humaines. Une forte croissance démographique, en particulier de la population urbaine, est observée depuis 30 à 40 ans. L'urbanisation anarchique et la détérioration de l'environnement urbain compliquent l'application des mesures de contrôle vectoriel. La migration des populations rurales vers les zones urbaines augmente encore le risque épidémique en amenant une population très réceptive, car peu immunisée contre le virus de la dengue, vers des zones à forte transmission. Les voyages aériens favorisent également l'essaimage rapide du virus dans le monde et augmentent les risques d'épidémie par introduction de nouveaux sérotypes (20).

La transmission endémique dans les pays du sud-est asiatique se caractérise habituellement par une transmission de l'infection durant toute l'année, avec un pic saisonnier pendant la saison des pluies. Ces épidémies surviennent de façon régulière et sont entrecoupées par des périodes inter-épidémiques pouvant durer jusqu'à 4 ans selon la nature des sérotypes circulant ou le niveau d'immunisation de la population.

Le profil épidémiologique de la dengue des pays du sud-est asiatique diffère notablement de celui de l'Amérique centrale et latine, l'autre grande région affectée par la dengue. Bien que les quatre sérotypes circulent aussi en Amérique, les formes sévères y sont moins fréquentes qu'en Asie du Sud-Est. Entre 1996 et 2005, l'incidence relative de la dengue hémorragique était 17,3 fois plus élevée en Asie que sur le continent américain (21). Les groupes d'âges les plus affectés par les formes sévères (DHF, DSS) dans les pays où la circulation est la plus forte (exemple : Cambodge, Laos, Vietnam, Indonésie, Philippines) sont les enfants âgés de 5 à 9 ans alors qu`à Singapour, où l'incidence de la dengue est plus faible, l'infection se présente plutôt sous une forme modérée et touche principalement les adultes. Au cours des 20 dernières années, la Thaïlande a connu un changement de profil marqué par un glissement du pic d'incidence des DHF vers des enfants de plus en plus âgés (22). Les cas de dengue classique tout comme les formes plus sévères commencent à toucher les adultes thaïs (23).

Ce phénomène serait attribuable à la longue histoire de transmission de la dengue en Asie du Sud-Est et à de probables différences de facteurs de risque (21). Dans les années cinquante, lors des premières épidémies de DHF aux Philippines et en Thaïlande, l'incidence annuelle de la dengue était de 15 %, et 50 % des enfants avaient déjà été infectés à l'âge de 5 ans. A l'âge de 20 ans, presque tous les individus avaient des marqueurs sérologiques de la dengue (24). Un niveau équivalent, voir même plus élevé, de transmission se retrouve encore de nos jours au Cambodge où les premiers cas ont été décrits en 1963 (25) et où à ce jour plus de 90 % des enfants âgés de quatre ans possèdent déjà des anticorps anti-dengue (Vong S et Buchy P, communication personnelle). Il existe malheureusement assez peu de données précises de surveillance permettant notamment de connaître l'incidence réelle de la dengue en Asie du Sud-Est, de détecter à temps les épidémies, d'adapter au mieux la lutte anti-vectorielle, de gérer plus efficacement les systèmes de soins, et de soutenir la recherche vaccinale qui donne des résultats encourageants.

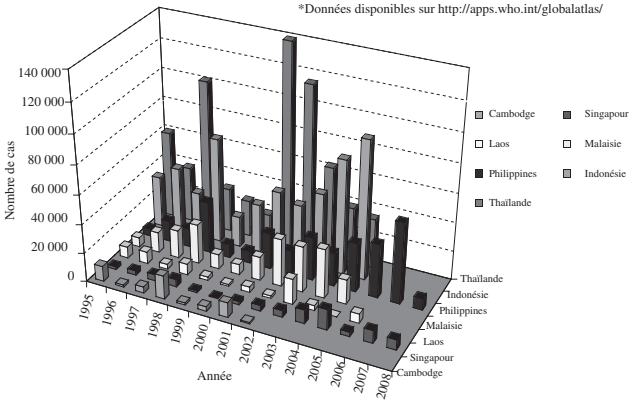

Figure 2. Nombre de cas de dengue notifiés par an au réseau DengueNet dans les pays du Sud-Est Asiatique de 1995 à 2008.*

L'Organisation Mondiale de la Santé (OMS) a été à l'initiative du réseau DengueNet, un système mondial de surveillance de la dengue disponible sur internet. Ce système a néanmoins montré ses limites car bien que la déclaration de la dengue soit obligatoire dans de nombreux pays du sud-est asiatique , seule une petite proportion des cas est signalée à l'OMS (Fig. 2). De plus, seuls quelques pays confirment tous les cas suspects de dengue par des examens de laboratoire (exemple : Singapour, Malaisie). D'autres ne testent que les cas hospitalisés (Philippines, Thaïlande). Le Cambodge, comme le Vietnam, ne réalisent le diagnostic sérologique ou virologique de la dengue que sur un échantillon plus ou moins représentatif de l'ensemble des cas suspects.

Certains pays de la région collaborent avec le PDVI (Pediatric Dengue Vaccine Initiative), un programme de l'International Vaccine Institute (IVI) basé à Séoul en Corée, pour créer des sites de surveillance active des fièvres dont le but est d'estimer plus précisément l'incidence de la dengue dans la communauté.

Environ 3 milliards de personnes vivent en zone d'endémie d'encéphalite japonaise (Asie et Pacifique) et l'incidence annuelle de la maladie est estimée entre 30 000 et 50 000 cas (26). Le nombre annuel de décès est évalué à environ 10 000 à 15 000 cas. Dans les régions tropicales rurales d'Asie du Sud-Est (sud du Vietnam et de la Thaïlande, Indonésie, Malaisie, etc.), la transmission s'effectue plutôt en saison des pluies. Les oiseaux migrateurs pourraient participer à l'essaimage du virus (17). On s'attend à une augmentation du nombre de cas dans des pays comme le Cambodge, l'Indonésie, le Laos, le Myanmar en raison de la croissance démographique élevée et de l'intensification de la riziculture. La majorité des pays du sud-est asiatique ne disposent pas de système de surveillance de l'encéphalite japonaise, ce qui est pourtant un pré-requis indispensable à toute prise de décision d'introduction du vaccin dans un programme national de vaccination (Anonyme). Aux Philippines, en Indonésie et en Malaisie, le vaccin anti-JEV ne fait pas partie des vaccins obligatoires. Néanmoins, en Malaisie, le vaccin est administré aux personnes travaillant dans les élevages commerciaux de porcs ainsi qu'aux habitants vivant dans un périmètre de 2 km autour de ces élevages. Au Vietnam, il existe un programme national basé sur la déclaration des cas cliniques d'encéphalites et un certain nombre de cas d'infection par le JEV sont confirmés par des tests sérologiques. Si officiellement le vaccin contre l'encéphalite japonaise produit au Vietnam sur cerveaux murins est inclus dans le programme élargi de vaccination, il est encore largement sous-utilisé.

Les pic épidémiques asiatiques de Chikungunya sont plutôt urbains et surviennent en saison des pluies, lorsque la densité vectorielle est la plus forte. Les taux d'attaque sont parfois élevés lorsque la population n'est pas immune (jusqu'à 37% en Inde en 1978) (27) puis l'amplitude des pics épidémiques diminue progressivement au fur et à mesure que les patients développent une immunité. En 2009, le sud de la Thaïlande rapportait depuis le début de l'année plus de 20 000 cas suspects (ProMed du 27 mai 2009), la Malaisie en comptait plus de 1 600 (ProMed du 15 Mai 2009), et l'Indonésie plus de 1 500 cas en une seule semaine dans un district de l'île de Sumatra (ProMed du 12 Janvier).

Epidémiologie moléculaire et évolution du virus de la dengue

L'origine du virus de la dengue est difficile à établir. Certains pensent que cet arbovirus provient d'Afrique, essentiellement parce que plusieurs des arbovirus les plus divergents sur le plan génétique circulent exclusivement sur ce continent, y infectant de surcroît surtout des primates (28). De plus, *Ae. aegypti* semble également avoir une origine africaine, même s'il n'est probablement devenu un vecteur de transmission de la dengue chez l'homme que depuis un passé relativement récent. Néanmoins, la coexistence en Asie des 4 sérotypes de la dengue chez l'homme et les primates fait plutôt pencher la balance vers une origine asiatique du virus.

Les 4 sérotypes de la dengue sont phylogénétiquement distincts, souvent autant que peuvent l'être 2 espèces différentes de *Flavivirus* (29). Holmes et Twiddy (30) ont montré que lorsqu'on réalise l'analyse phylogénétique de la protéine non-structurale NS5, les *Flavivirus* se rangent en 3 groupes correspondant généralement à leur mode de transmission : tiques, moustiques ou inconnue.

Les génotypes de chaque sérotype de virus de la dengue sont habituellement bien corrélés avec l'origine géographique des virus (Fig. 1). Néanmoins, dans une région donnée, de nouveaux variants peuvent apparaître à la suite de mutations, d'un phénomène de sélection ou à la suite de l'introduction de nouveaux virus à partir d'une autre région (par l'intermédiaire d'un patient virémique ou d'un virus infecté). Ainsi, l'analyse génotypique peut être un outil intéressant pour tenter d'identifier l'origine géographique d'une épidémie.

En calculant le taux de substitution nucléotidique par site, il est possible d'estimer la date d'apparition probable de la dengue. L'horloge moléculaire du virus de la dengue indique une naissance qui pourrait remonter à environ 1 000 ans, correspondant également à la date approximative des premiers écrits évoquant cette maladie (31). Le passage du virus DENV-2 du singe à l'homme pourrait s'être fait il y a environ 320 ans et celui de DENV-1 il y a quelques 125 années. La grande majorité de la diversité génétique que l'on observe à présent pour chaque sérotype pourrait s'être développée de manière simultanée et en un seul siècle (28). Cette récente histoire évolutive de la dengue suggère que cette virose se transmettait à l'homme initialement seulement selon un mode de cycle sylvatique. Les premières épidémies humaines ont dû survenir quand les hommes ont commencé à empiéter sur les domaines forestiers et ce n'est que l'augmentation rapide de la population, l'urbanisation et le développement des transports modernes qui a pu fournir un nombre suffisant d'hôtes susceptibles pour que la maladie passe définitivement à l'homme. Cette implantation chez l'homme a sans doute bénéficié du caractère bénin de l'infection, ce qui permet à un nombre limité d'hôtes de maintenir la circulation du virus dans la population humaine. La rapide et récente évolution génétique du virus de la dengue n'aurait pas pu avoir lieu si l'homme n'avait efficacement contribué à l'essaimage du virus de part le monde.

Se pose encore la question de l'origine des 4 sérotypes différents. Une des hypothèses évoquées, et à notre avis une des plus probables, serait une séparation du virus en différentes lignées en raison d'une partition géographique allopatrique ou écologique dans diverses populations de primates, permettant une évolution distincte de chacun des 4 sérotypes. L'autre hypothèse serait que le virus ait pu évoluer de manière sympatrique dans une seule et même population et que la présence de 4 sérotypes antigéniquement différents ait facilité la transmission de la maladie par l'intermédiaire du phénomène immunologique de facilitation dépendante des anticorps (ou ADE pour « Antibody-Dependant Enhancement ») (8).

Le virus de la dengue évolue très rapidement en raison d'un fort taux de mutation génétique (commun à tous les virus à ARN), d'un cycle réplicatif rapide et d'une immense population virale disponible. Dans l'exemple du sérotype DENV-2, il existe 6 génotypes dont 2 génotypes asiatiques et 1 génotype « cosmopolite » dont l'aire de distribution comprend la plupart des pays tropicaux, y compris asiatiques (Fig. 1). Ce génotype « cosmopolite » regroupe des virus d'origines géographiques très diverses et démontre à quel point l'hôte et le vecteur peuvent essaimer le virus. On ignore encore si certains génotypes ont un potentiel épidémique plus élevé que d'autres.

On assiste parfois à l'extinction de certaines souches au profit d'autres. Ce phénomène témoigne de l'effet de la sélection naturelle qui s'opère sur le virus de la dengue. Pour illustrer cela, on peut prendre l'exemple de la Thaïlande où, semble t-il, de nombreuses souches différentes de DENV-2 ont circulé entre 1980 et 1987 avant que la dengue 3 ne fasse son apparition dans les années 1990 (32, 33). Le remplacement d'une souche par une autre est prévisible lorsqu'il existe un avantage évolutif. Néanmoins, le phénomène d'extinction et de remplacement peut également être simplement le résultat de phénomènes stochastiques, tel qu'un effet de « goulot d'étranglement » au niveau de la population virale suivi du déclin du nombre de moustiques vecteurs en période inter-épidémique.

Conclusion

Ces données moléculaires et évolutives permettent d'envisager sous un jour un peu différent l'épidémiologie de la dengue. Elles ne viennent que renforcer l'idée que la dengue est une maladie complexe et que de nombreuses connaissances manquent encore à sa compréhension. Quant aux autres arboviroses d'Asie du Sud-Est, leur étude demeure encore bien plus incomplète.

Remerciements : nous souhaitons remercier le Dr J-L Sarthou, Directeur de l'Institut Pasteur du Cambodge, pour la relecture de ce manuscrit et le Dr Rani Manju du bureau régional du Pacifique occidental de l'Organisation Mondiale de la Santé à Manille pour les informations qu'elle a bien voulu nous fournir sur les programmes de vaccination contre l'encéphalite japonaise dans certains pays d'Asie.

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I.5 Diagnosis

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. Its clinical spectrum ranges from unapparent or mild febrile illness to severe and fatal hemorrhagic disease or vascular shock (Rigau-Perez et al., 1998). While most patients recover following a self-limiting non-severe clinical course, a small proportion progress to severe disease, mostly characterized by plasma leakage with or without haemorrhage (Figure 12).

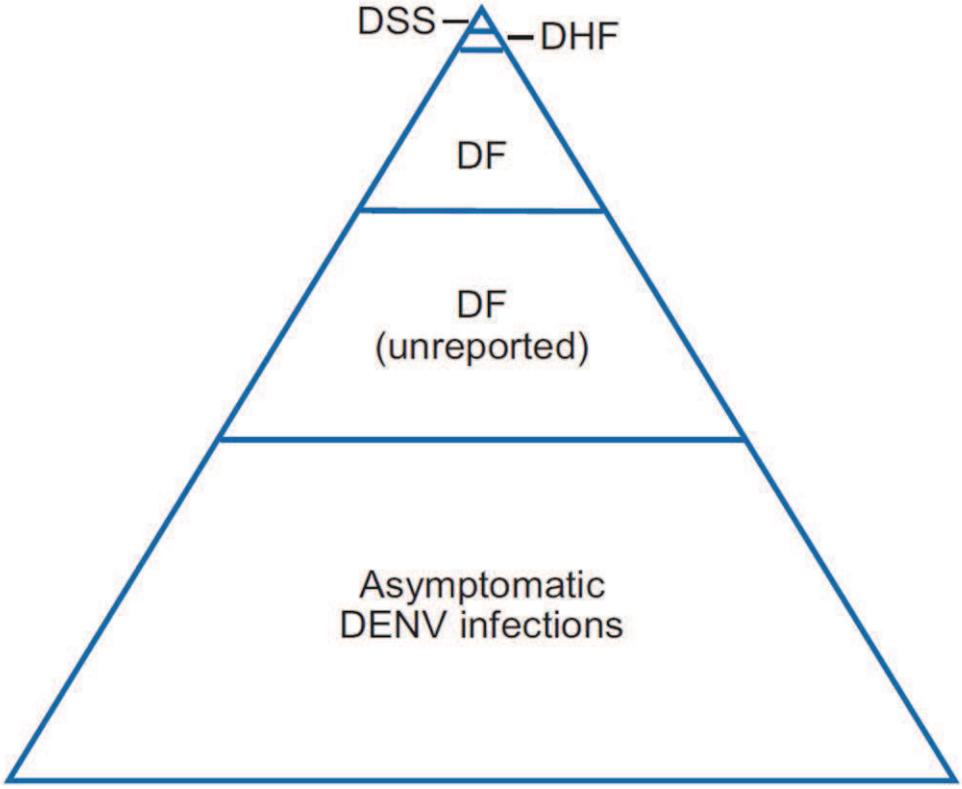

Figure 12: The pyramid representing the clinical spectrum of DENV infections. Among 100 million infections each year, 10%-50% are symptomatic (Kyle and Harris, 2008).

The following sections were mostly adapted from the World Health Organization "Dengue Guidelines for Diagnosis, Treatment, Prevention and Control published" (WHO, 2009).

I.5.1 Classification

According to the classification by WHO guidelines in 1999, dengue manifests in 3 clinical forms: undifferentiated fever, DF and DHF with plasma leakage that may lead to hypovolemic shock, DSS (WHO, 1997). This classification was repeatedly evaluated (Deen et al., 2006; Phuong et al., 2004; Setiati et al., 2007) and was often found inappropriate for clinical management of dengue infection as this classification could mistakenly classify dengue cases. In 2008, a WHO expert group

was organized and proposed a new classification scheme based on dengue disease entity which manifests in a broad spectrum of symptoms and often with unpredictable clinical evolution and outcome (WHO, 2009). The key of this classification is early recognition and understanding of the clinical problems during the different phases of the disease, leading to a rational approach to case management and a good clinical outcome. This new consensus grouped patients with non-severe dengue and those with severe dengue. These two entities can be recognized by a set of clinical and/or laboratory parameters (Figure 13). For practical reasons, the group of non-severe dengue was split into two subgroups: patients with warning signs and those without (Figure 13).

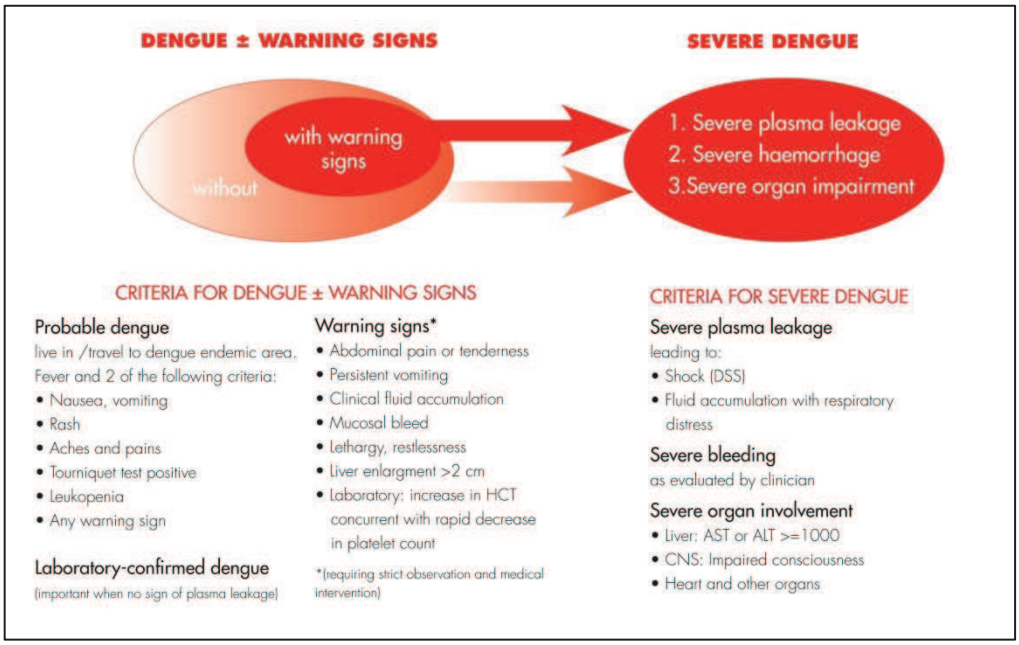

Figure 13: Clinical classification of dengue cases (WHO, 2009).
HCT : Hematocrit; AST : Aspartate Aminotransferase; ALT : Alanine Aminotransferase; CNS : Central Nervous System

I.5.2 Disease course

Dengue infection is a systemic and dynamic disease. After the incubation period, the illness begins abruptly and is followed by 3 phases: febrile, critical and recovery (Figure 14)

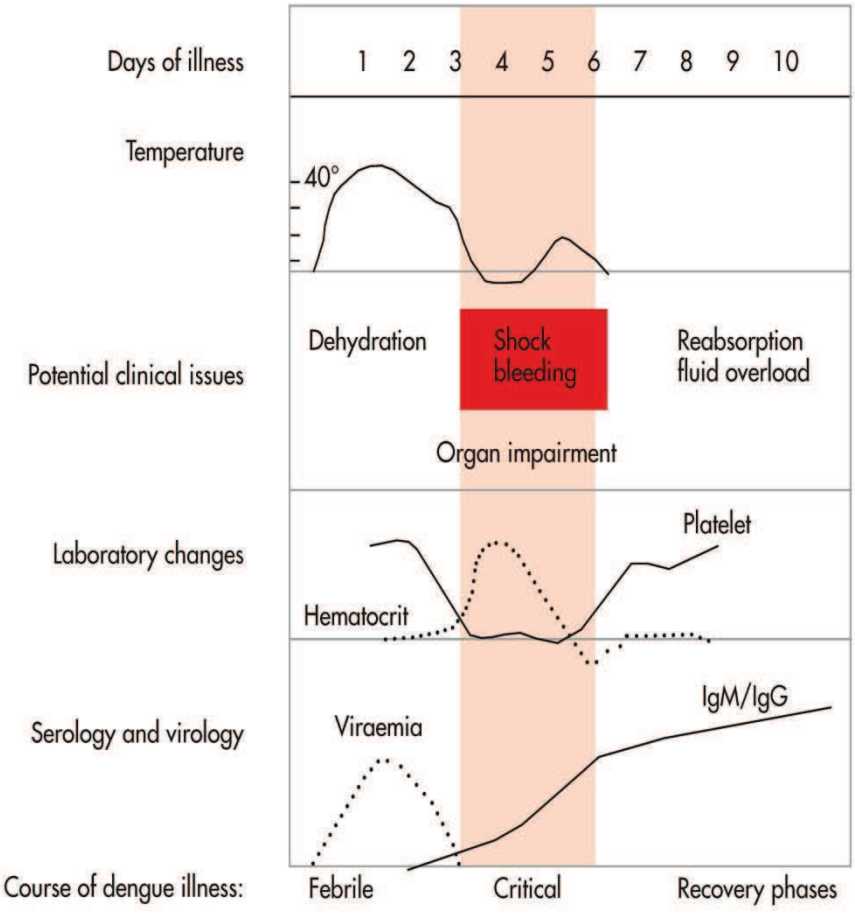

Figure 14: The course of dengue disease (WHO, 2009).

I.5.2.1 Febrile phase

The acute febrile phase usually starts with high fever and lasts 2–7 days (Figure 14). It is often accompanied by facial flushing, skin erythema, generalized body ache, myalgia, arthralgia and headache (Rigau-Perez et al., 1998). Some patients may have sore throat, injected pharynx and conjunctival injection. Anorexia, nausea and vomiting are common. It can be difficult to distinguish dengue clinically from non-dengue febrile diseases in the early febrile phase as these symptoms are not specific. A positive tourniquet test in this phase increases the probability of dengue (Cao et al., 2002; Kalayanarooj et al., 1997). In addition, these clinical features are indistinguishable between severe and non-severe dengue cases. Therefore monitoring for warning signs and other clinical parameters is crucial to recognizing progression to the critical phase.

Mild haemorrhagic manifestations like petechiae and mucosal membrane bleeding (e.g. nose and gums) may be seen (Hammond et al., 2005; Kalayanarooj et al., 1997). The liver is often enlarged and tender after a few days of fever (Kalayanarooj et al., 1997). The earliest abnormality in the full blood count is a progressive decrease in total white cell count, which should alert the physician to a high probability of dengue.

I.5.2.2 Critical phase

Around the time of defervescence, when the temperature drops to 37.5–38^{o}C or less usually on days 3–7 of illness (Figure 14), an increase in capillary permeability in parallel with increasing haematocrit levels may occur (Srikiatkhachorn et al., 2007). This marks the beginning of the critical phase. The period of clinically significant plasma leakage usually lasts 24–48 hours.

Progressive leukopenia (Kalayanarooj et al., 1997) followed by a rapid decrease in platelet count usually precedes plasma leakage. At this point patients without an increase in capillary permeability will improve, while those with increased capillary permeability may become worse as a result of lost plasma volume. Pleural effusion and ascites may be detectable depending on the degree of plasma leakage and the volume of fluid therapy and the investigation technique used (ex. clinical examination > ultrasonography > CT scan).

I.5.2.3 Recovery phase

If the patient survives the 24–48 hours critical phase, a gradual reabsorption of extravascular compartment fluid takes place in the following 48–72 hours (Figure 14). General well-being improves, appetite returns, gastrointestinal symptoms subside, haemodynamic status stabilizes and diuresis returns to normal. Some patients may have a rash of “isles of white in the sea of red” (Nimmannitya, 1987). Some may experience generalized pruritus.

The haematocrit stabilizes or may be lower due to the dilutional effect of reabsorbed fluid. White blood cell count usually starts to rise soon after defervescence but the recovery of platelet count is typically later than that of white blood cell count.

I.5.2.4 Sever dengue

Severe dengue is defined by one or more of the following: (i) plasma leakage that may lead to shock (dengue shock) and/or fluid accumulation, with or without respiratory distress, and/or (ii) severe bleeding, and/or (iii) severe organ impairment (Figure 13).

Shock occurs when a critical volume of plasma is lost through leakage. It is often preceded by warning signs. The patient is considered to have shock if the pulse pressure (i.e. the difference between the systolic and diastolic pressures) is ≤ 20 mm Hg in children or the patient presents signs of poor capillary perfusion (cold extremities, delayed capillary refill, or rapid pulse rate).

With prolonged shock, the consequent organ hypoperfusion results in progressive organ impairment, metabolic acidosis and disseminated intravascular coagulation. This in turn leads to

severe haemorrhage causing the haematocrit to decrease in severe shock. In addition, severe organ impairment such as severe hepatitis, encephalitis or myocarditis and/or severe bleeding may also develop without obvious plasma leakage or shock (WHO, 2009).

I.5.3 Laboratory diagnosis

A range of laboratory diagnostic methods has been developed to support patient management and disease control. The choice of diagnostic method depends on the purpose for which the testing is done (e.g. clinical diagnosis, epidemiological survey, and vaccine development), the type of laboratory facilities and technical expertise available, costs, and the time of sample collection. Laboratory diagnosis methods for confirming DENV infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques (Figure 15).

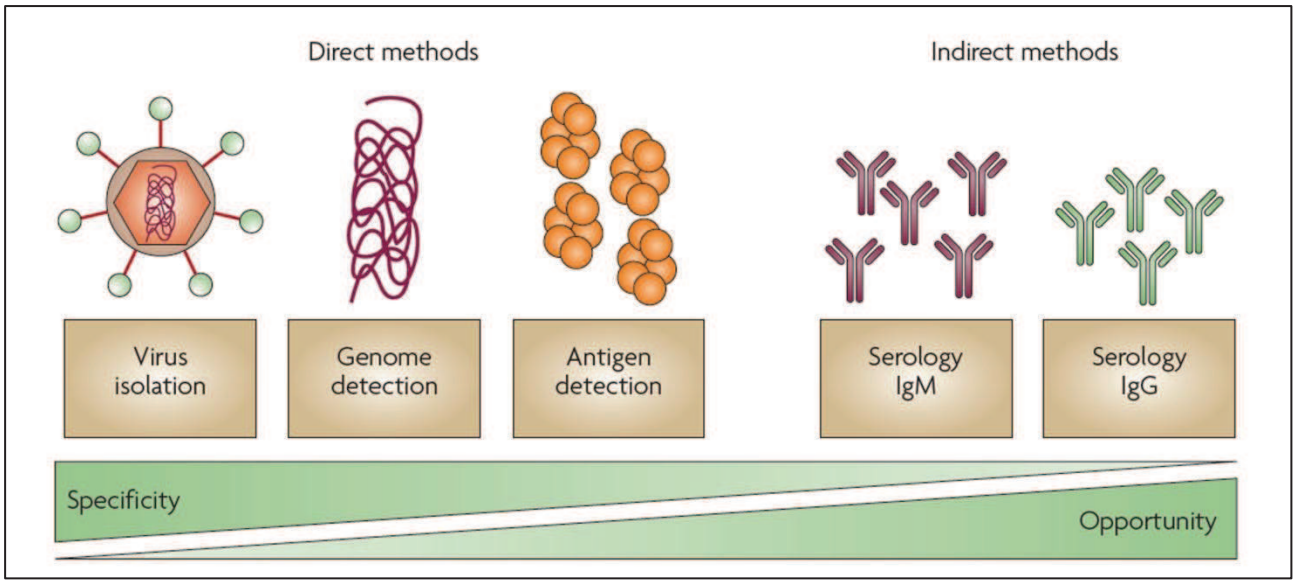

Figure 15: Comparative of direct and indirect laboratory methods for the diagnosis of dengue infections (Peeling et al., 2010).

I.5.3.1 Direct diagnosis methods

Before day 5 of illness, during the febrile period, dengue infections may be diagnosed by virus isolation in cell culture, by detection of viral RNA by nucleic acid amplification tests, or by detection of viral antigens by ELISA or rapid tests.

Virus detection

DENV can be isolated by the inoculation of a clinical specimen (serum, plasma or cerebro-spinal fluid) into mosquitoes, cell culture (using mosquito cell lines, such as C6/36 (cloned from *A.*

albopictus) and AP61 (cell line from *A. pseudoscutellaris*) or mammalian cell lines, such as Vero and LLC-MK2 cells) or intracerebral inoculation of suckling mice. For virus serotype identification, immunofluorescent assays using flavivirus group-reactive and serotype-specific monoclonal antibodies (mAbs) are carried out. The isolation and identification of DENV in cell cultures usually takes 1-2 weeks.

Viral RNA detection

Nucleic acid detection assays with excellent performance characteristics may identify DENV RNA within 24–48 hours. However, these tests require expensive equipment and reagents and, in order to avoid contamination, strict quality control procedures should be followed and the technique must be performed by experienced technicians. Various protocols have been developed that identify DENV using primers directed to serotype-specific regions of the genome (Lanciotti et al., 1992). Nested PCR techniques improve the sensitivity of detection because the initial amplification product is used as the target for a second round of amplification. However, it is crucial that laboratories performing nested PCR take every precaution to prevent false-positive results that can occur as a result of contamination. Finally, new PCR protocols and methodologies have been developed that allow the rapid detection and the quantification of RNA using TaqMan probe or SYBR Green. These methods demonstrate a high specificity and sensitivity. Additionally, it reduces the possibility of cross-contamination, allows the determination of viral load and gives result within few hours. Real-time RT-PCR assays are either “singleplex” (i.e. detecting only one serotype at a time) or “multiplex” (i.e. able to identify all four serotypes from a single sample).

Viral antigen detection

NS1 antigen detection kits are now becoming commercially available and can be used in laboratories with limited equipment and can yield results within a few hours. Enzyme-linked immunosorbent assay (ELISA) and rapid immunochromographic assays that target NS1 protein have shown that this antigen can be detected in patients with dengue infections up to 9 days after the onset of illness. Many studies have investigated the utility of NS1 detection as a diagnostic tool during the acute phase of a dengue infection and showed a good sensitivity (63-94%) and excellent specificity (98.4-100%) (Chuansumrit et al., 2008; Dussart et al., 2006; Guzman et al., 2010b; Hang et al., 2009) and the rapid dengue antigen detection tests can be used in field settings (i.e. point-of-care diagnostic test) and provide results in less than one hour (Tricou et al., 2010; Wang and Sekaran, 2010).

I.5.3.2 Indirect diagnosis methods

After day 5, DENV and antigens disappear from the blood coincidently with the appearance of specific antibodies. Dengue infection in a non-previously immune host produces a primary response of antibodies characterized by a slow and low titer antibody response (Figure 16). IgM antibody is the first immunoglobulin isotype to appear followed by anti-dengue IgG in a low titer. These antibodies are detectable in 50% of patients by days 3-5 after onset of illness, increasing to 80% by day 5 and 99% by day 10. IgM levels peak about two weeks after the onset of symptoms and then decline generally to undetectable levels over 2–3 months. Anti-dengue serum IgG is generally detectable at low titers at the end of the first week of illness, increasing slowly thereafter, with serum IgG still detectable after several months, and probably even for life (Innis et al., 1989; WHO, 1997). By contrast, during a secondary infection (dengue infection in a previously dengue or flavivirus immune host), antibody titers rise extremely rapidly and antibodies react broadly with many flaviviruses. High levels of IgG are detectable even in the acute phase and they rise dramatically over the following two weeks (Figure 16). The kinetics of the IgM response are more varied, appearing late during the febrile phase of illness, often preceded by IgG. Some anti-dengue IgM false negative reactions are observed in secondary infections due to low levels or absence of a detectable dengue IgM response.

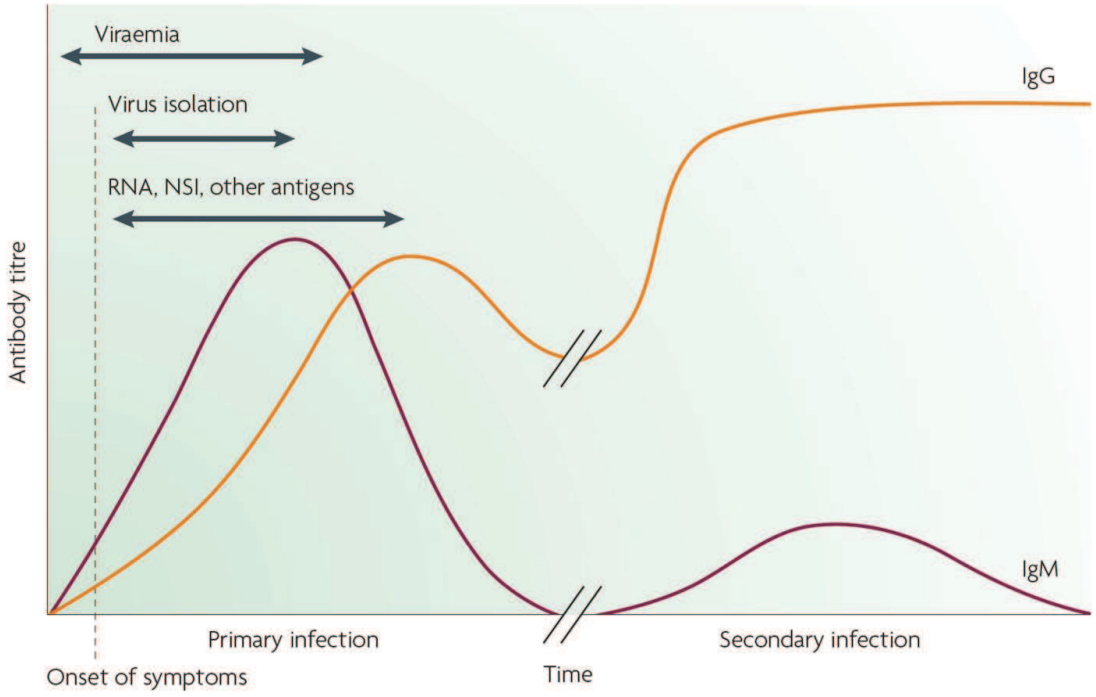

Figure 16: Major diagnostic markers for dengue infection and kinetic of antibodies IgM and IgG in dengue infection (Peeling et al., 2010).

IgM ELISA

The detection of dengue-specific IgM is a useful diagnostic and surveillance tool. The sensitivity and specificity of IgM based assays are strongly influenced by the quality of the antigen used (usually virus-infected cell culture supernatants or suckling mouse brain preparations) (Peeling et al., 2010). The IgM antibody capture ELISA (MAC-ELISA) is based on detecting IgM in serum using anti-human IgM that is bound to the solid phase. IgM detection is not useful for dengue serotype determination due to the cross-reactivity of the antibody observed even during primary infection and is not specific in countries with circulation of other flaviviruses such as JEV or YFV in most cases due to the presence of cross-reactive antigens shared by flaviviruses.

Rapid IgM-based dengue diagnostic tests have been developed as a quick and easy method for use at point of care or bedside. Most of these tests use recombinant antigens from all four DENV serotypes and the results are available within 15 to 90 minutes. However, the sensitivity (21%–99%) and specificity (77%–98%) vary greatly and are still poor (Peeling et al., 2010).

IgG ELISA

The IgG ELISA is used for the detection of recent or past dengue infections (if paired sera are collected within the correct time frame). This assay uses the same antigens E/M-specific capture (GAC) as the MAC-ELISA and allows detection of IgG antibodies over a period of 10 months after the infection. IgG antibodies are lifelong as measured by E/M antigen-coated indirect IgG ELISA. In general, IgG ELISA lacks specificity within the flavivirus serocomplex groups.

IgM/IgG ratio

A DENV E/M protein-specific IgM/IgG ratio can be used to distinguish primary from secondary DENV infections. IgM capture and IgG capture ELISAs are the most common assays for this purpose. In some laboratories, dengue infection is defined as primary if the IgM/IgG optical density ratio is greater than 1.2 (using patient's sera at 1/100 dilution) or 1.4 (using patient's sera at 1/20 dilutions). The infection is secondary if the ratio is less than 1.2 or 1.4. However, ratios may vary between laboratories (Falconar et al., 2006). In others, the threshold ratio of IgM/IgG is set to 1.78 (Dittmar et al., 1979).

Haemagglutination-inhibition test

The haemagglutination-inhibition (HI) test is based on the ability of dengue antigens to agglutinate red blood cells (RBC) of goose or trypsinized human O RBC. Anti-dengue antibodies in sera can inhibit this agglutination and the potency of this inhibition is measured. The assay does not discriminate between infections by closely related flaviviruses and between immunoglobulin isotypes. HI is time consuming and laborious to perform. However, HI titer can be used to interpret primary and secondary infection on paired sera with at least 5 days interval (WHO, 2009).

Clinically, diagnostic seroconversion is defined as a fourfold rise (or fall) in antibodies in paired sera by HI, or ELISA indicating acute or recent flavivirus infection (Gubler, 1997; Guzman and Kouri, 1996). However, waiting for the convalescent serum collected at the time of patient discharge is not very useful for diagnosis and clinical management and provides only a retrospective result. The ELISA format shows greater sensitivity in detecting dengue specific antibodies than the rapid tests, but the rapid tests are field friendly, with the results available in a short timeframe. Although, recent studies have shown the combination of IgM/IgG with NS1 antigen rapid diagnostic would increase the sensitivity compared to that of each method used alone and can be useful in clinical settings (Tricou et al., 2010; Wang and Sekaran, 2010).

The table below summarizes the interpretation of both the identification of virus/viral RNA/viral antigen and the detection of antibodies (Figure 17). When possible, combined approaches are preferable for dengue diagnosis.

Highly suggestive	Confirmed
One of the following: 1. IgM + in a single serum sample 2. IgG + in a single serum sample with a HI titre of 1280 or greater	One of the following: 1. PCR + 2. Virus culture + 3. IgM seroconversion in paired sera 4. IgG seroconversion in paired sera or fourfold IgG titer increase in paired sera

Figure 17: Interpretation of dengue diagnostic tests (WHO, 2009).
IgM: Immunoglobuline M; IgG: Immunoglobuline G; HI: Hemagglutination Inhibition test; PCR: Polymerase Chain Reaction.

I.5.4 Differential diagnosis

DF can easily be confused with non-dengue illnesses, particularly in non-epidemic situations. In Cambodia, other etiologies – including non-dengue flavivirus infections – should be ruled out: Japanese encephalitis, alphaviruses (i.e. Sindbis and Chikungunya), and other causes of fever such as malaria, leptospirosis, typhoid, rickettsial diseases, measles, influenza and influenza-like illnesses, etc.

I.6 DHF immunopathogenesis

I.6.1 *DHF pathogenesis*

The mechanisms leading to the severe manifestations of dengue infections are still not completely understood but are likely to be multifactorial (Martina et al., 2009). The genetic background of the host influences the way that the immune response reacts to dengue infection. Upon inoculation of DENV into the dermis, Langerhans cells and keratinocytes will primarily be infected. The virus subsequently spreads via the blood (primary viraemia) and infects tissue macrophages in several organs, especially the macrophages in the spleen. The replication efficiency of DENV in DC, monocytes, and macrophages, as well as its tropism for and replication efficiency in endothelial cells (EC), bone marrow stromal cells, and liver cells, collectively determine the viral load measured in blood. This viral load represents an important risk factor for development of severe disease. Infected cells die predominantly through apoptosis and to a lesser extent through necrosis. Necrosis results in release of toxic products, which activate the coagulation and fibrinolytic systems. Depending on the extent of infection of bone marrow stromal cells and the levels of IL-6, IL-8, IL-10, and IL-18, haematopoiesis is suppressed, resulting in thrombocytopenia. Platelets interact closely with EC, and a normal number of functioning platelets is necessary to maintain vascular stability. A high viral load in blood and possibly viral tropism for EC, severe thrombocytopenia, and platelet dysfunction may result in increased capillary fragility, clinically manifested as bleeding symptoms which is characteristic of DHF (Nachman and Rafii, 2008). At the same time, infection stimulates development of specific antibody and cellular immune responses to DENV. When IgM antibodies that cross-react with EC, platelets, and plasmin are produced, resulting in increased vascular permeability and coagulopathy is amplified. In addition, enhancing IgG antibodies bind heterologous virus during secondary infection and enhance infection of antigen presenting cells (APCs) and thereby contribute to the increased viral load that is seen during secondary viraemia in some patients. Furthermore, a high viral load overstimulates both low- and high-avidity cross-reactive T cells. In the context of certain HLA haplotypes, cross-reactive T cells produce high levels of proinflammatory cytokines and other mediators. Ultimately, these high levels of soluble factors, many of which still remain to be identified, induce changes in EC leading to the coagulopathy and plasma leakage characteristic of DSS.

I.6.2 DHF risk factors

DENV tropism

Cell and tissue tropism of DENV may have a major impact on the outcome of DENV infections. The absence of an appropriate animal model of dengue disease largely hampers our understanding of the role played by DENV tropism. In vitro data and autopsy studies suggest that three organ systems play an important role in the pathogenesis of DHF/DSS: (1) the **cells of immune system** such as immature Langerhans cells (epidermal DC), keratinocytes in epidermis and dermis (Limon-Flores et al., 2005), and in lymph nodes such as monocytes and macrophages; (2) the **liver** (hepatocytes and Kupffer cells) resulting in viral induced apoptosis and necrosis (Martina et al., 2009); and (3) **endothelial cell** specifically the microvessel in dermal papillae (Boonpucknavig et al., 1979) The tropism of DENV for cells of these systems and the corresponding pathological effects of DENV infection of these systems contribute to the pathogenesis of DHF.

Although increase of peripheral microvascular permeability has been shown in DHF/DSS patients, apoptosis of the microvascular EC in pulmonary and intestinal tissues has been detected in fatal cases (Limonta et al., 2007). This result provided one of the possible explanations for the profound plasma leakage seen in pleural and peritoneal cavities.

Virulence

Although DHF occurs more frequently in secondary infection than in primary infection, DHF also occurs in primary infection. This suggests that virulence of the virus strains may contribute to the development of DHF (Kurane, 2007; Leitmeyer et al., 1999; Tuiskunen et al., 2011a).

The most important evidence of DENV virulence was observed in the Americas during the first outbreak of DHF occurred in 1981 after the introduction of the possibly more virulent DENV-2 Southeast Asian genotype, as the original American genotype already present was only associated with DF (Guzman et al., 1999; Kouri et al., 1989). Interestingly, a recent study has shown that DENV isolated from patients with different degrees of severity could be characterized phenotypically and genetically in cell culture and in BALB/c mice (Tuiskunen et al., 2011a; Tuiskunen et al., 2011b). The result revealed that a virus isolated from a DSS patient showed unique features characterized by a lower level of replication in mammalian cells and extensive apoptosis in mosquito cells compared to those isolated from DF or DHF patients (Tuiskunen et al.,

2011a), while in mice, a virus isolate derived from a DSS patient persisted longer *in vivo* with extensive neuroinvasion in contrast to the other DENV-1 isolates that originated from milder human cases (Tuiskunen et al., 2011b). Genomic characterization of the three clinical isolates identified six amino acid substitutions unique for the DSS isolates that were located both in structural genes (M and E) and in non-structural genes (NS1, NS3, and NS5).

It has also been proposed that intraepidemic evolution of the circulating DENV might be responsible for increased severity of disease and more severe of disease manifestations and case-fatality rates were observed toward the end of the epidemic (Kouri et al., 1987; Kouri et al., 1989). This phenomenon suggested that the circulating DENV might have become more virulent through passage in hosts during the epidemic. In addition, epidemics with high incidences of DHF have been linked to primary infection with DENV-1 followed by infection with DENV-2 or DENV-3 (Guzman and Kouri, 2003; Halstead, 2007).

Activation of complement system

Activation of complement is another important clinical manifestation in DHF. It was reported that the levels of C3a and C5a, complement activation products, are correlated with the severity of DHF. The levels of C3a and C5a reached the peak at the time of defervescence when plasma leakage becomes most apparent and decreased in patients with DSS due to an accelerated consumption (Malasit, 1987; Nishioka, 1974). High levels of secreted NS1, pre-existing cross-reactive antibody, immune complexes were implied in mediating complement activation through classical and alternative pathways (Avirutnan et al., 2006; Malasit, 1987).

Transient autoimmunity

Antibodies produced during a DENV infection have been shown to cross-react with some self-antigens (Lin et al., 2006), but it is not clear if production of these antibodies is associated with secondary DENV infections. The presence of serum antibodies specific to NS1 also has been shown to correlate with disease severity (Libraty et al., 2002) (Shu et al., 2000). Cross-reaction of anti-NS1 with cells of the liver, EC, and platelets have been observed (Lin et al., 2003; Oishi et al., 2003; Sun et al., 2007). Anti-NS1 antibodies were shown to cross-react with human and mouse platelets leading to transient thrombocytopenia and hemorrhage in mice (Lin et al., 2008) and with EC causing cells apoptosis (Lin et al., 2003). It is not clear yet why the autoimmune phenomenon observed in some DENV-infected patients does not persist, though it is likely that the cross-

reactive antibodies to self-antigens are of the short-lived IgM isotype (Lin et al., 2001; Saito et al., 2004).

Host genetic factors

Differences in disease severity can be seen at both the individual and population levels. Several human HLA class I and II alleles are associated with development of DHF particularly HLA I (A*0207). Other HLA alleles such as A*0203 were associated with DF depending on DENV serotypes (Stephens et al., 2002). Polymorphism in the tumor necrosis factor alpha (TNF-α), Fcγ receptor, vitamin D receptor, CTLA-4, mannose-binding lectin and transforming growth factor β (TGF-β) genes has been associated with development of DHF/DSS (Rothman, 2003). Additionally, low incidence of severe disease was reported in populations of African origin which presents high prevalence of G6PD deficiency in studies conducted in Cuba and Haiti (de la et al., 2006; de la et al., 2007). Another study by Sakuntabhai et al. revealed a strong association between a promoter variant of CD209 (variant DCSIGN1-336.G allele) was associated with strong protection against dengue fever but showed no effect for DHF (Sakuntabhai et al., 2005).

Antibody-Dependent Enhancement

Epidemiology data have shown an increased risk of developing DHF after secondary infection. Studies conducted in Thailand demonstrated that up to 99% of DHF cases had heterotypic antibody to the serotype of DENV that caused DHF (Halstead, 1970). These DHF cases could be divided into 2 groups: (1) 90% of them were children who were older than 1 year and in a secondary infection and (2) the other 10% were less than 1 year-old and undergoing a primary dengue infection but were born from mothers with anti-DENV antibodies. Cross-reactive antibodies that lack neutralizing activity are induced in the primary infection. In secondary infection, DENV and non-neutralizing antibodies form virus–antibody complexes (Figure 18). This non neutralizing cross-reactive antibodies complexes bind to Fcγ receptors on target cells and result in enhancement of DENV infection leading to high viral load and to DHF (Halstead, 1970). This phenomenon is called "**antibody-dependent enhancement"**. Although some studies have shown a correlation between enhancing activity of serum, high levels of viraemia, and an increased risk for DHF/DSS, not all cases of severe disease are associated with ADE or preceded by infection with a heterologous serotype or by high viral loads (Martina et al., 2009).

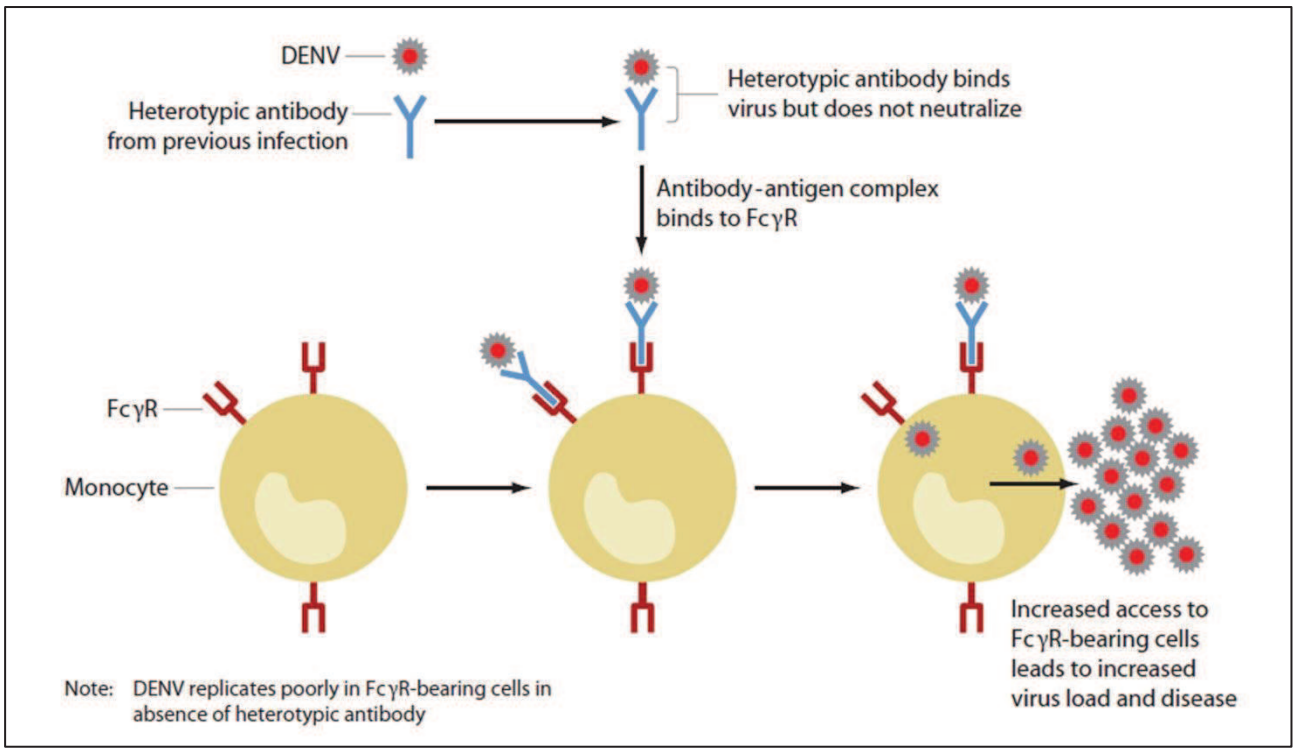

Figure 18: Model of antibodies-dependent enhancement (ADE) (Murphy and Whitehead, 2011).

Cross reactive T-Cell response

Infection of humans with DENV results in the development of dengue-specific CD4+ and CD8+ T cell responses with epitopes in multiple DENV antigens, primarily nonstructural proteins, being recognized by these T cells (Mathew and Rothman, 2008). However, this T cell responses cross-reactivity with heterologous virus can provide partial protective immunity as well as play a role in dengue pathogenesis.

T cell responses pattern is similar to the antibody response. After primary DENV infection, T cells responses are characterized by higher homotypic than heterotypic responses (Bashyam et al., 2006). T cell responses after secondary infections are highly serotype cross-reactive. Some studies have shown higher responses to the previously encountered DENV serotype and with higher avidity for that serotype than the new infecting serotype (Mongkolsapaya et al., 2003). This phenomenon referred to as "**original antigenic sin**". The majority of cross-reactive CD8+ T cells produce high concentrations of pro- and anti-inflammatory cytokines such as IFN-γ, TNF-α, Il-6 and IL-13 and other soluble factors that affect vascular permeability.

However, in accordance to what has been described for several other systems, it is possible that during a heterologous DENV infection, only a very small subset of cross-reactive memory T cells

will be stimulated to expand because of a narrowing TCR repertoire with unique specificity within individual. This could explain the variability seen in disease outcome upon secondary infection with heterologous DENV (Martina et al., 2009).

Soluble factors

Several studies have strongly attributed dengue pathogenesis to a "**storm**" **of inflammatory cytokines** and other mediators that lead to the increased plasma leakage in DHF/DSS (Basu and Chaturvedi, 2008). Higher plasma levels of IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, TGF-1β, TNF-α, and IFN-γ have been found in patients with severe DENV infections, in particular in patients with DSS (Martina et al., 2009). It is more likely that multiple cytokines contribute simultaneously in a complex way to the development of DHF/DSS. The fact that DSS patients recover extremely rapidly after appropriate fluid therapy suggests that cytokines do not cause tissue destruction like in many immunopathology models but rather cause a reversible EC dysfunction. Other mediators and soluble factors found to be increased in severe disease include vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibitory factor, thrombopoietin, soluble vascular cell adhesion molecule 1 (VCAM-1), soluble ICAM-1, von Willebrand factor antigen, thrombomodulin, E-selectin, tissue factor, plasminogen activator inhibitor 1, and tissue plasminogen activator (Martina et al., 2009).

I.7 Vaccines

Unlike other flaviviruses such as YFV, JEV and tick-borne encephalitis virus, no licensed vaccine for dengue exists. The difficulties to the development of a successful DENV vaccine include **(1)** the lack of an animal model that reproduces human disease, **(2)** the need to develop a separate vaccine for each DENV serotype (tetravalent) and **(3)** the risk of inducing enhanced disease upon subsequent natural infection if antibody to one or more serotypes wanes over time (Durbin and Whitehead, 2010). Therefore, an effective DENV vaccine must induce long-lasting and protective immunity against all four DENV serotypes (Whitehead et al., 2007). Despite these many obstacles, DENV vaccine development has made great strides in recent decades.

Up to present, the candidate vaccines that have been most extensively evaluated and are most advanced in development are live attenuated DENV vaccines from Sanofi Pasteur. The tetravalent dengue vaccine (TV) candidate utilizes the YFV (17D) backbone with DENV prM and E genes from each DENV type replacing those of YFV. Pre-clinical studies demonstrated genetic and phenotypic stability, no hepatotropism, less neurovirulence than YF17D, and failure to infect orally fed mosquitoes (Guirakhoo et al., 2004). NHP studies demonstrated TV to be highly immunogenic and induce protective immune responses (Guirakhoo et al., 2002). Data from an early phase I clinical trial comparing ChimeriVax-DEN2 to YF-VAXTM demonstrated that YFV primed volunteers had stronger, persistent and more cross-neutralizing antibodies (Guirakhoo et al., 2006). As of April 2009, >880 volunteers representing a spectrum of ages, genetic backgrounds (US, Mexico, Australia, Philippines) and flavivirus priming status had received at least one dose of a monovalent or TV. There were no overt safety signals in primed or unprimed volunteers and most clinical and biochemical reaction profiles were comparable to the licensed control vaccines. Seroconversion and neutralizing antibodies profiles were promising with 100% tetravalent seroconversion in a US adult cohort following three doses of TV. A flavivirus naive 2–45 years old cohort from Mexico experienced 77–92% seroconversion after three doses. As previously observed YFV and DENV priming did not alter the safety profile but improved the strength and breadth of the antibody response (Guy, 2009). The Phase II study in Mexico reported that the geometric mean titer in the JE primed group (3 injections of JE-VAX® at days -14, -7 and 0 followed by one dose of TV at day 105) were approximately twice (or in DENV-4 four-fold higher) those of the unprimed group (two injections of TV at days 0 and 105). Sanofi is currently completing vaccinations in a phase 2b efficacy trial in a cohort of 4,000 children (4–11 years) in Ratchaburi, Thailand. Phase 3 trials testing industrial scale vaccine lots are planned to begin by the end of 2011 (Guy et al., 2011).

The summary of current dengue vaccine candidates in their pre-clinical or clinical development stage are presented in the table 1 below.

Table 1: Summarize of current dengue vaccines (Thomas and Endy, 2011).

Institution/commercial partner	Vaccine Approach	Development stage	Reference
Carolina Vaccine Institute	**Viral replication:** Venezuelan equine encephalitis virus replicon expressing DENV E prot.	**Pre-clinical:** successful monovalent DENV3Es-VRP. Tetravalent planned.	(White et al., 2007)
GenPhar Inc.	**Viral replication:** non-replicating adenovirus-5 construct with prM and E DENV proteins	**Pre-clinical**: bivalent candidate (CAdVax-Den1,2 + CAdVax-Den3,4)	(Holman et al., 2007; Raja et al., 2007)
Pedro Kouri Tropical Medicine Institute, Havana, Cuba	**Recombinant:** Fusion EDIII of DENV-1 and DENV-2 into P64K protein of N. meningitidis	**Pre-clinical:** monovalent DENV-1 or DENV-2 + Freund's adjuvant.	(Bernardo et al., 2008)
WRAIR/GSK	**Inactivated:** Purified inactivated virus (PIV) produced in VERO cells inactivated by formalin	**Pre-clinical;** Phase 1: planning.	(Eckels and Putnak, 2003)
Inviragen Inc.	**Replicating:** DENV-DENV chimeria (DENV-2 PDK-53backbone; DENVax)	**Pre-clinical;** Phase 1: Tetravalent phase 1 Studies planned (U.S. and Columbia).	(Huang et al., 2003; Osorio et al., 2011)
Hawaii Biotech /Merck & Co.	**Recombinant:** Truncated recombinant E protein (DEN-80E) expressed in Drosophila S2	**Phase 1:** DENV-1 monovalent trial completed; Tetravalent phase 1 planned.	(Clements et al., 2010)
U.S. National Institutes of Health	**Replicating:** Recombinant live attenuated; Directed mutagenesis and DENV-DENV chimeras (DENV-4 backbone; TetraVax-DV)	**Phase 1:** 15 phase 1 studies of monovalent Vaccines completed; one tetravalent phase 1 study completed.	(Durbin et al., 2001; Durbin et al., 2006)
Naval Medical Research Center	**DNA:** prM and E DENV 1, CMV promoter (D1ME-VRP)	**Phase 1:** DENV-1 phase 1 completed; future testing unsure.	(Beckett et al., 2011)
WRAIR/GSK	**Replicating:** Live attenuated virus (LAV) (PDK passage)	**Phase 2:** Completed in U.S., Puerto Rico, Thailand, further Development in question (manufacturing complexities)	(Simasathien et al., 2008)
Sanofi Pasteur	**Replicating:** Chimera Yellow fever 17D-DENV (CYD)	Completing phase 2b. **Phase 3:** in Australia 2010, program expansion expected.	(Guy, 2009; Guy et al., 2011)

WRAIR: Walter Reed Army Institute of Research
GSK: GlaxoSmith-Kline

PART II

DENGUE IN CAMBODIA

II.1 CHAPITER 1: EPIDEMIOLOGY OF DENGUE IN CAMBODIA

II.1.1 Context of study

Cambodia is recognized as a country with poor health and economic indicators (ADB, 2009). The estimated population was 14.6 million in 2008 (NIS, 2009). The DENV was first detected in Cambodia in 1963 (Chastel, 1963) and is considered highly endemic with an epidemic seasonality during rainy season from May to November. The NDCP was established and dengue fever was reported through passive surveillance since 1980. This national surveillance was enhanced in 2001 with active hospital based surveillance in 5 sentinels sites (national hospitals) spreading across the country. Cases reported through the sentinel system included those among children in either paediatric hospitals or paediatric wards in sentinel hospitals. Patient's demographic as well as clinical data were collected. The enhanced surveillance program included laboratory diagnosis (virological and serological testing) for a sample of patient's suspected of dengue infection. The samples for laboratory diagnosis were selected throughout the year corresponding to about 10% of clinically notified dengue patients. The samples consisted of sera collected randomly from paediatric patients (less than 16 years old) on admission and on discharge (for serological purpose) and were tested at Institut Pasteur in Cambodia (IPC).

Since 2001, control of the dengue vector in Cambodia has consisted of biannual larvicide campaigns: 1% temephos sand granules distributed between April and July and between August and October. Medium-to-large water storage containers in households in districts identified by the NDCP as high-risk areas for epidemics were targeted. Targets were mainly in urban centers and densely populated areas. Other forms of prevention were also carried out through print media or vehicle with loud speakers as well as community-based clean-up campaigns. However, the effects of these campaigns covering mainly high-risk areas identified by the NDCP have not been evaluated.

II.1.2 Objectives

This report aims at describing the surveillance data on dengue collected in Cambodia since 1980 by the NDCP. Epidemiological trends were determined primarily using data from recent years (2001-2008). In addition, the impact of a 7-year vector control program on the incidence of the disease was also evaluated. Another aim of this article was to make the Cambodian surveillance data publicly available for comparison with other surveillance data in the hope that this will lead to a better understanding of the pattern of dengue transmission in the region.

II.1.3 Discussion and conclusions

This is the first published report of national dengue surveillance data in Cambodia covering a period of 28 years. As the data for 1980–2000 were not collected using a strict clinical case definition for suspected DENV infection, the data from 2002–2008 periods (during which more complete and reliable data on patients and the virus serotype were available) were more thoroughly studied. Of the 194 726 cases of dengue reported to the NDCP between 1980 and 2008, 74 947 (38.5%) were passively reported by public health-care facilities before 2001 using non-standardized clinical definitions of dengue. The epidemics occurred in cycles of 3–4 years, with the cycles subsequently becoming less prominent since the active surveillance system was improved in 2001. Two major epidemics occurred after 1997: there were 16,260 cases in 1998 and 39,618 in 2007

The estimated incidence of dengue nationally was high, varying from 0.7 to 3.0 per 1000 population during 2002–2008. DENV were reported throughout the year, with increases occurring during the rainy season between May and November. Generally there was no change in the overall age-adjusted annual incidence during 2002–2008, although there was a spike in case numbers in 2007. The data also showed that dengue remained prevalent among young children in Cambodia, with infants aged < 1 year and children aged 4–6 years being the most affected. Overall, from 2002 to 2008, the average proportion of clinical DENV infections classified as DHF was 41.5% (range: 20.5–54.0), while 6.6% (range: 3.0–8.7) were classified as DSS and the remainder, as dengue fever. The proportion classified as either DHF or DSS peaked in 2006 at 60.6% and in 2007 at 54.2%.

Between 2000 and 2008, paired serum samples were collected from an annual mean of 715 patients, who comprised 5.2% of all dengue cases reported based on clinical symptoms. All four DENV serotypes were permanently in circulation, though the predominant serotype has alternated between DENV-3 and DENV-2 since 2000. The predominant circulating serotype changed from DENV-3 to DENV-2 in 2002 and then switched back to DENV-3 four years later.

Between 2000 and 2008, dengue vector control interventions based on the distribution of temephos, community participation and the provision of educational messages were undertaken in 94 densely populated districts that the NDCP considered to be most affected by the disease. Of these, only 24 (35%) received interventions for 4 years or more (median: 2; range: 1–7). Logistic regression analysis showed no association between the intervention and dengue incidence.

The pattern of DENV circulating in Cambodia presented in this epidemiological study has raised question of what influenced the observed profile of evolution dynamic of DENV. Using the biobank of sera collected since the beginning of virological surveillance in NDCP, the work in the next chapter (Chapter 2) tried to look at this evolutionary process at the molecular level.

The results of this work are summarized in the article below which was published in « Bulletin of the World Health Organization » in 2010.

National dengue surveillance in Cambodia 1980–2008: epidemiological and virological trends and the impact of vector control

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Objective Dengue has been reportable in Cambodia since 1980. Virological surveillance began in 2000 and sentinel surveillance was established at six hospitals in 2001. Currently, national surveillance comprises passive and active data collection and reporting on hospitalized children aged 0–15 years. This report summarizes surveillance data collected since 1980.

Methods Crude data for 1980–2001 are presented, while data from 2002–2008 are used to describe disease trends and the effect of vector control interventions. Trends in dengue incidence were analysed using the Prais–Winsten generalized linear regression model for time series.

Findings During 1980–2001, epidemics occurred in cycles of 3–4 years, with the cycles subsequently becoming less prominent. For 2002–2008 data, linear regression analysis detected no significant trend in the annual reported age-adjusted incidence of dengue (incidence range: 0.7–3.0 per 1000 population). The incidence declined in 2.7% of the 185 districts studied, was unchanged in 86.2% and increased in 9.6%. The age-specific incidence was highest in infants aged <1 year and children aged 4–6 years. The incidence was higher during rainy seasons. All four dengue virus (DENV) serotypes were permanently in circulation, though the predominant serotype has alternated between DENV-3 and DENV-2 since 2000. Although larvicide has been distributed in 94 districts since 2002, logistic regression analysis showed no association between the intervention and dengue incidence.

Conclusion The dengue burden remained high among young children in Cambodia, which reflects intense transmission. The national vector control programme appeared to have little impact on disease incidence.

Une traduction en français de ce résumé figure à la fin de l'article. *Al final del artículo se facilita una traducción al español.* الترجمة العربية لهذه الخلاصة في نهاية النص الكامل لهذه المقالة.

Background

Over the past 30 years, dengue fever has emerged as the most important arthropod-borne viral disease of humans worldwide and is a major global public health problem, primarily in the tropics.[1] Infection with one of the four serotypes of the dengue virus often produces a self-limited but painful febrile illness. The illness may be asymptomatic or can involve severe manifestations such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), which may rapidly progress to death, particularly in children. To date, no drugs can cure the disease and no vaccine can prevent it. Dengue control and prevention have mainly relied on vector control and community action.

Dengue is considered endemic in Cambodia, a country with poor health and economic indicators.[2] The estimated population was 14.6 million in 2008.[3] The dengue virus was first detected in Cambodia in 1963[4] and dengue fever has been reported through passive surveillance since 1980. Surveillance was enhanced in 2000 to include laboratory diagnosis for a sample of patients with suspected dengue and, in 2001, with the introduction of active sentinel surveillance.

This report summarizes surveillance data on dengue collected in Cambodia since 1980. Epidemiological trends were determined primarily using data from recent years. In addition, the impact of a 7-year vector control programme on the incidence of the disease was also evaluated.

Methods

Cambodia has a tropical climate, with a rainy season occurring between May and November. Rainfall typically peaks between May and June. Some 80% of the population lives in the southern and north-western parts of the country, which together contain 24 provinces and 185 districts.

National surveillance

National surveillance of dengue was established in 1980 and involved passive reporting of clinically diagnosed cases by public-sector health centres and hospitals. In 2000, virological surveillance was introduced at five hospitals, as described below. Subsequently, in 2001, the system changed dramatically when the National Dengue Control Program (NDCP) implemented

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sentinel surveillance based on three public hospitals and three non-profit-making private hospitals in four provinces. Cases reported through the sentinel system include those among children in either paediatric hospitals or paediatric wards in sentinel hospitals. Thus, national data collected since 2001 were obtained by both passive and active reporting of cases.

Laboratory testing

Virological and serological surveillance is carried out at three of the public hospitals that serve as sentinel sites, a non-profit making private hospital in Siem Reap and an additional public provincial hospital. Paired serum specimens are collected on admission and at discharge from hospitalized patients with clinically diagnosed dengue. The specimens are centrifuged and sent weekly in liquid nitrogen to the Institut Pasteur–Cambodia for serological, virological and molecular testing. In theory, each site should send 5–10 paired serum specimens taken from a random sample of patients with suspected dengue each week throughout the year. In reality, patients are seldom randomly selected and only two sites regularly send specimens throughout the year. The paired serum specimens are tested using an immunoglobulin M (IgM)-antibody capture enzyme-linked immunosorbent assay (ELISA) and a haemagglutination inhibition assay. Because of possible cross-reactivity, all specimens are systematically tested for anti-dengue virus and anti-*Japanese* encephalitis virus IgM using an in-house IgM-antibody capture ELISA and a haemagglutination inhibition assay, as previously described.[5] The first sample is tested for viral ribonucleic acid using a modified version of the reverse-transcriptase polymerase chain reaction (PCR) procedure described by Lanciotti.[6] In addition, the virus is isolated by inoculating sera into C6/36 (*Aedes albopictus* mosquito) and Vero E-6 cell cultures and identifying the virus serotype by using a direct fluorescent antibody assay employing monoclonal antibodies, as described elsewhere.[5]

Case definition and data collection

Since 2002, clinical case definitions of dengue fever and its complications have been based on World Health Organization (WHO) definitions[7,8] and adapted for health centres and referral hospitals. Because resources were limited, the NDCP gathered data reported passively from referral hospitals and collected actively at sentinel sites on only a weekly basis. Data were collected on individual patients using a standard NDCP form, which recorded each patient's name, demographic characteristics, disease severity (i.e. dengue fever, DHF or DSS), district of residence, and vital status or status on transfer. The forms were stored centrally at the NDCP office and data were entered into a computerized database using statistical software (Epi Info 2000 version 3.3.1, Centers for Disease Control and Prevention, Atlanta, United States of America (USA)). A system was in place to check patients' names so that there was no duplication of those who were hospitalized at several different sites for the same illness episode.

Vector control interventions

In theory, since 2001 control of the dengue vector in Cambodia has consisted of biannual larvicide campaigns: 1% temephos sand granules distributed between April and July and between August and October. Medium-to-large water storage containers in households in districts identified by the NDCP as high-risk areas for epidemics were targeted. Targets were mainly in urban centres and densely populated areas. These campaigns were linked to nationwide publicity involving public service announcements on radio and television and in the print media, as well as the use of vehicles with loudspeakers and community meetings before each dengue season. However, because of budgetary constraints, some high-risk districts received only one round of larvicidal treatment between April and July or no treatment at all. Routine vector control activities were also limited and primarily involved community-based clean-up campaigns to remove and destroy small rain-filled containers and insecticide fogging to kill adult mosquitoes around houses close to locations where dengue cases had been reported.

Since the distribution of temephos has not been documented in detail, vector control coverage in each district in the years 2001–2008 was determined by ascertaining whether or not the NDCP intervened in that district in a specific year.

Data analysis

The analysis considered only data recorded and computerized from 2002 onwards because data for 1980–2000 were not collected using a strict clinical case definition for suspected dengue virus infection and data for 2001 were incomplete: 68% of demographic and district-of-residence data were missing. We calculated the age-specific incidence of dengue and the age-adjusted annual incidence per 1000 individuals using population data from the 1998 census.[9] Population estimates for other years were obtained from the Cambodian government's Institute of Statistics.[3] The annual number of cases was treated as a time series and the Prais–Winsten generalized linear regression model was used to calculate the significance of any increase or decrease in dengue incidence between 2002 and 2008, both overall and for each district. A change of slope was judged to be statistically significant using the F-statistic if the P-value was < 0.05. We assessed the impact of vector control interventions in individual districts by determining their effect on dengue incidence using a logistic regression model that controlled for the population density in each district. The number of vector control interventions was treated as a continuous variable, with the number per district being the number of years that interventions were used in that district. Results were expressed in odds ratios (ORs) and 95% confidence intervals (CIs). All statistical analyses were carried out using Stata 9.2 statistical software (StataCorp LP, College Station, USA).

Results

Secular trend and seasonality

Of the 194 726 cases of dengue reported to the NDCP between 1980 and 2008, 74 947 (38.5%) were passively reported by public health-care facilities before 2001 using non-standardized clinical definitions of dengue. The secular, or long-term, trend was characterized by a cyclical pattern of epidemics at intervals of about 3–4 years. Since the surveillance system was improved in 2001, the 3–4-year cycle has been less prominent. Two major epidemics occurred after 1997: there were 16 260 cases in 1998 and 39 618 in 2007 (Fig. 1).

Trends in incidence 2002–2008

In the period 2002–2008, the NDCP reported between 9006 and 39 618 cases of dengue per year (annual age-adjusted incidence range: 0.7–3.0 per 1000 population), with the case fatality rate ranging from 0.7 to 1.7% (Table 1). Dengue

cases were reported throughout the year, with increases occurring during the rainy season between May and November (i.e. weeks 17–48 in Fig. 2). After taking into account seasonal fluctuations and the major 2007 epidemic, analysis using the generalized linear regression model detected no significant trend in the annual age-adjusted incidence of reported clinical dengue virus infections.

Since the implementation of sentinel surveillance, the proportion of all dengue cases reported that came from sentinel sites has increased from 57.0% in 2002 to 89.1% in 2008 (Fig. 1). For example, in 2008 the two non-profit-making hospitals belonging to the Kantha Bopha Foundation in Siem Reap and Phnom Penh, respectively, accounted for 62.1% of all reported cases. These hospitals provide free medical care to Cambodian children and have large catchment areas.

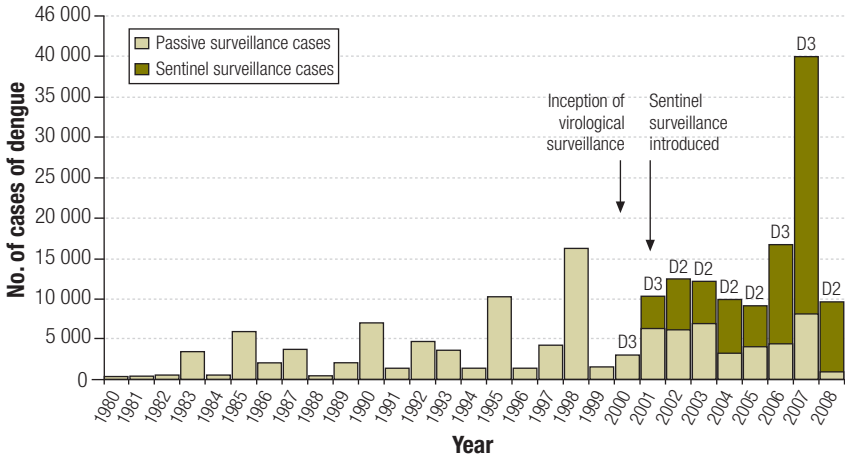
Overall, from 2002 to 2008, the average proportion of clinical dengue virus infections classified as DHF was 41.5% (range: 20.5–54.0), while 6.6% (range: 3.0–8.7) were classified as DSS and the remainder, as dengue fever (Table 1). The proportion classified as either DHF or DSS peaked in 2006, at 60.6%, and in 2007, at 54.2%.

The highest age-specific incidence of dengue fever occurred in infants aged less than 1 year, followed by those aged 4–6 years (Fig. 3). Some 79.0% of all reported cases were in children aged 9 years or younger (median: 6 years). The age distribution of dengue cases has been consistent since 2002. Moreover, no sex difference in incidence was observed in the period since 2002, during which the median proportion of males was 49.3% (range: 47.7–49.6).

Virological findings

Between 2000 and 2008, paired serum samples were collected from an annual mean of 715 patients, who comprised 5.2% of all dengue cases reported. Overall, 87.8% of samples were seropositive for dengue and there was little variation across sentinel sites. On average, 70.0% of seropositive samples also tested positive using PCR. Among seropositive patients aged < 1 year, 78% (i.e. 108 of 138) tested positive using PCR. Although most cases occurred during the rainy season, dengue virus infection was also identified during other times of the year, which confirms that dengue is endemic in Cambodia.

Fig. 1. **Number of cases of dengue fever reported nationally in Cambodia, 1980–2008**



D2, dengue virus type-2 (DENV-2) was predominant in the year; D3, dengue virus type-3 (DENV-3) was predominant in the year.

Since virological testing started in 2000, all four dengue virus serotypes have been observed to be in circulation each year, with DENV-2 and DENV-3 being predominant (Table 1). The predominant circulating serotype changed from DENV-3 to DENV-2 in 2002 and then switched back to DENV-3 4 years later (Fig. 1). Between 2000 and 2008, both the incidence of dengue and the proportion of cases with DHF were highest in 2006 and 2007, when the predominant serotype was DENV-3.

Impact of vector control

Between 2000 and 2008, dengue vector control interventions based on the distribution of temephos, community participation and the provision of educational messages were undertaken in 94 densely populated districts that the NDCP considered to be most affected by the disease. Of these, only 24 (35%) received interventions for 4 years or more (median: 2; range: 1–7). Linear regression analysis showed that the incidence of dengue declined in only 5 (2.7%) of all 185 districts studied between 2000 and 2008, while it remained unchanged in 162 (86.2%) and increased in 18 (9.6%). Two (40.0%) of the five districts in which the incidence declined had received interventions during the previous 7 years compared with 47.5% of districts where the incidence was unchanged and 33.3% where it increased. Logistic regression models, whether controlling for the district population density or not, failed to find any significant association between the use of interventions and decreased incidence.

Discussion

This is the first published report of national dengue surveillance data in Cambodia covering a period of 28 years. As the data for 1980–2000 were not collected using a strict clinical case definition for suspected dengue virus infection, we focused on the 2002–2008 period, during which more complete and reliable data on patients and the virus serotype were available.[10] The estimated incidence of dengue nationally was high, varying from 0.7 to 3.0 per 1000 population during 2003–2008. Generally there was no change in the overall age-adjusted annual incidence during 2002–2008, although there was a spike in case numbers in 2007. The data also show that dengue remains prevalent among young children in Cambodia, with infants aged < 1 year and children aged 4–6 years being the most affected. The age distribution of dengue cases in other countries in the region showed wide variations. In Thailand and Viet Nam, dengue has become more common in older children.[11–14] A prospective cohort study of children aged 3–15 years in southern Viet Nam found that the incidence was highest in those aged 6–10 years (L Pollissard, personal communication, 2007). In Malaysia and Singapore, in contrast, most cases were seen in adults aged over 18 years.[15,16] The reasons for these differences may include the level of development,[17] the effectiveness of vector control programmes,[15] the predominance

Table 1. **Cases of dengue fever, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) reported by the National Dengue Control Programme, Cambodia, 2000–2008**

Parameter	Year of surveillance								
	2000	2001	2002	2003	2004	2005	2006	2007	2008
DF cases, no.	3145	10266	12441	12099	9991	9006	16635	39618	9546
DHF, %[a]	ND	ND	27.8	20.5	41.5	34.6	54.0	51.2	42.9
DSS, %[a]	ND	ND	5.4	7.0	8.7	8.4	6.6	3.0	3.6
DHF and DSS, %[a]	ND	ND	33.2	27.5	50.2	43.0	60.6	54.2	46.5
DF case fatality rate, %	ND	ND	1.2	1.6	0.9	1.7	0.9	1.0	0.7
Age-adjusted incidence (per 1000 population)									
Of DF	ND	ND	1.02	0.97	0.78	0.69	1.27	2.96	0.70
Of DHF and DSS	ND	ND	0.34	0.27	0.39	0.30	0.77	1.60	0.32
Age-specific incidence of DHF and DSS (per 1000 population)									
<1 yr	ND	ND	0.45	0.25	0.59	0.61	2.43	5.49	1.01
1–4 yr	ND	ND	0.58	0.71	1.00	0.72	2.15	4.07	0.77
5–9 yr	ND	ND	1.22	0.77	1.14	0.91	2.21	4.41	0.86
10–14 yr	ND	ND	0.54	0.44	0.65	0.42	0.98	2.30	0.54
15–19 yr[b]	ND	ND	0.00	0.01	0.01	0.03	0.05	0.27	0.07
Clinical cases tested									
No.	415	748	809	677	680	527	575	1400	598
%	13.2	7.3	6.5	5.6	6.8	5.9	3.5	3.5	6.3
Clinical cases that tested positive for DF									
No.	324	603	736	617	611	467	510	1315	509
%	78.1	80.6	91.0	91.1	89.9	88.6	88.7	93.9	85.1
Clinical cases that tested positive with PCR[c]									
No.	191	375	468	444	374	310	381	1095	358
%	59.0	62.2	63.6	72.0	61.2	66.4	74.7	83.3	70.3
Specific virus serotype, %									
DENV-1	5.6	23.3	21.0	10.4	3.3	5.5	5.7	4.3	10.8
DENV-2	24.4	20.1	41.0	61.2	74.1	45.3	9.2	9.1	44.1
DENV-3	58.9	45.0	18.0	15.5	16.7	39.4	82.2	83.6	19.7
DENV-4	11.1	11.6	20.0	12.9	5.9	9.7	2.9	3.1	25.4

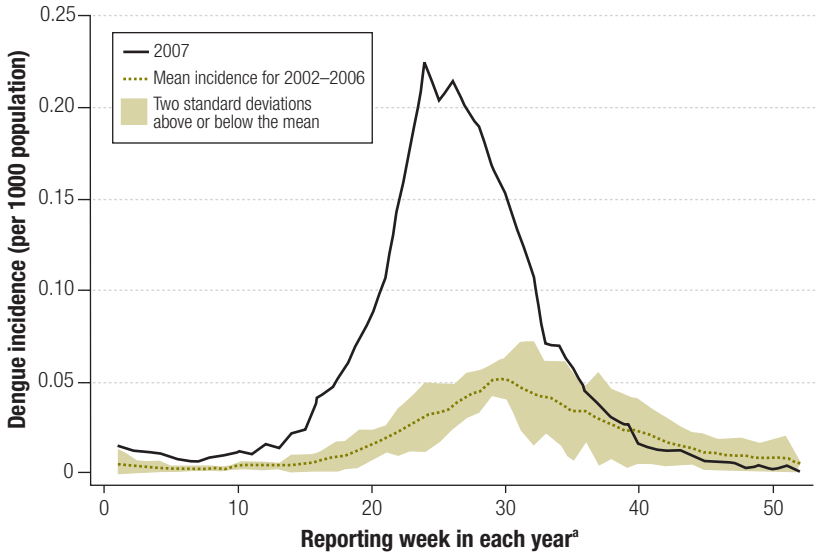
ND, not determined; DENV, dengue virus; DF, dengue fever; DHF, dengue haemorrhagic fever; DSS, dengue shock syndrome; PCR, polymerase chain reaction.
[a] This represents the percentage of cases among all reported cases of dengue fever.
[b] There was no requirement to report dengue in those aged 15–19 years.
[c] Among cases that tested positive for DF.

of different virus genotypes[18] and a demographic transition or shift.[19]

Significantly, the alert system for detecting epidemics established by the NDCP and modelled on the early warning system for malaria[20] predicted the occurrence of the 2007 epidemic. As shown in Fig. 2, the weekly dengue incidence was consistently above the alert threshold of two standard deviations above the mean in early 2007, while it remained below the threshold in other years. Although the authorities were quickly alerted, the response to the outbreak, which included vector control interventions, educational messages and providing public hospitals with sufficient medical supplies, came too late. Unfortunately, the NDCP has too few human resources and too little funding to implement these interventions in a timely manner.

Although all four dengue virus serotypes were circulating in the country throughout the reported surveillance period, illness was predominantly caused by DENV-2 and DENV-3. The change in the predominant serotype from DENV-3 to DENV-2 in 2002 resulted in only a small increase in incidence, possibly because DENV-2 had been circulating in earlier years and many Cambodians had developed immunity. The incidence declined steadily between 2002 and 2005, until a large-scale epidemic due to DENV-3 occurred in 2006–2007. We speculate that there is a 3–4 year cyclical pattern of epidemics in Cambodia involving different serotypes, with epidemics of the same serotype possibly occurring every 8–9 years (e.g. in 1998 and 2006–2007). Indeed, unpublished laboratory data from the Institut Pasteur–Cambodia and the large-scale DENV-3 epidemic observed regionally in 1998[21–26] indicate that the serious 1998 epidemic which disrupted the Cambodian health system by overloading hospitals[27] was due to DENV-3. Moreover, in the 2006–2007 epidemics in Cambodia, the DENV-3 virus was associated with a high proportion of severe complications (i.e. DHF and DSS). In Thailand, DENV-3 was also predominant during the severe dengue years of 1987 and 1998.[21] Further studies, which should include full genome sequencing, are needed to explore the association

Fig. 2. **Incidence of dengue fever in 2007 and mean incidence for 2002–2006, by reporting week, Cambodia**



[a] The first reporting week is the first week in January.

between dengue serotype, virus virulence and disease severity.

Several studies have shown that vector control interventions reduce larval indices though the reduction must be substantial to influence dengue transmission.[28–30] In Cuba and Singapore, the incidence of dengue was dramatically reduced only after anti-vector legislation was introduced and aggressive vector control measures had been used for years.[31] Nevertheless, dengue has re-emerged because new dengue viruses are constantly being introduced from neighbouring countries.[15,32] In this study, we evaluated the impact of many years of vector control interventions and educational messages on the incidence of dengue in individual districts. No association between such interventions and disease incidence was observed. Clearly, as dengue transmission is highly localized, a more rigorous assessment of interventions would consider outcomes at the village level, but this information was not available.[33] We are not suggesting that temephos does not prevent transmission at the household level, but rather that, given the limited resources, it is unlikely that current interventions in Cambodia will affect disease incidence.

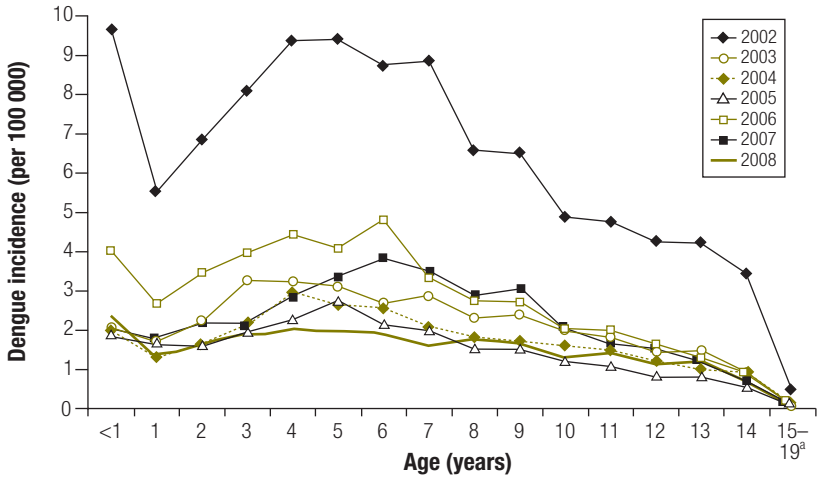
In Cambodia, dengue viruses are transmitted primarily by *Aedes aegypti* mosquitoes (C Paupy, personal communication, 2002), which are abundant in populated rural areas.[34] Over 80% of larval foci for this species are in the ubiquitous, concrete jars filled with rainwater used in most homes. Unfortunately, the quantity of parricides available to the NDCP has been insufficient to cover all high-risk areas thoroughly and there was little reduction in dengue transmission. The long-term strategy for implementing vector control programmes in Cambodia needs to be re-examined.

The use of surveillance data to describe epidemiology and evaluate disease burden has several limitations. In particular, there are weaknesses in the design of the surveillance system resulting from the need to balance limited resources and data quality. For example, surveillance only covered patients hospitalized at major public and non-profit-making paediatric hospitals and paediatric wards to ensure the accuracy of dengue diagnosis. Moreover, clinicians at our surveillance sites often had difficulty in classifying disease severity using standard WHO definitions (Institut Pasteur–Cambodia, unpublished data, 2007). Improving diagnosis by obtaining complete blood counts or carrying out radiographic or ultrasound imaging is often too technically difficult or too expensive for most health-care facilities in Cambodia. The presence of haemoconcentration, suggestive of DHF, is also difficult to detect because Cambodian clinicians tend to administer fluids intravenously as soon as dengue is suspected.[35]

The size of the patient samples used in virological surveillance was small. Moreover, patients suspected of having dengue were not selected randomly but rather because there was a high level of suspicion that they had severe dengue. Another limitation was that dengue was frequently overdiagnosed during epidemics and underdiagnosed during the intervening periods. The use of laboratory testing in dengue diagnosis is clearly vital when resources permit. We believe that, in the absence of systematic laboratory diagnosis of dengue, surveillance programmes should exclude patients with undifferentiated febrile illnesses to increase the specificity of diagnosis by avoiding the inclusion of those with, for example, influenza, typhoid or leptospirosis.[36,37]

Despite these limitations, our observation that dengue activity patterns for different ages and genders have remained consistent over time indicates that the

Fig. 3. **Age-specific incidence of dengue fever, Cambodia, 2002–2008**



[a] There was no requirement to report dengue in those aged 15–19 years.

surveillance data are reliable. Moreover, no other data available match the completeness or cover the same timescale as the Cambodian national dengue surveillance data.

Another aim of this article was to make the Cambodian surveillance data publicly available for comparison with other surveillance data in the hope that this will lead to better understanding of the pattern of dengue transmission in the region. Currently, however, descriptive national data are difficult to obtain. Differences in the surveillance systems used in other countries must be taken into account. For example, in Malaysia and Singapore, all suspected dengue cases are confirmed by laboratory testing, whereas only hospitalized patients are tested in the Philippines and Thailand. In Viet Nam, as in Cambodia, only a sample of patients suspected of having dengue undergo serological or virological testing and it is not clear whether these patients are representative of the general population. In contrast, in the Philippines and Viet Nam, all clinically diagnosed dengue cases at all health-care facilities, including health centres and hospitals, are reported.[38]

With the development of dengue vaccines expected in the near future,[39,40] there is an urgent need to accurately estimate the true disease burden. Several countries are collaborating with the Paediatric Dengue Vaccine Initiative of the International Vaccine Institute in Seoul, the Republic of Korea, to set up community-based surveillance sites to measure the incidence of dengue accurately. ■

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Competing interests: None declared.

ملخص

الوطني لحمى الدنج في كمبوديا 1980 – 2008: الاتجاهات الوبائية والفيروسية وتأثير مكافحة الناقل

الغرض يجري التبليغ عن حمى الدنج في كمبوديا منذ عام 1980. وبدأ الترصد الفيروسي هناك عام 2000، وتأسس الترصد المخفري في ستة مستشفيات عام 2001. ويتكون حالياً الترصد الوطني من جمع معطيات فاعل ولا فاعل والتبليغ عن الأطفال في المستشفيات في عمر 0-15 سنة. ويلخّص هذا التقرير معطيات الترصد التي جمعت منذ عام 1980.

الطريقة عُرضت المعطيات الخام خلال الفترة 1980-2001، بينما استخدمت المعطيات خلال الفترة 2002-2008 لوصف اتجاهات المرض وتأثير التدخلات الخاصة بمكافحة الناقل. وحُللت اتجاهات وقوع حمى الدنج باستخدام نموذج التحوف الخطي العام لباريس-ونستون Prais–Winsten للتسلسل الزمني.

الموجودات خلال الفترة 1980-2001، وقعت أوبئة في دورات استمرت لمدة 3-4 سنوات، وعقب ذلك أصبحت الدورات أقل وضوحاً. وبالنسبة للمعطيات خلال الفترة 2002-2008، كشف تحليل التحوف الخطي عن عدم وجود اتجاه ملموس في معدل الوقوع السنوي المصحح حسب العمر المبلغ عنه لحمى الدنج (مدى معدل الوقوع: 0.7-3.0 لكل 1000 فرد من السكان). وتراجع معدل الوقوع في 2.7% من 185 منطقة خاضعة للدراسة، ولم يتغير في 86.2% من المناطق، وازداد في 9.6% من المناطق. وكان أعلى معدل وقوع خاص بعمر معين بين الرضّع أقل من عمر سنة واحدة والأطفال في الفئة العمرية 4-6 سنوات. وكان معدل الوقوع أعلى في مواسم الأمطار. وكانت جميع الأنماط المصلية الأربعة لفيروس الدنج في دوران مستمر، ولكن النمط المصلي السائد كان يتراوح بين النمط الثالث DENV-3 والنمط الثاني DENV-2 منذ عام 2000. وبالرغم من القيام بتوزيع مبيدات اليرقات في 94 منطقة منذ عام 2002، لم يُظهر تحليل التحوف اللوجستي ارتباطاً بين هذا التدخل ومعدل وقوع حمى الدنج.

الاستنتاج ظل عبء حمى الدنج مرتفعاً بين صغار الأطفال في كمبوديا، مما يعكس انتقالاً مكثفاً للعدوى. ويبدو أن البرنامج الوطني لمكافحة الناقل ذو تأثير قليل على معدل وقوع المرض.

Résumé

Surveillance nationale du dengue au Cambodge 1980–2008 : tendances épidémiologiques et virologiques, et impact du contrôle des vecteurs

Objectif La dengue est une maladie à déclaration obligatoire au Cambodge depuis 1980. La surveillance virologique a commencé en 2000 et une surveillance sentinelle a été établie dans six hôpitaux en 2001. Actuellement, la surveillance nationale comprend un recueil passif et actif des données et la déclaration des enfants de 0-15 ans hospitalisés. Le présent rapport résume les données de surveillance recueillies depuis 1980.

Méthodes Les données brutes de 1980-2001 sont présentées, alors que les données de 2002-2008 sont utilisées pour décrire des tendances de la maladie et l'effet des interventions au niveau du contrôle du vecteur. Les tendances sur l'incidence de la dengue ont été analysées par la méthode des moindres carrés généralisés (Prais-Winsten) pour séries chronologiques.

Résultats Pendant les années 1980-2001, les épidémies se sont produites en cycles de 3-4 ans, les cycles devenant moins évidents par la suite. Pour les données de 2002–2008, l'analyse de régression linéaire n'a détecté aucune tendance significative de l'incidence annuelle déclarée de la dengue ajustée selon l'âge (fourchette d'incidence: 0,7-3,0 par 1 000 habitants). L'incidence a décliné dans 2,7% des 185 districts étudiés, elle a été inchangée dans 86,2% et elle a augmenté dans 9,6%. L'incidence spécifique de l'âge a été plus élevée chez les nourrissons de < 1 an et les enfants de 4-6 ans. L'incidence a été plus élevée pendant la saison des pluies. Les sérotypes des quatre virus de la dengue (DENV) ont circulé en permanence. Toutefois, le sérotype prédominant a alterné entre DENV-3 et DENV-2 depuis 2000. Bien qu'un larvicide ait été distribué dans 94 districts depuis 2002, l'analyse de régression logistique n'a montré aucune association entre l'intervention et l'incidence de la dengue.

Conclusion Le fardeau de la dengue est resté élevé parmi les jeunes enfants au Cambodge, ce qui reflète une transmission intense. Le programme national de contrôle du vecteur apparaît comme ayant peu d'impact sur l'incidence de la maladie.

Resumen

Vigilancia nacional del dengue en Camboya entre 1980 y 2008: tendencias epidemiológicas y virológicas e impacto del control de vector

Objetivos La declaración del dengue ha sido obligatoria en Camboya desde 1980. La vigilancia virológica se inició en el año 2000 y la vigilancia centinela se fijó en 2001 en seis hospitales. En la actualidad, la vigilancia nacional comprende la recopilación de datos activos y pasivos y la presentación de informes de niños hospitalizados de entre 0 y 15 años. Este informe resume los datos de vigilancia recopilados desde 1980.

Métodos Se presentan los datos brutos desde 1980 hasta 2001 y los datos obtenidos entre 2002 y 2008 se emplean para describir las tendencias de la enfermedad y el efecto de las intervenciones para el control del vector. Las tendencias de la incidencia del dengue se analizaron con el modelo básico de regresión lineal de Prais-Winsten para las series temporales.

Resultados Entre 1980 y 2001 se produjeron epidemias en ciclos de 3-4 años, siendo los ciclos siguientes menos destacados. El análisis de regresión lineal no detectó, en los datos comprendidos entre 2002 y 2008, ninguna tendencia significativa en la incidencia anual del dengue comunicada y ajustada por edades (intervalo de incidencia: 0,7–3,0 por 1000 habitantes). La incidencia disminuyó en un 2,7% de los 185 distritos estudiados, se mantuvo sin cambios en el 86,2% y aumentó en el 9,6%. La incidencia específica por edades fue mayor en los lactantes menores de un año y en los niños de entre cuatro y seis años. La incidencia fue mayor en épocas de lluvia. Los cuatro serotipos del virus del dengue (VDEN) estuvieron en circulación de forma permanente, si bien el serotipo predominante se fue alternando entre el virus DEN-3 y el DEN-2 desde el año 2000. A pesar de que se han distribuido larvicidas en 94 distritos desde el año 2002, el análisis de regresión logística no mostró relación alguna entre dicha intervención y la incidencia del dengue.

Conclusión La carga del dengue siguió siendo elevada entre los niños pequeños en Camboya, lo que refleja su elevada transmisión. El programa nacional para el control de vectores tuvo poco impacto sobre la incidencia de la enfermedad.

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CHAPTER 2: MOLECULAR EPIDEMIOLOGY OF DENV

II.1.4 Context of study

Several studies have tried to decipher the evolution dynamic of DENV using both epidemiological and molecular level data. Several evidences and/or hypothesis have been raised attempting to explain this evolutionary process namely: (1) positive selection (Twiddy et al., 2002a; Twiddy et al., 2002b), (2) purify selection result of negative selection (Zhang et al., 2005), (3) natural selection induced by difference in fitness (Vu et al., 2010), (4) stochastic processes (Sittisombut et al., 1997; Wittke et al., 2002), (5) herd cross-immunity protection (Adams et al., 2006; Wearing and Rohani, 2006) or (6) recombination event (Holmes et al., 1999; Tolou et al., 2001).

In Cambodia, the long-term trend was characterized by a cyclical pattern of epidemics at intervals of about 3–4 years (Huy et al., 2010b). Since virological testing started in 2000, all four DENV serotypes have been observed to be in circulation each year, with DENV-2 and DENV-3 being predominant (Figure 19).

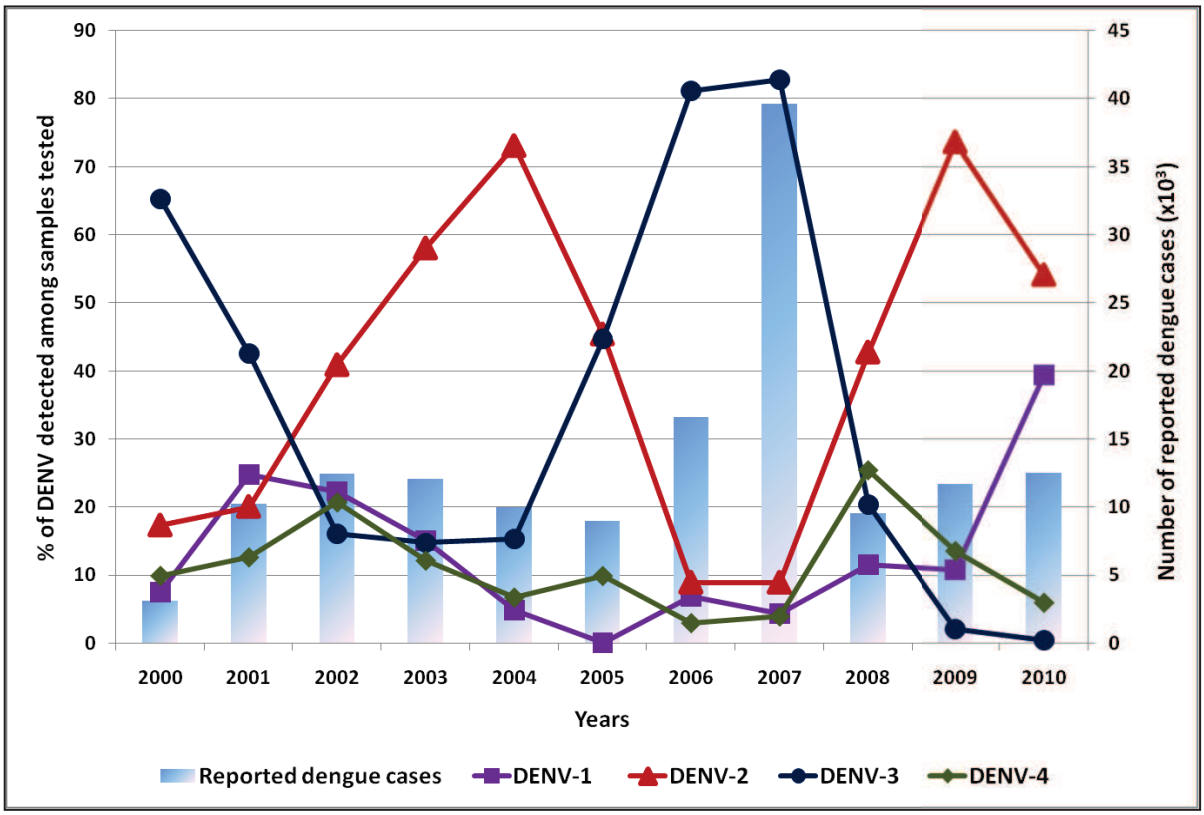

Figure 19: Number of dengue cases reported nationally (clinical surveillance) and percentage of each DENV serotype detected in Cambodia.
Number of clinical dengue cases declared to National Dengue Control Program per year in 5 sentinel sites (right-hand axis, bar) and the percentage of DENV serotypes detected among samples tested by RT-PCR (left-hand axis, line).

II.1.5 Objectives

Although it is 50 years since DENV was first isolated in Cambodia, relatively little is known about the molecular evolution and epidemiology of the virus in this population. In particular, which virus genotypes contribute most to the genetic diversity of the virus, and how they have changed through time, is unclear. Using DENV strains from the collection of NDCP and IPC's own collection, we present the first in-depth study of the evolution of the 4 DENV serotypes in Cambodia sampled over a period of 12 years. In this study, we wanted to explain how DENV evolved in the pattern observed in the figure 19, why there was a shift in dominant strains every 3 or 4 years, and test whether Cambodian DENV have undergone any selection or recombination throughout their evolution in the last 12 years period. As extinction or replacement event is frequently observed previously in DENV, we aimed to demonstrate whether this event was due to natural selection (different in fitness of DENV) or purely stochastic process (random bottleneck).

II.1.6 Results and Conclusions

All 4 DENV serotypes have been found in circulation in Cambodia since 2000. The prevalence of each serotype differed each year and was characterized by shifting of predominant serotype between DENV-2 and DENV-3. The phylogenetic analysis classified Cambodian DENV into: genotype I for DENV-1, Asian/American and Asian 1 for DENV-2, genotype II for DENV-3 and genotype I for DENV-4. These genotypes are commonly found in other neighboring countries such as Vietnam and Thailand.

Although different models have been developed to explain the evolutionary complex of DENV (i.e. immunological cross-protection or enhancement of transmission) it is unclear which of these models best explains the epidemiological dynamics of dengue in Cambodia. Nonetheless, our phylogenetic analysis revealed that the patterns of intra-serotype genetic diversity of DENV varied greatly on temporal scale and exhibited a strong spatial clustering. Additionally, it was shown that the individual lineages or genotypes of DENV arisen frequently, persisted for a period of time, and then disappeared. In the case of DENV-2, the high of prevalence observed in 2004 coincided with the introduction of genotype Asian 1 that replaced the Asian/American genotype completely after 2004. This phenomenon is suggestive of better fitness of the newly introduced genotype, which was well demonstrated by Vu TT in Vietnam (Vu et al., 2010). There are as well some evidences of population bottlenecks that may have assisted in the lineage extinction and emergence. These bottleneck events were marked during at least two distinct circumstances: (1) stochastic process

in 2003 caused by one of the worst flood in 2000 and drought in 2003 and 2004; and (2) herd immunity induced by DENV-3 which was the predominant serotype during the large outbreaks in 2006-2007 countrywide. Our findings have also revealed a clear evolutionary relationship between DENV in Cambodia and that in neighboring countries, particularly in Vietnam and Thailand. Our viruses often clustered closely with either Vietnamese or Thai strains. In addition, Thai strains tend to fall basal to the Cambodian viruses in most of our phylogenetic trees and this suggests that many of the viral lineages circulating in Cambodia are of Thai origin. Another striking observation is that some Cambodian DENVs fall within the genetic diversity of Thai or Vietnamese viruses, which is indicative of relatively frequent viral gene flow at the national levels through probably human migration and commercial exchanges between these 3 countries. Lastly, we demonstrated that recombination and selection were most likely not involved in the evolutionary process of DENVs in Cambodia.

The results of this work are summarized in 3 manuscripts. One article published in « Infection, Genetics and Evolution » and 2 other 'in preparation" manuscripts are presented below.

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Genetic diversity and lineage dynamic of dengue virus serotype 1 (DENV-1) in Cambodia

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ABSTRACT

In Cambodia, dengue virus (DENV) was first isolated in 1963 and has become endemic with peak epidemic during raining season. Since 2000, the Dengue National Control Program has reported from 10,000 to 40,000 cases per year with fatality rates ranging from 0.7 to 1.7. All four dengue serotypes are found circulating in Cambodia with alternative predominance of serotypes DENV-2 and DENV-3. The DENV-1 represents from 5% to 20% of all circulating viruses, depending upon the year. In this work, 79 clinical strains of DENV-1 were isolated between 2000 and 2009 and their genome fully sequenced. Four distinct lineages with different dynamics were identified. The main evolutionary drive was negative selective pressure but each lineage was characterized by the presence of specific mutations acquired through evolution. Coexistence, extinction and replacement of lineages occurred over the 10-year period. Lineages 1, 2 and 3 were all detected since 2000–2002 and disappeared in 2003, 2004–2005 and 2007, respectively. Lineages 1 and 2 displayed different dynamics. Lineage 1 was very diverse whereas lineage 2 was very homogeneous. Lineage 4 which derived from lineage 3 in 2003 remained the only one at the end of the sampling period in 2008–2009 owing to a selective sweep. The lineages dynamic of DENV-1 viruses and consequences for molecular epidemiology are discussed.

1. Introduction

Dengue is the most rapidly spreading mosquito-borne viral disease with a 30-fold rise in the number of human cases reported in the last 50 years associated with a geographic expansion of the disease to new countries (WHO/TDR, 2009). Globally, about two-thirds of the world's lineage live in area at high risk for infection (more than 75% in the WHO South-East Asia and Western Pacific regions) and an estimated 50–100 million cases of dengue infection occur every year (WHO/TDR, 2009). Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. While most patients recover following a self-limiting non-severe clinical course, a small proportion progress to severe disease, mostly characterized by plasma leakage with or without hemorrhage (Duong et al., 2009; WHO/TDR, 2009).

Dengue virus (DENV) which comprises four genetically and antigenically distinct serotypes (DENV-1, -2, -3, and -4) belongs to the family *Flaviviridae*, genus *Flavivirus* (Calisher et al., 1989). DENV genome is a single stranded positive-sense RNA virus and has about 11 kb in length encoding a single open reading frame (Lindenbach and Rice, 2003). The translated protein is cleaved by host and virus derived proteases to produce structural proteins (capsid, premembrane/membrane, envelope; C, prM/M, E) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2003; Weaver and Vasilakis, 2009). The single open reading frame (ORF) is flanked by 5′ untranslated region (UTR) capped with type I 7-methyl guanosine structure and by 3′ UTR lacked of poly(A) region (Lindenbach and Rice, 2003).

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Nucleic acid sequencing has allowed the classification of each of the DENV serotype into genotypes (Rico-Hesse, 1990). Rico-Hesse defined these genotypes as clusters of DENV viruses having nucleotide sequence divergence not greater than 6% within a given genome region (in this case the E/NS1 junction). Various phylogenetic analyses based on partial E/NS1 or complete E nucleotide sequences indicated that (1) DENV-1 are organized into five genotypes (I–V), (2) DENV-2 comprises six genotypes: South-East Asian/American, Asian I, Asian II, Cosmopolitan, American and Sylvatic, (3) DENV-3 comprises of four genotypes (I–IV) and (4) DENV-4 are classed into four genotypes (I, II, III and Sylvatic) (Rico-Hesse, 2003; Vasilakis and Weaver, 2008; Weaver and Vasilakis, 2009).

Previous studies have shown that dengue genotypes are not fixed entities as there is evidence of recombination within all four serotypes (Holmes et al., 1999; Tolou et al., 2001; Worobey et al., 1999) and evidence of selection pressure showing lineage turnover (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002; Zhang et al., 2005). Although, the mechanism of lineage replacement's occurrence is unclear and two hypothesis have been raised (Zhang et al., 2005): (1) a ladder-like phylogenetic trees showing a strong temporal topology. This event may be introduced by the elimination of deleterious mutation strains by purifying selection (Holmes, 2003) and/or a regular random population bottleneck perhaps due to decline in mosquito population and density during annual dry season (Scott et al., 2000); (2) or a more dramatic change by entire clade replacement by a new clade of viruses. Although the evolutionary process is unclear, the pattern could reflect the action of either dramatic population bottleneck or natural selection such as clades with a better fitness out-compete another or clades with mutations that allow them to evade cross-protective herd immunity (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002). In Cambodia, all the four DENV serotypes are co-circulating each year although the predominant serotype has alternated mainly between DENV-2 and DENV-3 during the last decade (Huy et al., 2010). Each dengue serotype corresponds to distinct emergence event from sylvatic viruses (Wang et al., 2000) with specific evolutionary traits. Therefore, separate in depth analyses of the lineage structure and dynamics of each serotype were undertaken over this decade. We report in this study the first of this series of separate analyses: the characterization of the genetic diversity and lineage dynamic of the complete genome of DENV-1 viruses isolated in Cambodia between 2000 and 2009.

2. Material and methods

2.1. Virus strains

Dengue virus strains were isolated during the national surveillance established by the National Dengue Control Program (NDCP), Ministry of Health Cambodia in collaboration with the Institut Pasteur in Cambodia and the World Health Organization, in five sentinel sites and by a dengue cohort study in Kampong Cham province (Vong et al., 2010). This study was approved by the National Ethics Committee for Health Research in Cambodia and a written informed consent was given by the patients (or their parents/guardians) included in the cohort study. Samples collection methods and diagnostic testing including serological, molecular and virological tests were described elsewhere (Buchy et al., 2005; Huy et al., 2010; Vong et al., 2010). All strains were isolated in C6/36 mosquito cells (*Aedes albopictus* clone) and cultures were not passaged more than two times. After complete anonymization, the strains included in the study were selected randomly by geographical origin, year of sampling and severity of the disease according to former WHO criteria (WHO/TDR, 1997).

2.2. DENV genome sequencing

Viral genomes were sequenced using the Broad Institute's capillary sequencing (Applied Biosystems) directed amplification viral sequencing pipeline (http://www.broadinstitute.org/annotation/viral/Dengue; Vu et al., 2010). Briefly, viral RNA was extracted from low passage cell culture supernatants using the QIAmp viral RNA mini kit (Qiagen). The genome was reverse-transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen, California, USA), random hexamers (Roche Diagnostics GmbH, Mannheim, Germany) and a specific oligonucleotide targeting the 3′ end of the target genome sequences (5′-AGA ACC TGT TGA TTC AAC AGC AC-3′; nt 10,700–10,722). The cDNA obtained was then amplified using a high fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and a pool of specific primers to produce 14 overlapping amplicons of 1.5–2 kb in size for a physical coverage of 2×. Amplicons were then sequenced in the forward and reverse direction using primer panels consisting of 96 specific primer pairs, tailed with M13 forward and reverse primer sequence, that produce 500–700 bp amplicons from the target viral genome. Amplicons were then sequenced in the forward and reverse direction using M13 primer. Total coverage delivered post amplification and sequencing was about 8-fold. Resulting sequence reads were assembled *de novo* and annotated using the Broad Institute's in-house viral assembly and annotation algorithms.

2.3. Sequence alignment and phylogenetic analysis

Multiple sequence alignment was conducted with Muscle (Edgar, 2004) available in Seaview version 4.2.5 package (Galtier et al., 1996; Gouy et al., 2010). Phylogenetic analyses were performed using maximum likelihood (ML) method for the complete coding region (10,182 nucleotides), for each locus separately and for concatenated data set. In addition, to genotype Cambodian DENV-1 isolates, 51 reference strains of DENV-1, i.e. genotypes I, II, IV and V (complete genome of genotype III was not available), obtained from GenBank were included in the alignment and phylogenetic analysis. Jmodeltest (Posada, 2008) was used to select the optimal evolution model by evaluating the selected parameters using the Akaike Information Criterion (AIC). A corrected version of the AIC (AICc) was used for each locus separately sequence data set because sample size (n) was small compared with the number of parameters ($n/K < 40$). This approach suggested the following models: GTR + G4 for complete coding region, HKY + I + G for C; GTR + G for E, NS1 and NS2A; GTR + I + G for NS2B, NS3, NS4A, NS4B, NS5 and concatenated data set; and K80 + I for PrM. Under the selected models, the parameters were optimized and ML analyses were performed with PhyML (version 2.4.4) (Guindon and Gascuel, 2003). The robustness of nodes was assessed with 1000 bootstrap replicates for complete coding region, 100 bootstrap replicates for each gene and 500 bootstrap replicates for concatenated data set. Finally, trees were edited using FigTree v1.3.1 (BEAST softwares).

2.4. Genetic similarity and DNA polymorphism analysis

Genetic similarity and difference matrices were constructed from ClustalX2 alignments (Thompson et al., 1997) using BioEdit 7.0.9.0 (Hall, 1999). DNA sequence polymorphism and all subsequent tests were investigated using several functions from the DnaSP5.00.02 package (Librado and Rozas, 2009). Haplotypes (alleles) were calculated according to Nei (Nei, 1987). Nucleotide diversity, Pi (π), the average number of nucleotide differences per site between two sequences was calculated according to Nei (1987), using the Jukes and Cantor (1969) correction. Theta (Watterson's mutation parameter) was calculated for the whole

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sequence from *S* (Watterson, 1975). Eta (η) is the total number of mutations, and *S* is the number of segregating (polymorphic) sites. *Ka* (the number of non-synonymous substitutions per non- synonymous site), and *Ks* (the number of synonymous substitutions per synonymous site) for any pair of sequences were calculated according to Nei and Gojobori (1986). Tajima's *D* test (Tajima, 1989) was used for testing the hypothesis that all mutations are selectively neutral (Kimura, 1983). Other tests of neutrality are Fu and Li's tests D^* and F^* (Fu and Li, 1993) and Fu's Fs statistic (Fu, 1997). ZnS statistics (Kelly, 1997) is the average of R^2 (Hill and Robertson, 1968) overall pairwise comparisons. It reflects the excess of linkage disequilibrium compared with that expected under neutrality. Wall's *B* and *Q* statistics (Wall, 1999) were also considered. Significant pairwise *Ka*/*Ks* biases were assessed by Fisher exact test with Bonferroni multiple testing correction.

2.5. Multiple Correspondence Analysis (MCA)

Multiple Correspondence Analysis (MCA) is an exploratory statistical technique adapted to multivariate categorical data (Greenacre, 1984; Greenacre and Blasius, 2006; Lebart et al., 1984). It can be viewed either as an extension of Correspondence Analysis (CA) to more than two variables or as an adaptation of Principal Component Analysis (PCA) when the variables are categorical instead of quantitative and the metrics is the chi-square rather than the euclidean distance (Tenenhaus and Young, 1985). Its application to the analysis of co-mutations in multiple alignments has already been described elsewhere (Pazos et al., 2006). In this work, a 'site' is defined as a position in the multiple alignment, a 'state' denotes a particular nucleotide (A,C,G,T) and a 'trait' is a particular state at a particular site (e.g. trait 'A50' means state A at position 50). As a first step the multiple alignment was encoded into a boolean table (called an indicator matrix) where rows correspond to the sequences and columns to the traits. Entry will be 1 if the trait (column) is observed for this sequence (row) and 0 otherwise. The second step is to perform a standard Correspondence Analysis (CA) on this table using the ADE4 R package (Thioulouse et al., 1997; Chessel et al., 2004). As with any Principal Component Analysis, the purpose is to reduce the space dimension to the first eigenvectors (usually 2 or 3) and to interpret the plot of row points (sequences) or columns points (traits) in lower dimensional maps. Two row points (sequences) are close to each other when they tend to select the same traits (i.e. the same states at the same sites). In the same way, two columns points (traits) are close to each other when they tend to be observed on the same set of sequences. In a third step, row points (sequences) were clustered by k-means (R package cluster:pam) (Kaufman and Rousseeuw, 1990) into four groups, using the coordinates on the three main axes. Finally, a Naive Bayes approach was used to identify which traits were specific of each group of sequences previously identified. A trait 't' (i.e. a state at a given site) is affected to a group of sequences *i* (i = 1,2,3,4) according to the observed frequency of 't' in this group.

2.6. Lineage demography

Lineages history was inferred using the Bayesian skyline plot (BSP) method implemented in BEAST v 1.6 (Drummond et al., 2005). The analysis was conducted on all of the 79 sequences and on separate lineages. However, only population 4 had a sufficient number of sequences (45 sequences) and sampling time frame (2003–2009) to yield significant results. We use the HKY85 substitution model (accounting for transition/transversion only) with codon positions partitioned in two classes (1 + 2, 3) (Shapiro et al., 2006). Tests with more general models (GTR, site heterogeneity) yielded similar results. In the same way, both strict and relaxed (uncorrelated lognormal) molecular clocks gave similar results. We used $m = 10$ steps in the BSP model for the whole population analysis and $m = 5$ for the analysis of population 4. MCMC analyses were run for 10. 10^6 states and posterior analysis were carried out with a 10% burn-in.

3. Results

3.1. Clinical feature and distribution of samples

Of 101 DENV-1 strains isolated between 2000 and 2009, 79 isolates were sequenced and the full genome sequences used in this study are available in GenBank (Supplementary Table 1). The average age of the 79 patients was 8 years (standard deviation: 5.2, range: 1–27). The severity of the disease and the general demographical data of the patients are described in Supplementary Table 1. The relative importance of each of the four DENV serotypes varied depending on the year, DENV-1 representing between 5% and 25% of the circulating strains among the diagnosed cases. The temporal distribution and relative importance of each of the four serotypes is shown in Supplementary Fig. 1.

3.2. Coalescence analysis and demography

Coalescent analysis of the whole set of genomes yielded an estimated nucleotide substitution rate of 7.2×10^{-4} substitutions/site/year, a value consistent with previous reports for DENV-1 (Twiddy, 2003) and an estimated year of the most recent common ancestor (MRCA) at 1987. Bayesian skyline plot (Supplementary Fig. 1b) shows a bottleneck starting in 2002–2003 to 2006. The same analysis restricted to lineage 4 yielded a similar substitution rate of 6.4×10^{-4} substitutions/site/year and an MRCA at year 2002–2003. The plot (Supplementary Fig. 1c) shows an increase in genetic diversity (hence effective population) in early 2006 consistent with the increased number of cases at that time. Unfortunately data for lineages 1, 2 and 3 were insufficient to carry out the same analysis on these lineages.

3.3. Lineage structure and clustering of sample sequences

The whole set of 79 full length sequences was analyzed through Multiple Correspondence Analysis (MCA). The alignment had a total length of 10,392 sites (with only three gapped sites (1 codon) which were further removed) out of which only 1332 (13%) sites were variables (average of 98.6% identity between pairs of sequences) and 650 (6%) sites were informative in the sense of parsimony (at least two states with an occurrence of at least two). Only informative sites were considered for MCA. These 650 sites gave rise to 1328 different traits, indicating that most sites (624 out of 650) contained only two states. As a consequence, the two disjunctive traits associated to a site corresponded to one majority state (i.e. the state observed on the consensus sequence) and one mutated state. The first three principal axes accounted for 22%, 18% and 12%, summing up to 53%, of the total inertia. However, these were underestimated (uncorrected) values. Applying the Benzecri (1979) eigenvalues correction yielded 46%, 30% and 15% respectively, summing up to 91%, of the total inertia. The projection of sequence points on the first and second axis is shown on Supplementary Fig. 2. Sequence points clustering by k-means yielded four lineages associated with a color. Lineage 1 (black), lineage 2 (red), lineage 3 (green) and lineage 4 (blue) contained 7, 8, 17 and 47 sequences, respectively. Traits (i.e. mutations) specific to each group of sequences, or lineages, were identified using a Naive Bayes approach. The threshold condition ($P_{best} \geqslant 0.7$) applied on the probability allowed selection of the most significant traits per group. These traits can be seen as mutational features

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(SNPs) specific of each lineage. An overall 314 traits were selected: 104 for lineage 1, 68 for lineage 2, 90 for lineage 3 and 52 for lineage 4. These 314 traits corresponded to 312 different sites, indicating that with the exception of two sites occurring twice, most sites occur only once. Examination of the distribution of these sites on the alignment showed that they were evenly distributed along the genome (data not shown) and not related to particular proteins or genome segments. In order to display the blocks of mutations characteristic of each lineage, sequences (rows) and sites (columns) in the alignment were re-ordered according to the group they belong to (Supplementary Fig. 3). For clarity, the majority state in each column was colored in white, so that only minority states were depicted in colors. Except for the two sites indicated by triangles, the four lineages harbored completely different mutational patterns. These patterns were very homogeneous within each lineage but there was clearly no mutational relation between groups, suggesting that they evolved independently. The isolate KHD1_HM181952_07_KCH displayed a hybrid structure with blocks 1, 2, 3 corresponding clearly to lineage 4, although some sites in block 3 corresponded to lineage 3, whereas block 4 was undoubtedly similar to that of lineages 1, 2 and 3. Sequence alignment showed that this isolate is a hybrid between lineage 3 and lineage 4 (Supplementary Fig. 4). Hybrid features between lineages 3 and 4 are also found in isolate KHD1_FJ639688_07_KCH, although to a lower extent (Supplementary Fig. 4).

3.4. Spatiotemporal distribution of traits

The year of sampling was represented as a supplementary categorical component in MCA (Fig. 1a). The sequence points in Fig. 1a were the same as in Supplementary Fig. 2 and points representing the component 'year of sampling' have been superimposed and joined for clarity (with the exception of year 2002 which was represented only by one sequence). Years were clearly ordered along the first axis, with group 4 associated to recent samples and the other groups to late samples. This effect, known as Guttman or

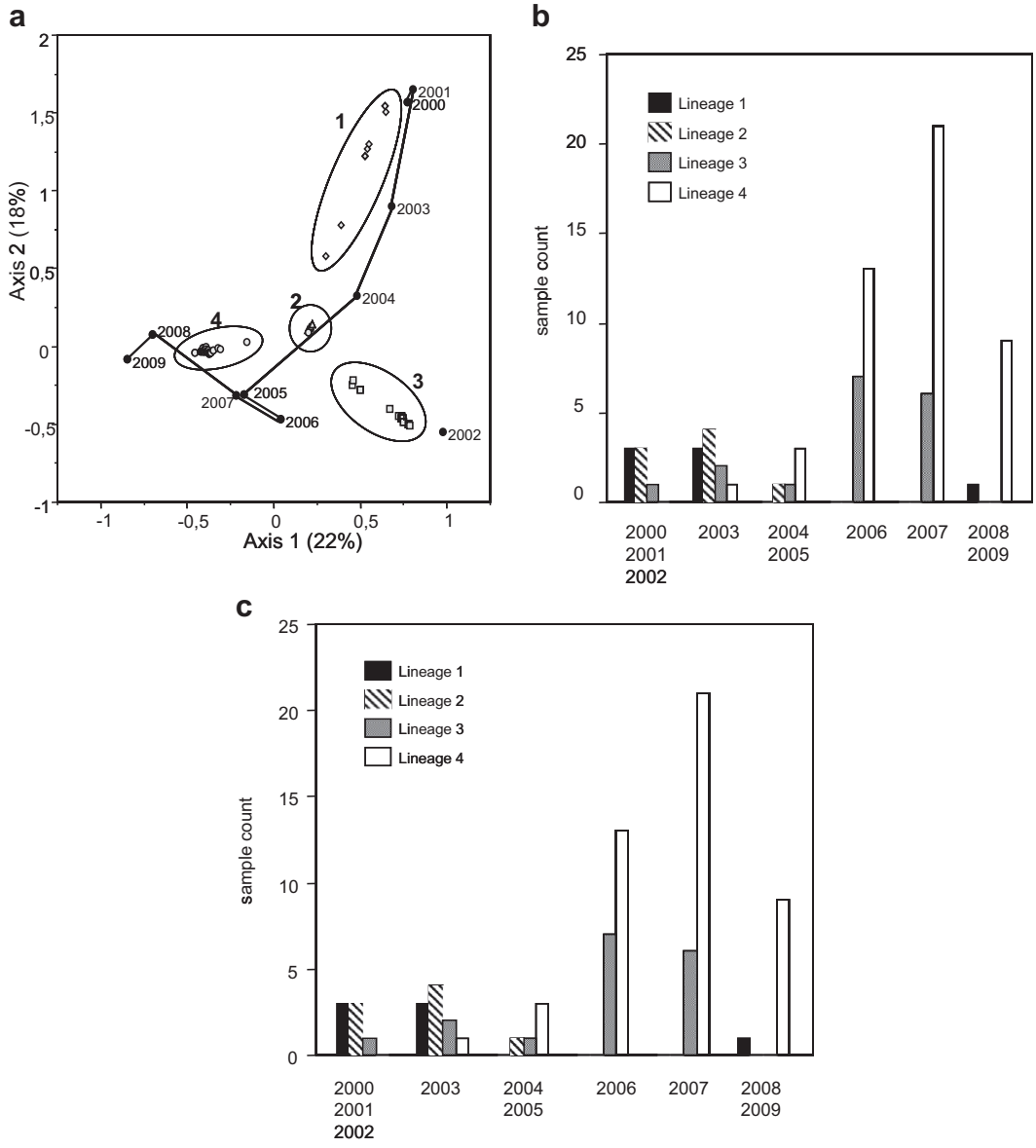

Fig. 1. (a) Multiple correspondence analyses of co-mutations superimposed with years of sampling. Superimposition of the sequences points (same as in Supplementary Fig. 2) with supplementary characters 'year of sampling' (open circles). The character points are almost totally ordered along the first axis, showing that the year of sampling is strongly related to the mutational patterns. ◇ Lineage 1, △ lineage 2, □ lineage 3, ○ lineage 4. (b) Distribution of DENV-1 populations over the sampling period. Years are shown on the *x* axis and number of isolates is shown on the *y* axis. ■ Lineage 1, ▨ lineage 2, ▩ lineage 3, □ lineage 4. ∗: Isolate of population 1 from Vietnamese origin.

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horse-shoe effect, suggested that the underlying one-dimensional variable, i.e. the year of sampling, enforced a strong structure on the data and that the mutations patterns were thus mostly organized by year. Since some years, i.e. 2002, 2004 and 2009, fewer DENV-1 sequences were available leading to statistical weaknesses, data were pooled and distributed as follows: 2000–2002, 2003, 2004–2005, 2006, 2007 and 2008–2009 (Fig. 1b). Lineage replacement was clearly visible. Lineages 1, 2 and 3 were all detected since 2000–2002 and disappeared in 2003, 2004–2005 and 2007, respectively. Lineage 4 which appeared in 2003 remained the only one at the end of the sampling period in 2008–2009. The only apparent exception was the isolate KHD1_GQ868636_08_KOP which belonged to lineage 1 and was isolated in 2008. However the sequence of this isolate was shown by blast to cluster with the sequences of known Vietnamese strains and not with Cambodian strains. In addition, the strain KHD1_GQ868636_08_KOP was obtained from a patient originated from Kampot province, at the border of Vietnam. An important limitation of this analysis is the overrepresentation of Kampong Cham (KCH) province in the geographical distribution of the samples and this may introduce a bias. Another limitation to consider in this analysis is that DENV-1 represented only a minor fraction, i.e. from 5% to 25% depending upon the year, of the overall DENV circulating serotypes (Supplementary Fig. 1).

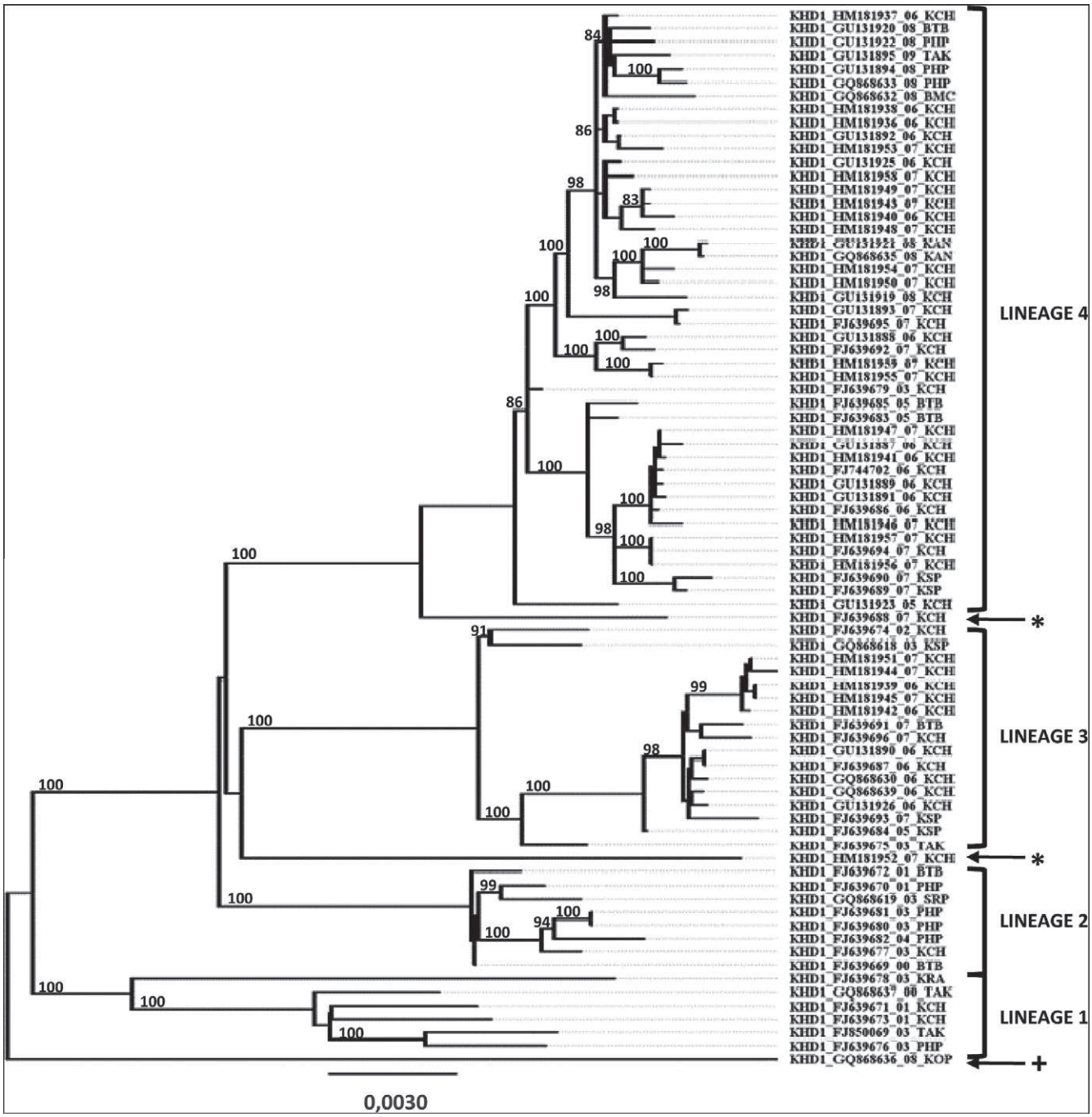

Fig. 2. Distribution of DENV-1 sequences based on the nucleotide sequences of the whole coding region of the genome. The tree was built using the Maximum Likelihood method with a GTR + I + G evolutionary model. Bootstrap values <70% are not shown.

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3.5. Phylogenetic analysis of the complete coding region

All the isolates considered in this work were strongly clustering with the genotype 1 reference strains of DENV-1 (Supplementary Fig. 5). The distribution in four distinctive lineages was confirmed by the phylogenetic analysis of the complete coding region (Fig. 2). The isolate KHD1_GQ868636_08_KOP most probably introduced from Vietnam was used as outgroup for rooting the tree. Lineages 1 and 2 were clustering separately either at the root (lineage 1) or with a bootstrap value of 100 (lineage 2) confirming they were distinct genetic entities. On the contrary, lineages 3 and 4 appeared genetically related, with a weaker bootstrap value of 50, and belonged to the same main cluster. Furthermore, lineage 4 appeared divided into two sub-lineages. The isolate KHD1_HM181952_07_KCH was found separated from lineage 3 branching at the origin of the cluster at an intermediate position between lineage 3 and lineage 4. A similar situation observed for isolate KHD1_FJ639688_07_KCH but closer to lineage 4 and to a lower extent for isolate KHD1_GU131923_05_KCH (Fig. 2).

3.6. Phylogeny of individual genes

Analyzing the phylogeny of the individual genes systematically confirmed the distribution into the same four distinct lineages (Supplementary Fig. 6a–j). The separation of lineage 4 into two sublineages could also be seen for each individual gene. The intermediate position of isolate KHD1_FJ639688_07_KCH between lineage 3 and 4 was systematically confirmed. Three other isolates were found at this same intermediate position: KHD1_GU131923_05_KCH, KHD1_GU131920_08_BTB and KHD1_GQ868632_08_BMC. The position of isolate KHD1_HM181952_07_KCH varied depending on the gene. Although in most genes, this isolate was located at the node between the four lineages. For the gene C, encoding the capsid protein, this strain was associated with lineage 2 (Supplementary Fig. 6a).

3.7. Lineage structure based on amino-acid similarity

The distribution of the isolates based on amino-acid similarity for each individual protein was slightly different than that observed for the individual coding sequences illustrating the selective pressures specific to each protein (Supplementary Fig. 6). NS2B seemed highly conserved through the years with branches isolating the four lineages not very supported suggesting a negative selective pressure. The radial view showed a distribution centered on a single point (Supplementary Fig. 6f). Other proteins displayed a partial conservation characterized by an overall round shape of the radial distribution but with some lineages extending outside. This is the case for NS3 (Supplementary Fig. 6g) and NS4A (Supplementary Fig. 6h) and to a lower extent for prM (Supplementary Fig. 6b). However, the diverging lineages varied depending on the protein. In the case of prM, both lineages 1 and 3 diverged from the central core whereas only lineage 1 diverged in the case of NS4A (Supplementary Fig. 6h) and only lineage 3 differed in NS3 (Supplementary Fig. 6g). Interestingly, in the latter two cases, the same isolate from lineage 2 (KHD1_GQ868619_03_SRP) diverged along either lineage 1 or lineage 3. If the divergence of lineage 4 was always seen with the other proteins, i.e. C, E, NS1, NS2A, NS4B and NS5, it was not always possible to discriminate the other three lineages. Each one of the lineages 1, 2 and 3 were clearly diverging in proteins E, NS1, NS2A and NS5. In the case of NS4B, only lineage 1 branched separately, lineages 2 and 3 being associated (Supplementary Fig. 6i) and no discrimination could be observed for the capsid protein C (Supplementary Fig. 6a). Unlike what was observed at the DNA level, the intermediate isolates displayed differing positions depending upon the protein considered. The same distribution as in DNA trees was observed for proteins C, NS2A, NS2B, NS4A, NS4B and NS5. Both isolates were associated with lineage 4 in protein E, NS1 and prM whereas positions differed in proteins E and NS3. Isolate KHD1_FJ639688_07_KCH was associated with lineage 2 in protein E and lineage 4 in protein NS3 whereas KHD1_HM181952_07_KCH was associated with lineages 3 and 4 in protein E and lineage 3 in protein NS3. Significant non synonymous mutations associated to these variations in topology are summarized in Supplementary Table 2. The protein E is playing a key role in virus host interaction, especially receptor binding, membrane fusion and host immunity response (Chavez et al., 2010; Crill and Roehrig, 2001; Lisova et al., 2007; Gromowski et al., 2010). Out of 29 non-synonymous mutations identified when considering the whole 79 sequences only three were significantly associated with a specific lineage. G163A (domain II) was associated to lineage 4, A508T (domain I) was associated to lineage 3 and A511T (domain I) was associated to lineage 1. All the other non synonymous mutations were borne only by one or few sequences. All together, eight non-conserved mutations were located on the structural domain I, six on domain II and 15 on domain III (data not shown). In protein NS3, discrimination of lineage 3 is correlated with a major conformational mutation in which a proline at position 119 (P119) is replaced by a serine (S119).

3.8. DNA polymorphism and selective pressure

The analysis of DNA polymorphism displayed differing results depending upon the gene and lineage considered (Supplementary Table 3). The first noticeable feature was the limited diversity of lineages 2 and 3. Whatever gene considered, lineage 2 remained the least variable. Lineage 3, although also poorly diverse, displayed a more variable level of diversity depending upon the gene considered. Although being the smallest, lineage 1 was the most diverse whatever gene was considered. Lineage 4 displayed the highest diversity among the four lineages considered. However, lineage 4 was the largest with 47 isolates. A feature common to all lineages is the systematically largely higher number of synonymous substitutions than that of non synonymous substitutions and the very low ratio (Supplementary Table 4). Only lineage 4 displayed significant neutrality tests. The lack of significance in other lineages might be related to the low number of isolates or to their limited diversity. Putative recombination events were detected using a four gametes test but mostly in lineages 1 and 4 (Supplementary Table 4). Interestingly, although limited in size, lineage 1 gave rise to detection of recombination events in several genes (i.e. E, NS1, NS3, NS4A, NS4B and NS5). These putative recombination events were detected in all genes in lineage 4 with the exception of NS4A. NS5 was the gene in which the highest number of events was detected (Supplementary Table 3).

4. Discussion

The data reported here on the genetic diversity of DENV-1 isolates in Cambodia indicate the existence of four lineages with specific dynamics over a 10-year period. All isolates from lineage 1 share clear and specific conserved mutations meaning that this lineage is most likely a true one and not a “default” lineage gathering isolates which could not fit in other lineages. But lineage 1 was not detected after 2003 in Cambodia and is probably no more in circulation in this country. The only exception is the isolate KHD1_GQ868636_08_KOP which was isolated in 2008 from a Cambodian patient who was probably infected by a Vietnamese DENV-1 strain. The presence of this Vietnamese isolate within lineage 1 indicates that lineage 1 was still circulating in this neighboring country at least until 2008. This in turns suggests that this

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lineage is therefore probably established in South East Asia outside Cambodia as for the Asian 1 genotype of DENV-2 (Hang et al., 2009). The higher diversity exhibited by lineage 1 might thus be an indication that it is an ancient lineage having accumulated mutations over time.

As lineage 1, lineages 2 and 3 were present at the beginning of the study period, thus it is not possible to know when they appeared and for how long they have coexisted and co-circulated. However, they displayed differing traits. Lineage 2 was very homogeneous with a limited genetic diversity and was no longer found after 2005. The evolution of lineages 3 and 4 suggests similar trends but with a time shift and evidence of selective sweep, indicating thus that these two lineages are genetically related and derive one from the other. Lineage 3 was found since at least 2000 whereas lineage 4 was first identified only in 2003. They followed the same initial pattern of expansion and after 2005, when lineages 1 and 2 disappeared, underwent a phase of expansion. However, lineage 3 apparently disappeared after 2007 and lineage 4 remained until the end of the sampling period.

Two isolates of the data set appeared to be hybrids with mutational markers specific of both lineages 3 and 4: KHD1_HM181952_07_KCH and KHD1_FJ639688_07_KCH although to a lower extent, the similarity with lineage 3 being more limited. These strains are most likely not resulting from a major recombination event between both lineages since the corresponding mutational markers are scattered all along the genome (Supplementary Fig. 3). However, the occurrence of more discrete recombination events cannot be excluded. These two strains most likely represent various intermediate evolutionary steps between lineage 3 and lineage 4, isolate KHD1_HM181952_07_KCH representing probably a most ancient intermediate than isolate KHD1_FJ639688_07_KCH. The presence of these intermediates indicates the existence of a possible continuous, larger lineage of DENV-1 prior to 2007 and the occurrence at that date of a selective event which led to a bottleneck and the survival of only a fraction of this initial DENV-1 lineage, i.e. lineage 4 (Supplementary Fig. 1). The presence of only lineage 4 after that date is therefore probably the result of a selective sweep. In accordance with that conclusion is the significant Tajima's D test for all genes in lineage 4 which associated with an excess of synonymous substitutions and low Ka/Ks, indicates a lineage in expansion.

An interesting feature of all the lineages analyzed here is the differing and apparently contradictory trends displayed by the genes polymorphism and the proteins they encode. A first trait is the negative selective pressure observed in this work, as well as in previous reports (Wittke et al., 2002; Holmes, 2003; Thu et al., 2005). Despite the lack of proof reading action from the RNA-dependent RNA polymerase found in dengue virus (Steinhauer et al., 1992), which is expected to generate a large diversity of viral lineages, the ratio between synonymous and non-synonymous mutations is largely biased towards synonymous substitutions, as shown by the low *Ka*/*Ks* ratio observed whatever gene was considered. The selective pressure observed, at least over the 10-year period considered in this work, is strongly purifying indicative of strict and homogeneous adaptation of all the lineages described to core function with no significant host-driven selection.

A second and apparently contradictory trend is the occurrence of signals of positive selection suggested by the protein polymorphism analysis. Adaptive evolution, although limited, has indeed been reported in dengue virus (Bennett et al., 2003, 2006; Holmes and Twiddy, 2003; Twiddy et al., 2002a, 2002b; Thu et al., 2005; Zhang et al., 2006). The distribution of isolates, especially the intermediates between lineages 3 and 4, differs from that of DNA sequence analysis. These differences in tree topology indicate that although very limited because of the high negative selective pressure some non-synonymous mutations may be very important in term of protein structure and function. In DENV-1 lineages from Myanmar, Thu et al. (2005) showed that only NS5 displayed some limited sign of selective pressure and some specific amino-acid replacements with potential functional significance. In the case of the Cambodian isolates of DENV-1 described in this work, several potentially important amino acid substitutions were detected. The most important one might be the replacement of a proline by a serine at position 119 in the NS3 protein from lineage 3. Proline has a major role in the folding of a protein and therefore this mutation is very likely to modify the conformation and thus the function of NS3 in lineage 3. NS3 plays a major role in dengue virus life cycle. Indeed, NS3 is a multifunctional protein (Perera and Kuhn, 2008) in which 184 N-terminal fraction, or NS3pro, is involved in the cleavage of the capsid C protein and at NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 boundaries (Perera and Kuhn, 2008), whereas the C-terminal fraction codes for a nucleotide triphosphatase, an RNA triphosphatase and a helicase (Perera and Kuhn, 2008). Furthermore, NS3 protein is interacting with NS2B which acts as a cofactor. Specific recognition and interaction are therefore essential for the proper action of NS3 and these functions could be altered by a change like the one observed in NS3 from lineage 3 at position 119. The amino acid replacements observed in the other proteins and associated with differential tree topologies between protein and gene might also be of importance in term of protein function. However, it is not possible at this stage to predict what could be the functional modifications. Further specific structure–function investigations will be needed to confirm and characterize functional changes.

The presence of conserved mutations highly specific to each lineage brings other element of understanding of this complex evolutionary pressure. These lineage-specific mutations did not originate from recombination but instead were accumulated through time as an adaptive response to a specific environment. This is a strong indication of host driven selection. These data suggest the occurrence of separate adaptation to different host lineages under a strong purifying selection to maintain core functions. A plausible explanation is that these separate adaptations took place in distinct mosquito lineages and are representative of specific virus–vector coevolution and organism to organism interaction as already proposed (Lambrechts et al., 2009). A balanced coevolution in alternate hosts leading to the presence of conserved adaptive mutations was also demonstrated through experimental evolution (Coffey et al., 2008; Vasilakis et al., 2009). Owing to the high prevalence of dengue in Cambodia (Huy et al., 2010), adaptation to specific human immunological reaction is also expected. However, since no major signal for positive selection can be detected such mutations are probably present in limited number making difficult the detection of a scheme of positive selection.

The main question is therefore how to combine these apparently diverging and contradictory observations. This can easily be done if one considers that the main drive behind extinction and replacement of lineages of dengue virus is the occurrence of major stochastic events as reported in Myanmar (Thu et al., 2005). However the definition of "stochastic events" must be understood also as the successive or simultaneous occurrence of separate events at the level of the human hosts and insect vector. In a larger scale study in Thailand, Zhang et al. (2005) found that genetic diversity within DENV-1 peaked at the time of high prevalence and that clade replacement was associated with periods of low prevalence. These findings are in correlation with our result showing a similar fluctuation in diversity of DENV-1 according to epidemiologic dynamics. The data obtained from the National Dengue Control Program showed that prevalence of DENV-1 was high between 2001 and 2003 and then started to decline from 2004 and stayed low until 2007 (Huy et al., 2010). The high peak prevalence of DENV-1 was associated with the co-circulation of the three

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lineages of DENV-1 in year 2000–2002 and four lineages in 2003 (Supplementary Fig. 1 and 1b). The disappearance of lineages 1 and 2 occurred during the low prevalence period (2004–2007) demonstrating a lineage bottleneck. Inversely, this period corresponded to very high peak of DENV-2 circulation between 2003 and 2005 and DENV-3 between 2006 and 2007. This lineage (clade) survival event could be explained partially by the "herd" immunologic profile (Zhang et al., 2005). The lineage 4 that survived during the decline of DENV-1 prevalence could have been more antigenically distinct from DENV-2 and/or DENV-3 than population 1 and 2, which could have been subjected to some cross-protective immune activity. This mechanism was described as neutralization-escape mutants by Guzman et al. (2000). However, this observation could be biased by the limited number of provinces where our isolates were obtained (only and mostly from Kampong Cham province in 2006 and 2007 respectively, Supplementary Table 1) and this hypothesis will require rigorous experimental testing. The envelope protein (protein E) is of particular interest since it is the protein responsible for binding to cellular receptor and for membrane fusion, but is also the primary antigen inducing protective immunity (Crill and Roehrig, 2001; Chavez et al., 2010; Lisova et al., 2007; Butrapet et al., 2011). The three dimensional structure of the envelop protein was determined and shown to be in a trimeric postfusion conformation even without prior exposure to lipids (Modis et al., 2004; Nayak et al., 2009). The E protein is organized into three structural domains. Domain 2 bears the fusion loop involved in membrane insertion domain. Although the E protein is highly conserved among flaviviruses, a specific structure is found in the envelop protein of dengue virus: a tight cluster of four polar residues between domains I and III which stabilizes the protein and is involved in the pH sensor triggering of the fusogenic conformational change of the protein (Nayak et al., 2009). This polar cluster and the fusion loop display differences between DENV-1 and DENV-2 E proteins (Nayak et al., 2009). A key domain, however, is domain III which the main antigenic domain of the protein E and interacts with host immune system. Residues essential for both human and insect specificity, stability and epitope binding have been mapped (Chavez et al., 2010; Crill and Roehrig, 2001; Lisova et al., 2007; Gromowski et al., 2010). None of these key residues was bearing a non-synonymous mutation in the samples analyzed in this work and only three mutations, all located outside domain III were associated with a lineage. Nevertheless, most of the non-synonymous mutations in protein E are located in domain III which could in line with the interaction of this domain with the host immunity. However, since these mutations are borne by only one or few sequences, it is not possible to infer any correlation and conclude on adaptive evolution.

On another hand, the reported bottlenecks can find an explanation in stochastic events affecting the vector populations. Vectorial capacity was shown to be dependent on virus infectivity and fitness indicating thus that virus-vector interaction is a key issue for dengue dissemination (Anderson and Rico-Hesse, 2006; Armstrong and Rico-Hesse, 2003; Hanley et al., 2008). Organism-to-organism specific interactions were also recently demonstrated using mosquito isogenic female lines (Lambrechts et al., 2009). However the resulting diversity is reduced by the very high negative pressure imposed for efficient conservation of the core function in both the human and the invertebrate hosts. Indeed, the negative pressure observed in this work and previously reported (Wittke et al., 2002; Holmes, 2003; Thu et al., 2005) seems to be the main factor in terms of virus evolution. A given mosquito population has only a very limited range of spread and displays a limited life span, restricting thus the spread of a dengue virus (Harrington et al., 2005). The long distance spread of dengue was indeed shown to be associated to human's movements (Huber et al., 2004; Harrington et al., 2005; Santana de Melo et al., 2007; Honorio et al., 2009) and is therefore independent from the distribution of related mosquito populations. A strong positive selection would not be a favorable adaptive strategy since there is a low chance for a virus to come into contact with the same or a very close *Aedes* population. A negative selection making possible survival in a majority of *Aedes* population appears more favorable in terms of adaptation while some variations might turn selectively advantageous in some cases, i.e. when the mutations confer a higher infectivity rate as previously observed (Hanley et al., 2008). A highly efficient virus lineage will not be selected if associated with a less efficient mosquito population or if human mobility brings into contact with such a lower-efficiency mosquito population. A less invasive virus lineage could be in the contrary selected for the same reason. A recent survey conducted over seven Cambodian provinces in both urban and rural areas and showed *Aedes aegypti* makes an average of 95.5% of the dengue vector population in all the zones considered. The remainder is made by *A. albopictus* (Seng et al., 2009). Mosquito control programs did not affect the incidence of dengue nation-wide which remained the similar over the last decade with a rather high level ranging from 0.7/1000 to 3/1000 (Huy et al., 2010). There is no clear identification for the events which led to the population structure and temporal dynamics described in this work but it could be related to a change in vector populations resulting from an environmental, climatic or anthropic change and/or the dissemination of a novel population of vectors.

Therefore, these data must also be considered in the light of previous works on the influence of the alternate replication in human host and insect vectors on fitness and patterns of genome evolution. In vitro artificial evolution analysis of DENV-2 virus comparing multiple passages in the same cell line versus passages in alternating hosts, i.e. human cell lines and mosquito cell lines showed differing fitness gains end mutational patterns (Vasilakis et al., 2009). Dengue virus rapidly specialized and acquired higher fitness when multiplied on a single cell line while losing fitness for the other cell line. Similar results were observed with Venezuelan equine encephalitis virus (VEEV) as a model (Coffey et al., 2008). Alternately passaged viruses did not display any detectable fitness gains in either host. This indicates that adaptation and evolution is constrained by obligate host alternation. Adapting these conclusions to this work might bring some hypotheses to explain lineage replacements. Dengue is a hyperendemic infection in South East Asia and the seroprevalence is high in the population (Thu et al., 2005; Thai et al., 2005, 2007; Hiscox et al., 2010; Vong et al., 2010). Dengue virus is therefore expected to be exposed to selective pressure from the human host in order to overcome immunological defenses. Dengue virus can most likely quickly adapt to the human host however as shown by in vitro experimental evolution (Coffey et al., 2008; Vasilakis et al., 2009). However, these studies also showed that there was a trade-off and that this higher adaptation to one host resulted in a lower fitness for the other host. In the situation reported here, it is expected that a dengue virus lineage evolves with the human host to become more adapted, capable of avoiding immunological defenses. However, this is accompanied by a decrease in fitness for the mosquito host. Owing to the limits imposed by the obligate host alternation, this natural evolution for adaptation to the most constraining host, i.e. the human host, is expected to reach a break point. This limit is when the lineage is no longer competitive for replication in the insect host due to loss of fitness and/or cannot evolve anymore for further adaptation to human host. There is thus an expected loss of adaptation to the obligate host alternation which imposes a stalemate. At this point, another viral lineage will be more adapted to the new state of evolution of both the human and mosquito hosts resulting from this recent evolutionary history and will therefore outcompete the previous lineage. However, this replacement event is purely stochastic

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and can result either in the replacement by a totally different lineage or by a specific subset of the current lineage through selective sweep. Both situations are observed in this work. Nevertheless, the stochastic nature of the replacement imposed by the alternating host context is worsen by the mobility of the human host which brings the virus into contact both with different mosquito populations and human populations, both displaying different genetic backgrounds. Lineage replacement is a major driver in dengue evolutionary pattern which was extensively demonstrated in South America where dengue extended since the late 1970's (Carrington et al., 2005; Ramírez et al., 2010; Mendez et al., 2010; Carrillo-Valenzo et al., 2010). An interesting feature in the Cambodian context is the simultaneous presence of the four serotypes with differing dynamics. The replacement process might therefore be a complex system in which the different serotypes cross interact on both human and insect hosts leading thus to intricate patterns. The data reported in this work are limited to DENV-1 in order to do investigate the intra-serotype dynamics and therefore do not allow for such an integrated inert-serotype analysis. However combining individual specific investigations on each of the circulating serotypes within a single integrated analysis may bring valuable information on the dynamics of dengue. A complex succession of balanced coevolution, unpredictable stochastic events and genetic drift seems to drive dengue evolution. It is therefore important to address evolution and spread of dengue virus through a comprehensive approach associating both the human host dimension and the environmental and vector component. This would be a major step toward a better understanding of the complex dynamic of a disease which remains the most important human arboviral disease worldwide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.06.019.

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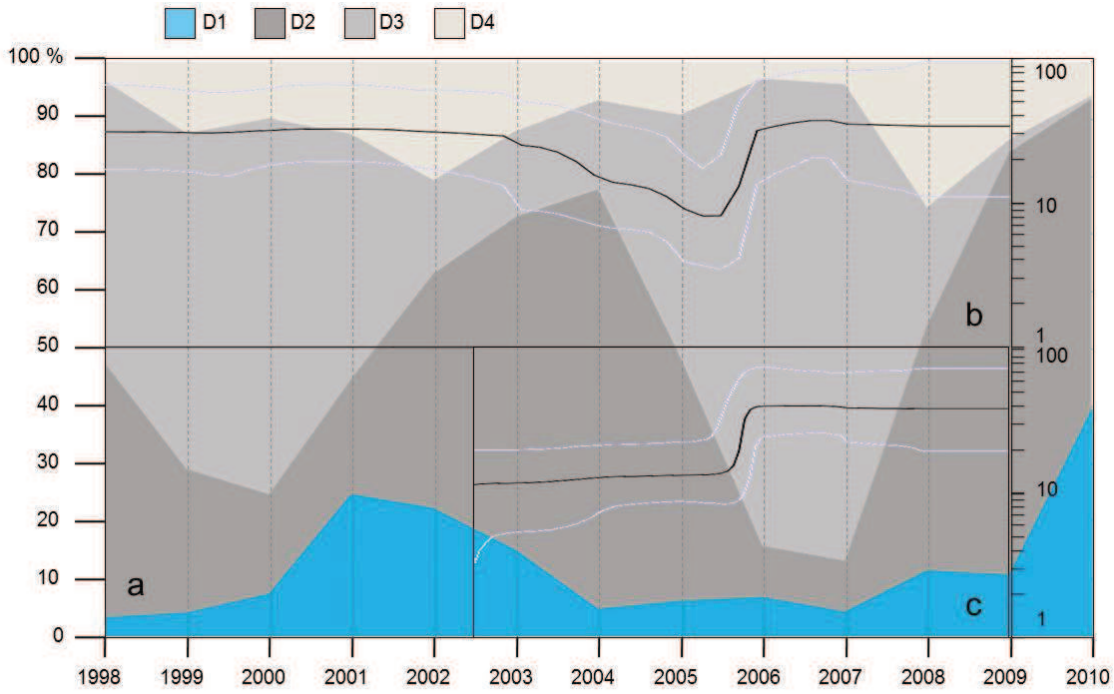
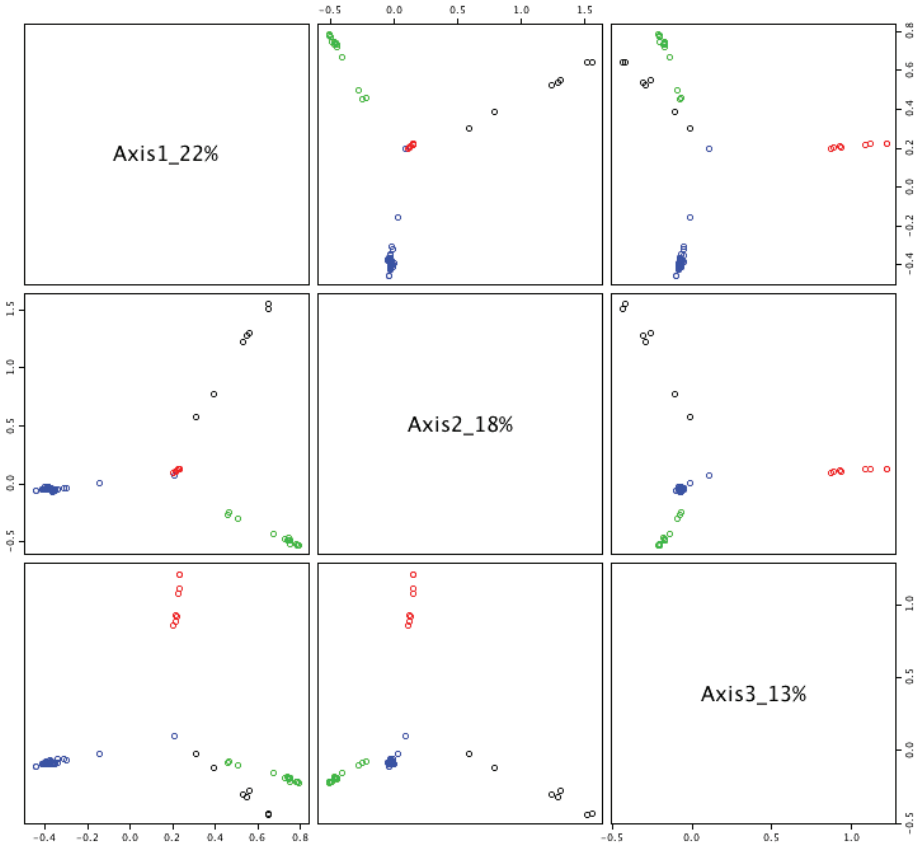
Supplementary Figure 1



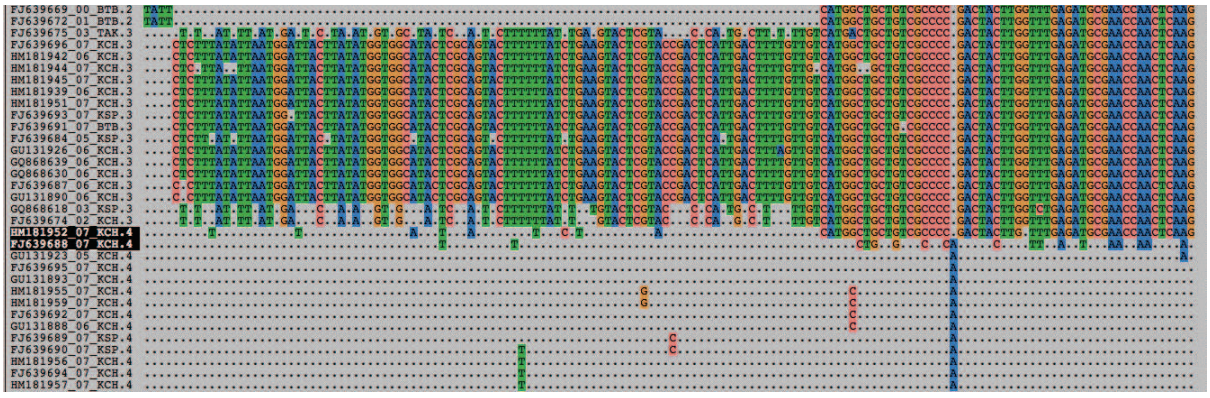
Supplementary Figure 1

Supplementary Figure 2

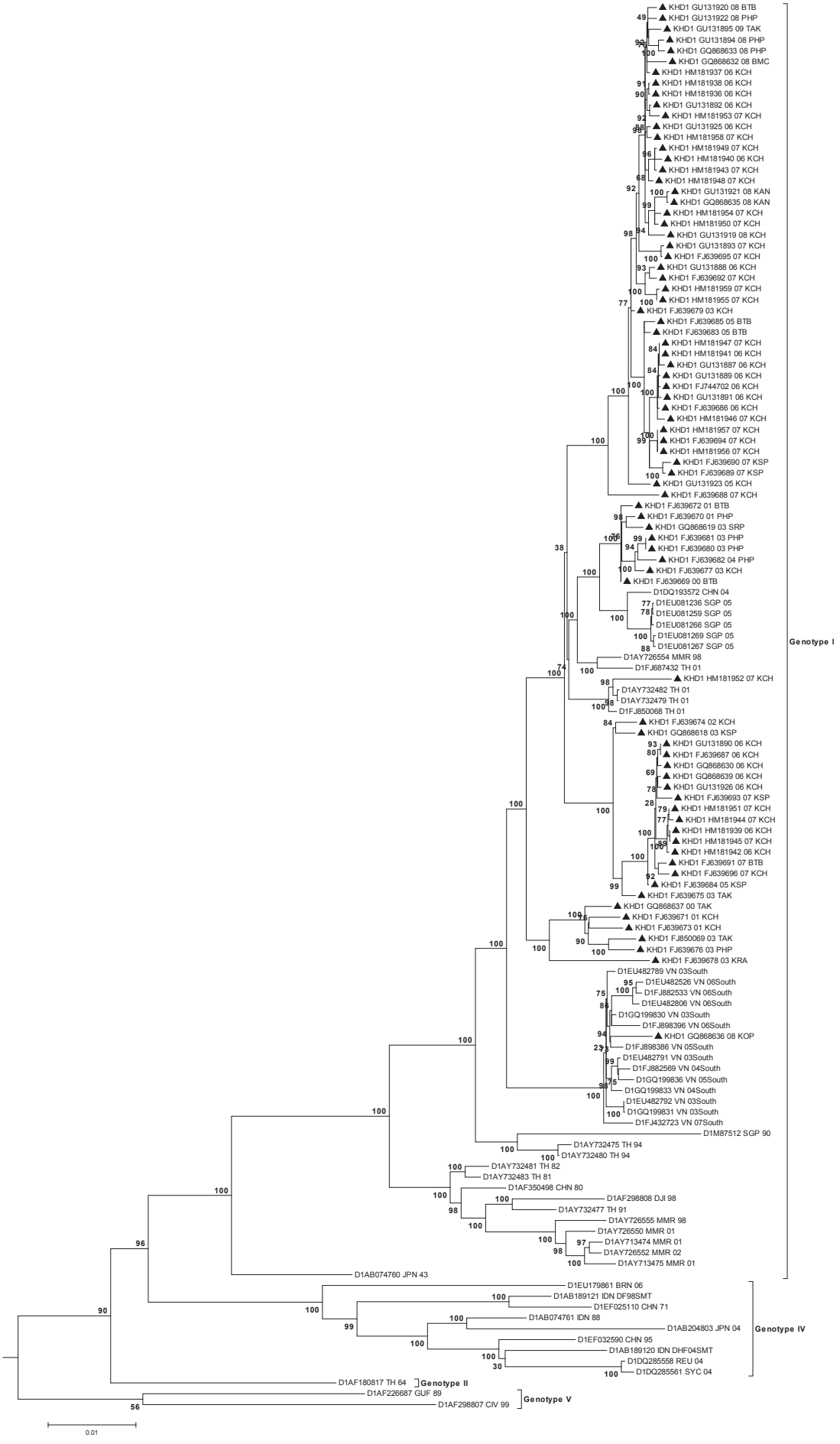


Supplementary Figure 3

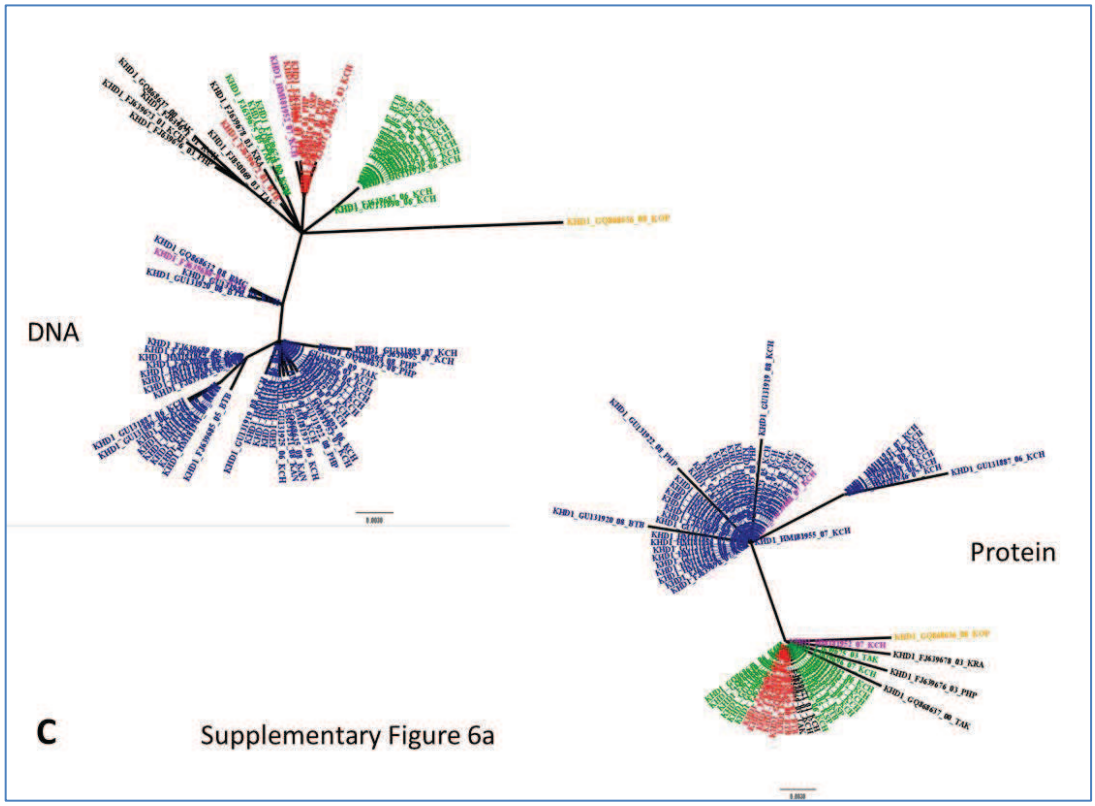
Supplementary Figure 4

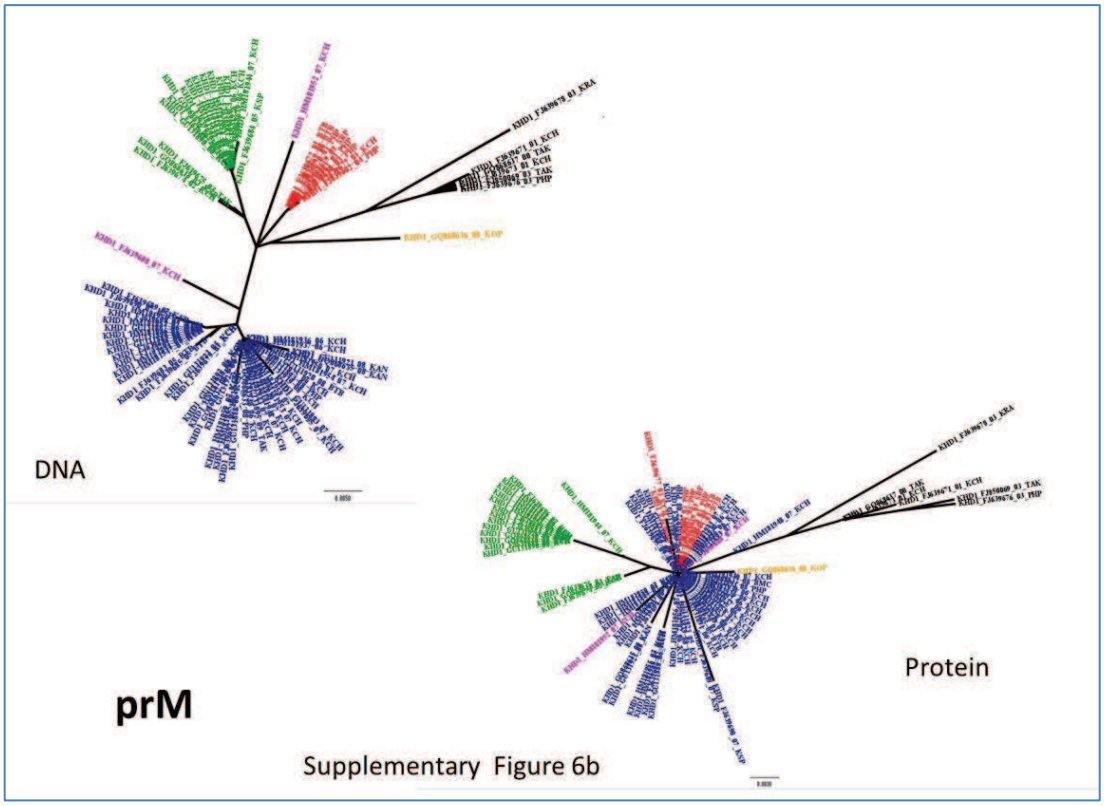


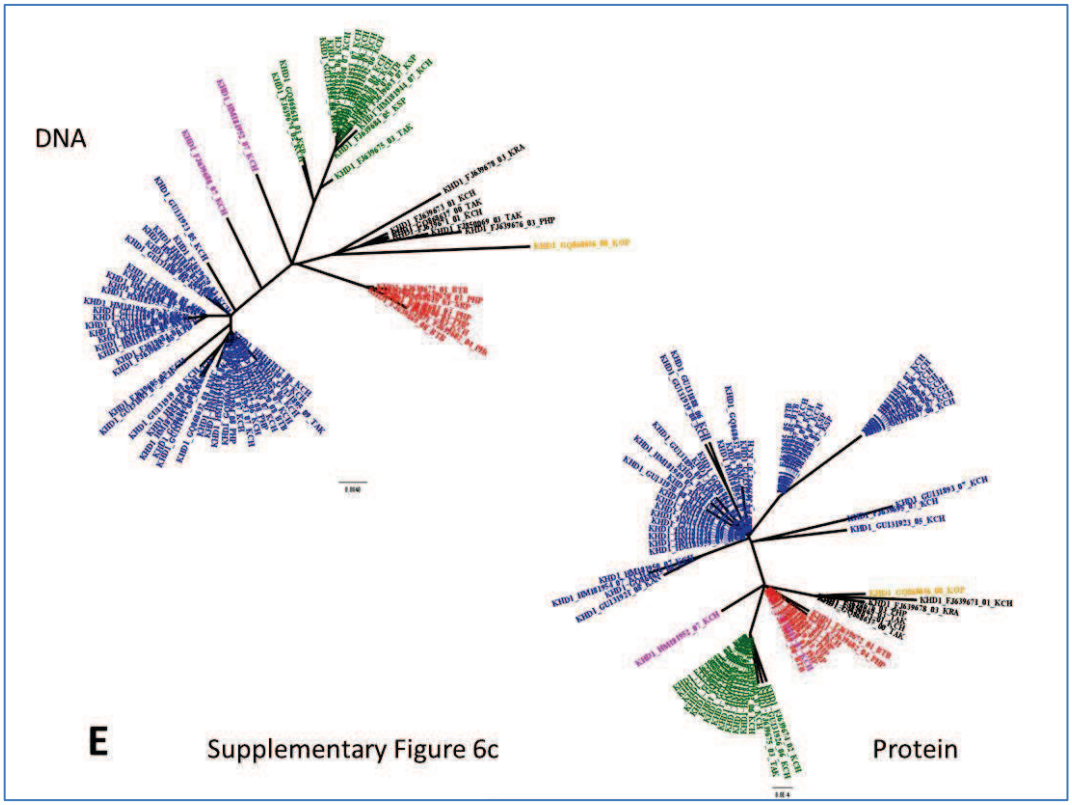
Supplementary Figure 4

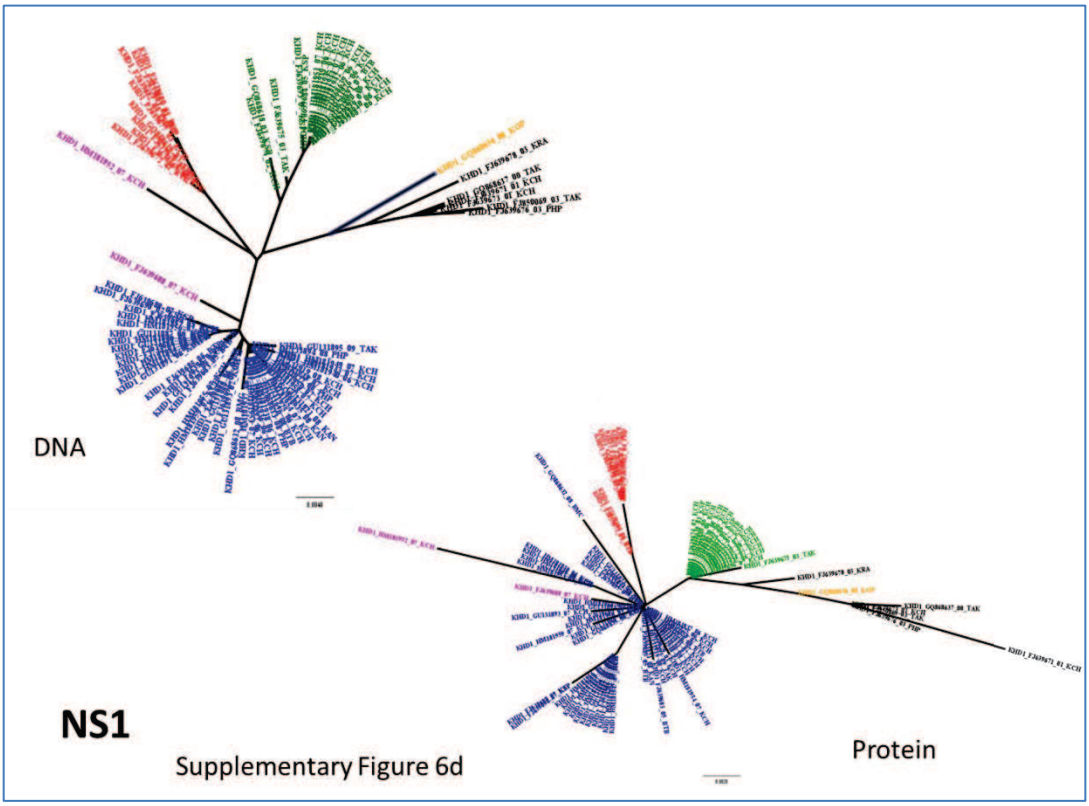


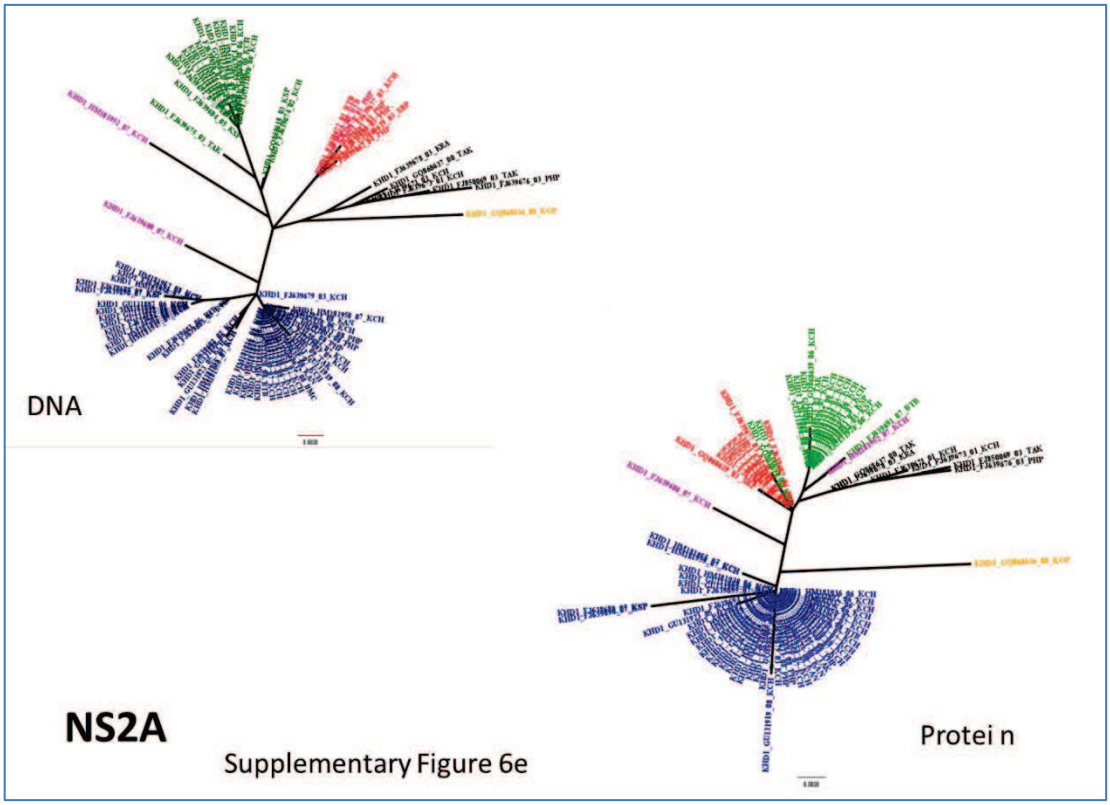
Supplementary Figure 6



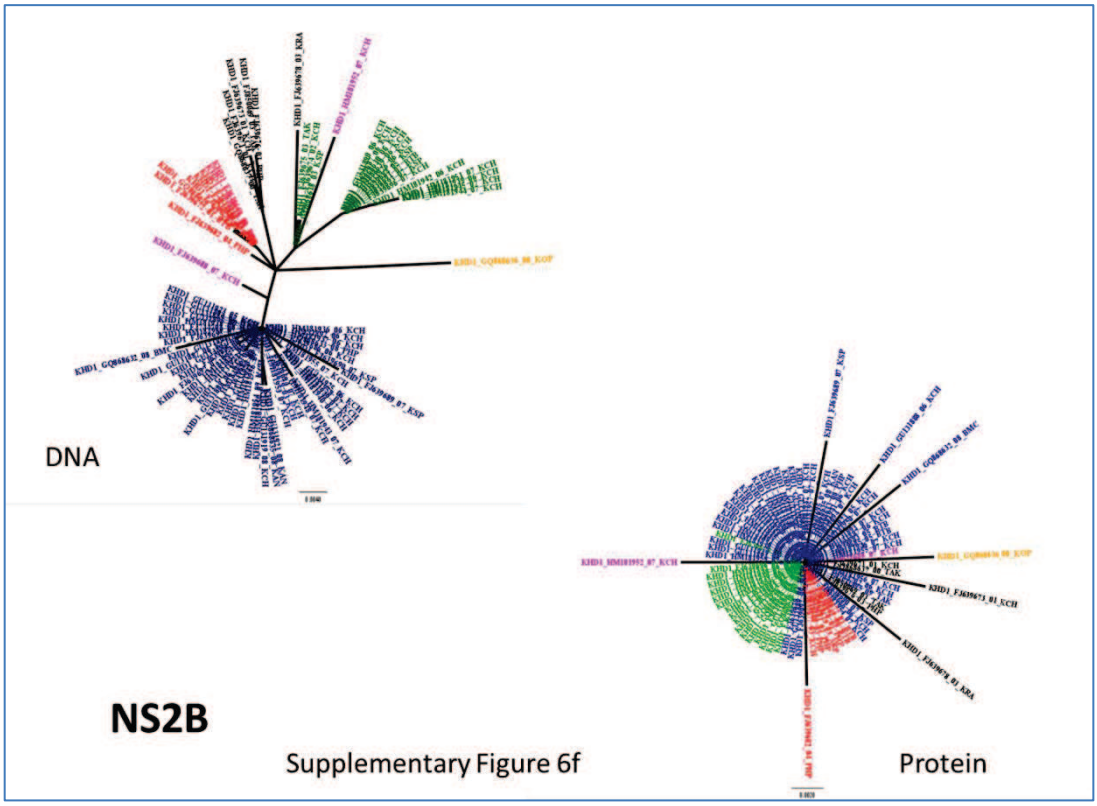



DNA
Protein
E
Supplementary Figure 6c

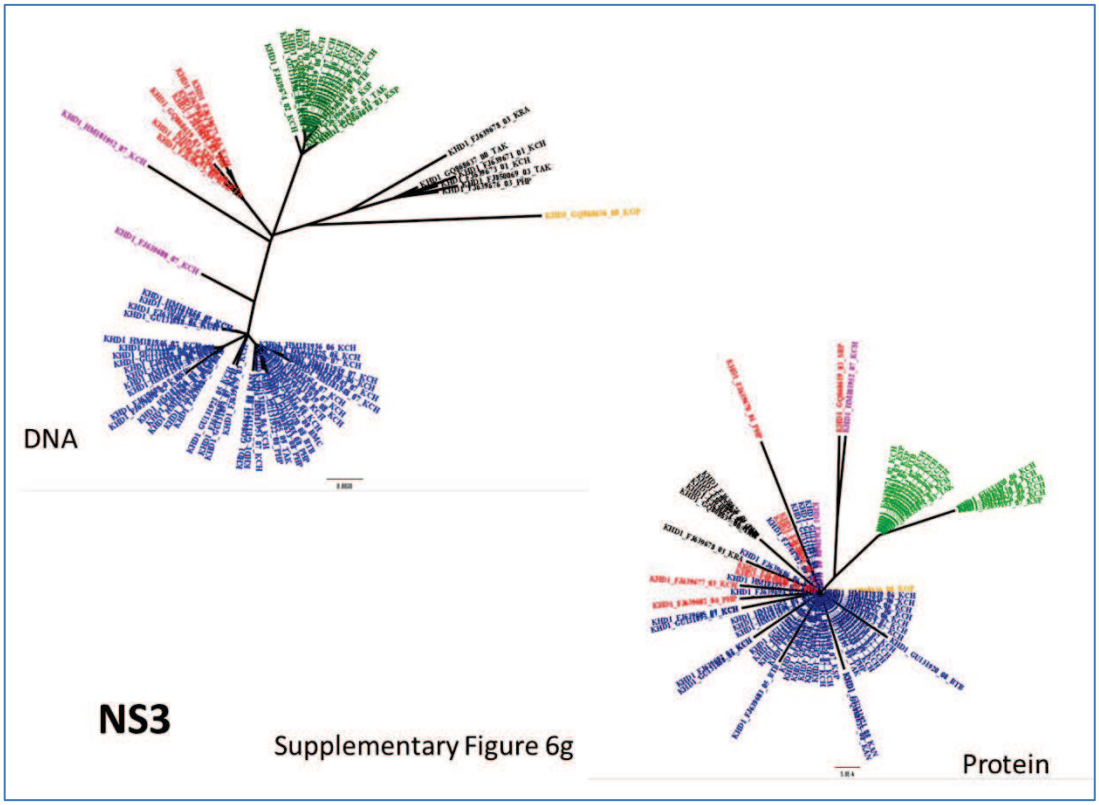

DNA
Protein
NS1
Supplementary Figure 6d



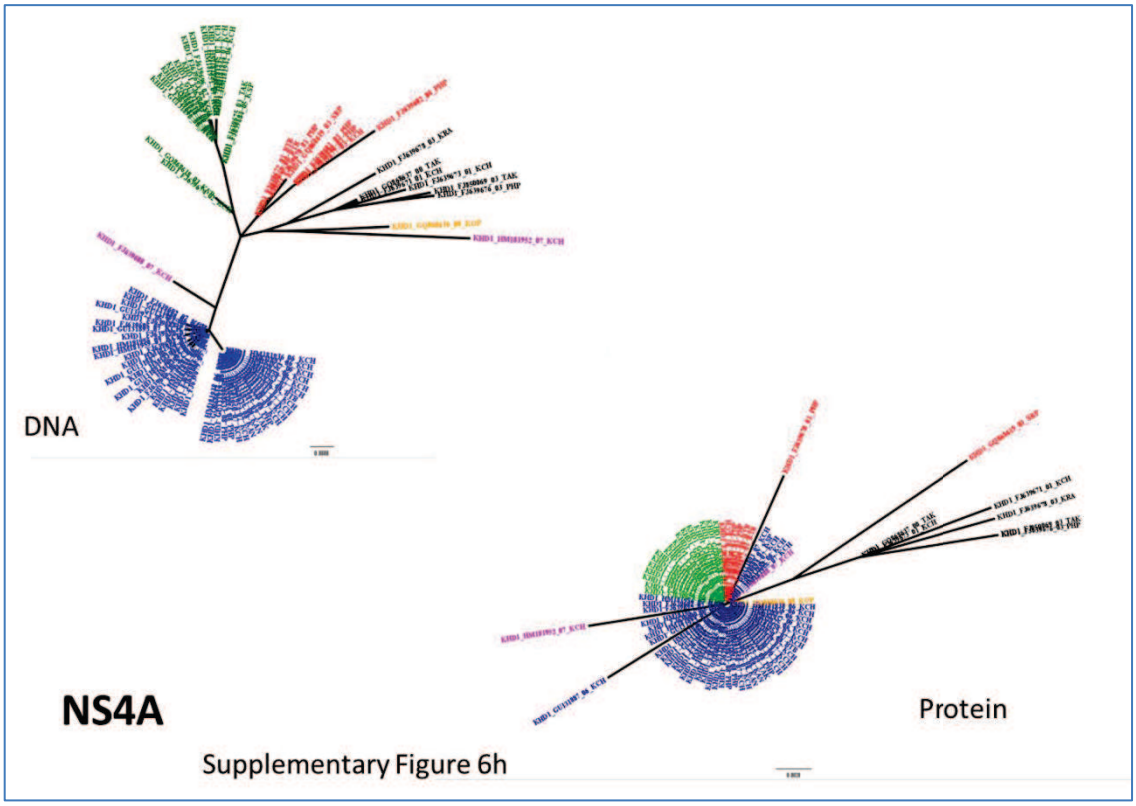
Supplementary Figure 6e



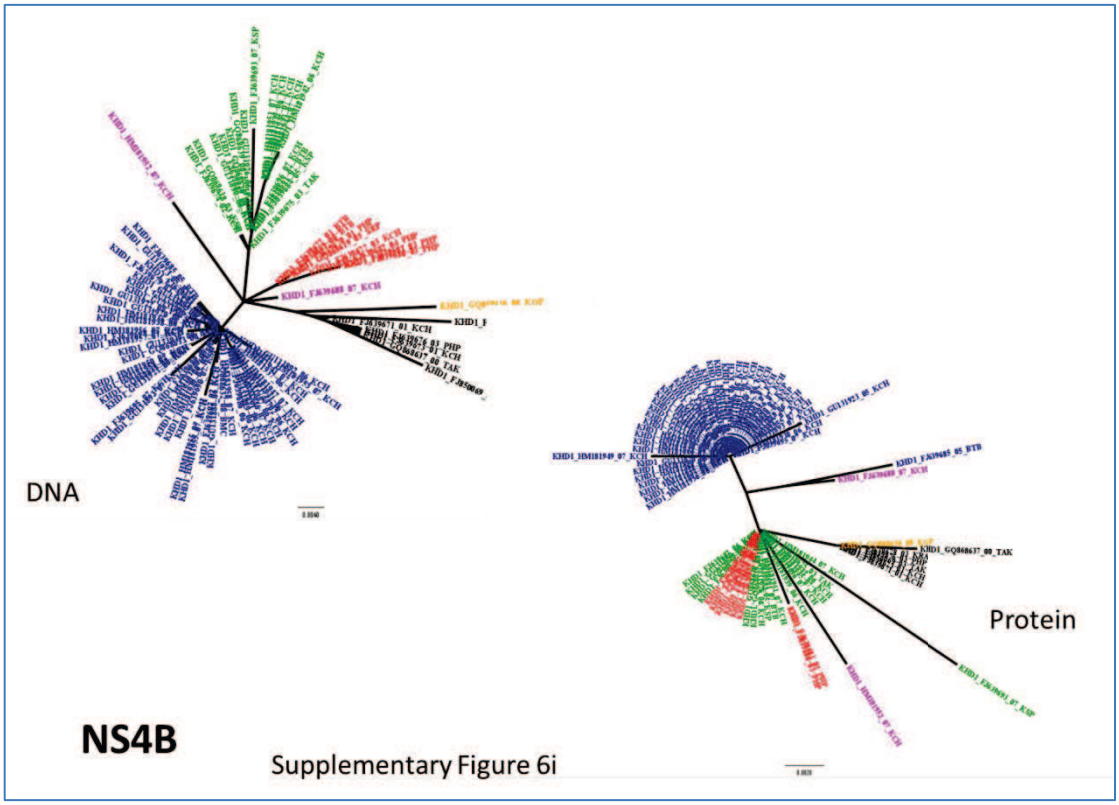
Supplementary Figure 6f



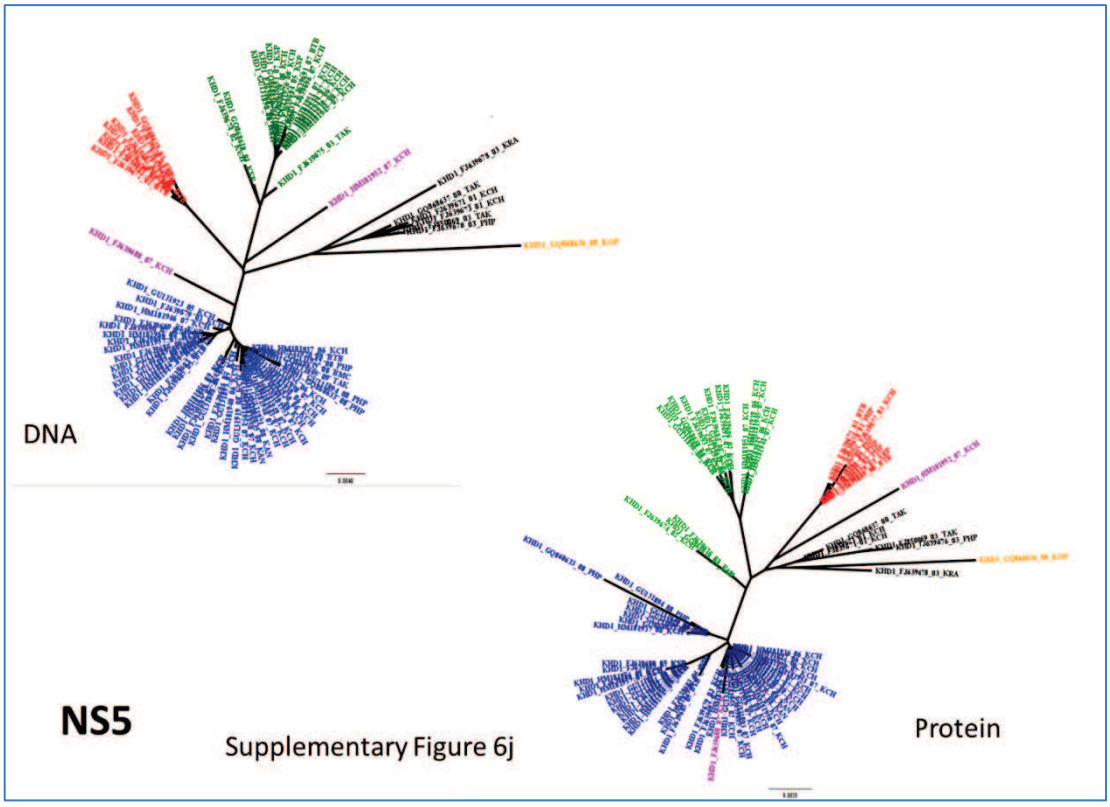
Supplementary Figure 6g



Supplementary Figure 6h



Supplementary Figure 6i



Supplementary Figure 6j

Supplementary table 1. General demographic data, severity of disease, distribution of the 79 samples obtained from patients infected with DENV-1 and the GenBank accession number.

Sequence ID	GenBank accession number	Age (year)	Gender	Province of patient's origin	Year of sample collection	DOF	WHO severity classific-ation
KHD1_GQ868636_08_KOP	GQ868636	24	Male	KOP: KAMPOT	2008	3	DF
KHD1_FJ639671_01_KCH	FJ639671	10	Male	KCH: KAMPONG CHAM	2001	2	DF
KHD1_GQ868637_00_TAK	GQ868637	1	Female	TAK: TAKEO	2000	3	DHF
KHD1_FJ639673_01_KCH	FJ639673	10	Female	KCH: KAMPONG CHAM	2001	2	DHF
KHD1_FJ850069_03_TAK	FJ850069	8	Female	TAK: TAKEO	2003	3	DF
KHD1_FJ639676_03_PHP	FJ639676	25	Female	PHP: PHNOM PENH	2003	3	DHF
KHD1_FJ639678_03_KRA	FJ639678	13	Female	KRA: KRATIE	2003	5	DHF
KHD1_FJ639669_00_BTB	FJ639669	12	Male	BTB: BATTAMBANG	2000	4	DHF
KHD1_FJ639670_01_PHP	FJ639670	24	Female	PHP: PHNOM PENH	2001	4	DF
KHD1_FJ639672_01_BTB	FJ639672	1	Male	BTB: BATTAMBANG	2001	5	DHF
KHD1_FJ639677_03_KCH	FJ639677	1	Female	KCH: KAMPONG CHAM	2003	2	DF
KHD1_FJ639680_03_PHP	FJ639680	11	Female	PHP: PHNOM PENH	2003	1	DF
KHD1_FJ639681_03_PHP	FJ639681	5	Female	PHP: PHNOM PENH	2003	1	DF
KHD1_GQ868619_03_SRP	GQ868619	6	Female	SRP: SIEM REAP	2003	4	DHF
KHD1_FJ639682_04_PHP	FJ639682	27	Male	PHP: PHNOM PENH	2004	2	DHF
KHD1_FJ639674_02_KCH	FJ639674	7	Female	KCH: KAMPONG CHAM	2002	4	DF
KHD1_FJ639675_03_TAK	FJ639675	8	Female	TAK: TAKEO	2003	5	DF
KHD1_GQ868618_03_KSP	GQ868618	5	Male	KSP: KAMPONG SPEU	2003	5	DF
KHD1_FJ639684_05_KSP	FJ639684	11	Male	KSP: KAMPONG SPEU	2005	3	DF
KHD1_HM181939_06_KCH	HM181939	6	Male	KCH: KAMPONG CHAM	2006	3	unknown
KHD1_HM181942_06_KCH	HM181942	12	Male	KCH: KAMPONG CHAM	2006	3	unknown
KHD1_GU131926_06_KCH	GU131926	6	Male	KCH: KAMPONG CHAM	2006	2	DF
KHD1_FJ639687_06_KCH	FJ639687	7	Male	KCH: KAMPONG CHAM	2006	1	DHF
KHD1_GQ868630_06_KCH	GQ868630	8	Male	KCH: KAMPONG CHAM	2006	N/A	AS
KHD1_GQ868639_06_KCH	GQ868639	6	Male	KCH: KAMPONG CHAM	2006	4	DHF
KHD1_GU131890_06_KCH	GU131890	10	Female	KCH: KAMPONG CHAM	2006	4	DF
KHD1_HM181944_07_KCH	HM181944	4	Male	KCH: KAMPONG CHAM	2007	1	unknown
KHD1_HM181945_07_KCH	HM181945	9	Male	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181951_07_KCH	HM181951	4	Female	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_FJ639691_07_BTB	FJ639691	3	Male	BTB: BATTAMBANG	2007	2	DHF
KHD1_FJ639693_07_KSP	FJ639693	3	Female	KSP: KAMPONG SPEU	2007	2	DHF
KHD1_FJ639696_07_KCH	FJ639696	12	Male	KCH: KAMPONG CHAM	2007	1	DF
KHD1_FJ639679_03_KCH	FJ639679	7	Female	KCH: KAMPONG CHAM	2003	2	DF
KHD1_FJ639683_05_BTB	FJ639683	12	Female	BTB: BATTAMBANG	2005	2	DHF
KHD1_FJ639685_05_BTB	FJ639685	6	Male	BTB: BATTAMBANG	2005	3	DHF
KHD1_GU131923_05_KCH	GU131923	6	Female	KCH: KAMPONG CHAM	2005	5	DHF
KHD1_FJ639686_06_KCH	FJ639686	9	Female	KCH: KAMPONG CHAM	2006	5	DHF
KHD1_FJ744702_06_KCH	FJ744702	7	Male	KCH: KAMPONG CHAM	2006	N/A	AS

KHD1_GU131887_06_KCH	GU131887	8	Male	KCH: KAMPONG CHAM	2006	3	DF
KHD1_GU131888_06_KCH	GU131888	5	Male	KCH: KAMPONG CHAM	2006	3	DHF
KHD1_GU131889_06_KCH	GU131889	12	Female	KCH: KAMPONG CHAM	2006	3	UD
KHD1_GU131891_06_KCH	GU131891	3	Female	KCH: KAMPONG CHAM	2006	N/A	AS
KHD1_GU131892_06_KCH	GU131892	6	Female	KCH: KAMPONG CHAM	2006	3	DF
KHD1_GU131925_06_KCH	GU131925	6	Male	KCH: KAMPONG CHAM	2006	3	DF
KHD1_HM181936_06_KCH	HM181936	5	Male	KCH: KAMPONG CHAM	2006	2	DF
KHD1_HM181937_06_KCH	HM181937	1	Female	KCH: KAMPONG CHAM	2006	4	DF
KHD1_HM181938_06_KCH	HM181938	13	Female	KCH: KAMPONG CHAM	2006	4	DF
KHD1_HM181940_06_KCH	HM181940	3	Male	KCH: KAMPONG CHAM	2006	4	DHF
KHD1_HM181941_06_KCH	HM181941	6	Male	KCH: KAMPONG CHAM	2006	3	unknown
KHD1_FJ639688_07_KCH	FJ639688	10	Female	KCH: KAMPONG CHAM	2007	1	DHF
KHD1_FJ639689_07_KSP	FJ639689	3	Male	KSP: KAMPONG SPEU	2007	1	DHF
KHD1_FJ639690_07_KSP	FJ639690	6	Male	KSP: KAMPONG SPEU	2007	2	DHF
KHD1_FJ639692_07_KCH	FJ639692	5	Female	KCH: KAMPONG CHAM	2007	3	DHF
KHD1_FJ639694_07_KCH	FJ639694	9	Female	KCH: KAMPONG CHAM	2007	4	DSS
KHD1_FJ639695_07_KCH	FJ639695	5	Female	KCH: KAMPONG CHAM	2007	2	DF
KHD1_GU131893_07_KCH	GU131893	8	Female	KCH: KAMPONG CHAM	2007	5	DF
KHD1_HM181943_07_KCH	HM181943	2	Female	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181946_07_KCH	HM181946	6	Female	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_HM181947_07_KCH	HM181947	4	Female	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_HM181948_07_KCH	HM181948	5	Female	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181949_07_KCH	HM181949	6	Male	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_HM181950_07_KCH	HM181950	5	Male	KCH: KAMPONG CHAM	2007	4	unknown
KHD1_HM181952_07_KCH	HM181952	5	Male	KCH: KAMPONG CHAM	2007	1	unknown
KHD1_HM181953_07_KCH	HM181953	8	Female	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181954_07_KCH	HM181954	12	Male	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181955_07_KCH	HM181955	6	Female	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_HM181956_07_KCH	HM181956	6	Male	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181957_07_KCH	HM181957	1.7	Female	KCH: KAMPONG CHAM	2007	2	unknown
KHD1_HM181958_07_KCH	HM181958	17	Female	KCH: KAMPONG CHAM	2007	3	unknown
KHD1_HM181959_07_KCH	HM181959	6	Male	KCH: KAMPONG CHAM	2007	1	unknown
KHD1_GQ868632_08_BMC	GQ868632	8	Female	BMC: BANTEAY MEANCHEY	2008	4	DHF
KHD1_GQ868633_08_PHP	GQ868633	9	Male	PHP: PHNOM PENH	2008	5	DHF
KHD1_GQ868635_08_KAN	GQ868635	10	Female	KAN: KANDAL	2008	5	DF
KHD1_GU131894_08_PHP	GU131894	8	Male	PHP: PHNOM PENH	2008	4	DHF
KHD1_GU131919_08_KCH	GU131919	8	Female	KCH: KAMPONG CHAM	2008	4	DHF
KHD1_GU131920_08_BTB	GU131920	12	Female	BTB: BATTAMBANG	2008	3	DHF
KHD1_GU131921_08_KAN	GU131921	4	Female	KAN: KANDAL	2008	5	DHF
KHD1_GU131922_08_PHP	GU131922	15	Male	PHP: PHNOM PENH	2008	6	DHF
KHD1_GU131895_09_TAK	GU131895	7	Male	TAK: TAKEO	2009	3	DHF

DOF: number of days after onset of the fever at the time of blood collection; N/A: not available; DENV1: Dengue virus serotype 1; DF: Dengue fever; DHF: Dengue hemorrhagic fever; DSS: Dengue shock syndrome; AS: Asymptomatic; UD: Unable to determine

Supplementary table 2. Significant non-synonymous mutations

Protein	Position	Consensus amino acid	Mutated amino acid	Lineage
prM	44	Leucine (L)	Phenylalanine (F)	3
prM	56	Valine (V)	Alanine (A)	1
prM	57	Serine (S)	Threonine (T)	3
NS1	86	Phenylalanine (F)	Leucine (L)	2
NS1	111	Tyrosine (Y)	Histidine (H)	1
NS1	324	Argnine (R)	Lysine (K)	3
NS3	119	Proline (P)	Serine (S)	3
NS4	93	Valine (V)	Alanine (A)	1

Supplementary table 3. Polymorphism analysis and neutrality tests for the different DENV-1 lineages (Lin.)

Gene C

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	34	37	37	20	17	7.489	4	15	666	-1,61185^{NS}	-3,26998**	-3,14891**	-24,090	1.000
Lin.1	7	7	16	16	13	3	6.531	0	0	120	-1,09826^{NS}	-1,13318^{NS}	-1,23869^{NS}	-2,898	1.000
Lin.2	8	4	4	4	3	1	1.543	0	0	6	-1,02972^{NS}	-0,92081^{NS}	-1,03990^{NS}	-0,903	0.916
Lin.3	17	4	3	3	1	2	0.887	0	0	3	-0,76702^{NS}	-0,06265^{NS}	-0,28740^{NS}	-1,153	0.923
Lin.4	47	19	19	19	11	8	4.302	2	2	171	-1,80192*	-2,49572*	-2,67565*	-14,411	1.000

Gene E

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	61	189	198	97	92	38.257	23	452	16290	-1,63782^{NS}	-3,92454**	-3,57109**	-25.596	1.000
Lin.1	7	7	62	67	51	11	25.306	2	5	1653	-1,34870^{NS}	-1,27234^{NS}	-1,42825^{NS}	-0,395	1.000
Lin.2	8	6	10	10	7	3	3.857	0	0	45	-0,68712^{NS}	-0,88379^{NS}	-0,92853^{NS}	-1.373	0.951
Lin.3	17	12	32	32	20	12	9.465	1	2	496	-1,42063^{NS}	-1,62105^{NS}	-1,80965^{NS}	-2.798	0.983
Lin.4	47	36	94	95	58	36	21.283	9	56	4278	-2,14234*	-3,50557**	-3,58668**	-21.688	1.000

Gene prM

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	37	76	81	37	39	15.384	10	86	2556	-1,54884^{NS}	-3,18396*	-3,02801**	-11,392	1.000
Lin.1	7	7	38	38	33	5	15.510	0	0	703	-1,37074^{NS}	-1,37096^{NS}	-1,51597^{NS}	-1.273	1.000
Lin.2	8	3	2	2	2	0	0.771	0	0	1	-1,31009^{NS}	-1,40980^{NS}	-1,51361^{NS}	-0,999	0.938
Lin.3	17	7	11	11	8	3	3.254	0	0	57	-1,49685^{NS}	-1,88998^{NS}	-2,05258^{NS}	-1.658	0.941
Lin.4	47	20	34	36	15	14	7.698	3	7	496	-1,95413*	-3,07596*	-3,18582*	-8.851	1.000

Gene NS1

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	51	142	145	66	76	28.743	15	332	9591	-1,44829^{NS}	-3,07372*	-2,88532*	-15,811	1.000
Lin.1	7	7	52	52	43	9	21.224	2	8	1326	-1,26844^{NS}	-1,23515^{NS}	-1,37548^{NS}	-0737	1.000
Lin.2	8	7	13	13	6	7	5.014	0	0	78	0,02216^{NS}	-0,07256^{NS}	-0,05602^{NS}	-1.892	0.980
Lin.3	17	10	17	17	8	9	5.29	3	10	136	-1,11707^{NS}	-0,76426^{NS}	-0,99798^{NS}	-2.726	0.981
Lin.4	47	27	65	65	45	20	14.717	4	12	2080	-2,17503*	-4,01661**	-3,99158**	-12,881	1.000

Gene NS2A

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	40	97	99	51	46	19.634	11	119	4465	-1,50949^{NS}	-3,60836**	-3,30507**	-10,872	1.000
Lin.1	7	7	37	37	31	6	15.102	0	0	666	-1,25163^{NS}	-1,26312^{NS}	-1,39378^{NS}	-1,273	1.000
Lin.2	8	5	7	7	6	1	2.700	0	0	21	-1,35929^{NS}	-1,36041^{NS}	-1,50298^{NS}	-1,232	0.939
Lin.3	17	11	18	18	12	6	5.324	0	0	153	-1,59260^{NS}	-1,73554^{NS}	-1,95839^{NS}	-4,556	0.998
Lin.4	47	17	41	42	22	19	9.283	3	7	780	-1,74088^{NS}	-2,61936**	-2,74471**	-3,192	0.982

Gene NS2B

	N	Hp	S	η	Si	Pa	θ	Rm	4TG	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	32	47	47	28	19	9.514	5	15	1081	-1,91083*	-3,98219**	-3,79862**	-19,494	1.000
Lin.1	7	7	20	20	18	2	8.163	0	0	190	-1,42234^{NS}	-1,44578^{NS}	-1,58801^{NS}	-2.550	1.000
Lin.2	8	4	4	4	4	0	1.543	0	0	6	-1,53470^{NS}	-1,66525^{NS}	-1,79736^{NS}	-1,236	0.943
Lin.3	17	6	6	6	2	4	1.775	0	0	15	-0,18576^{NS}	-0,07668^{NS}	-0,12249^{NS}	-1,053	0.893
Lin.4	47	15	22	22	16	6	4.981	1	1	231	-2,33946**	-3,67616**	-3,81289**	-10,630	1.000

Gene NS3

	N	Hp	S	η	Si	Pa	θ	Rm	4GT	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	60	222	225	120	102	44.936	30	802	23871	-1.56663^{NS}	-4.03327**	-3.60879**	-19.542	1
Lin.1	7	7	90	90	77	13	36.735	5	33	4005	-1.32664^{NS}	-1.34771^{NS}	-1.48830^{NS}	0.008	1
Lin.2	8	7	22	22	17	5	8.485	0	0	231	-0,95956^{NS}	-1,20169^{NS}	-1,27429^{NS}	-1,185	0.954
Lin.3	17	11	18	18	13	5	3.368	0	0	153	-1,44098^{NS}	-2,00916^{NS}	-2,13546^{NS}	-4,246	0.997
Lin.4	47	35	107	107	61	46	9,865	6	62	5671	-2,12809*	-3,07182*	-3,25268*	-17,266	1

Gene NS4A

	N	Hp	S	η	Si	Pa	θ	Rm	4GT	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	29	49	53	24	25	9.918	7	42	990	-1.47481^{NS}	-3.36138**	-3.13625**	-8.708	1
Lin.1	7	7	22	22	17	5	8.980	1	2	231	-1.09359^{NS}	-1.01624^{NS}	-1.14008^{NS}	-2.182	1
Lin.2	8	5	9	9	7	2	3.471	0	0	36	-0.81600^{NS}	-1.13595^{NS}	-1.17530^{NS}	-0.361	0.845
Lin.3	17	7	7	7	3	4	2.071	0	0	21	-0.75571NS	-0.47731NS	-0.63731NS	-2.237	0.970
Lin.4	47	10	22	22	18	4	4.981	0	0	231	-2,25963**	-4,35003**	-4,30372**	-3,306	0.988

Gene NS4B

	N	Hp	S	η	Si	Pa	θ	Rm	4GT	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	43	88	92	47	41	17.813	5	36	5386	-2.13148*	-3.90645**	-3.82289**	-25.367	1.000
Lin.1	7	7	29	29	26	3	11.837	1	2	406	-1.38872^{NS}	-1.45683^{NS}	-1.59231^{NS}	-1.764	1.000
Lin.2	8	5	5	5	0	5	1.928	0	0	10	1.42820^{NS}	1.36768^{NS}	1.52219^{NS}	-0.640	0.882
Lin.3	17	9	15	16	10	5	4.437	0	0	91	-1.38325^{NS}	-1.81023^{NS}	-1.95135^{NS}	-2.254	0.968
Lin.4	47	22	37	39	18	19	8.377	2	4	595	-2.28578**	-2.30706^{NS}	-2.73671*	-13.873	1.000

Gene NS5

	N	Hp	S	η	Si	Pa	θ	Rm	4GT	PW	Tajima's D	D*	F*	Fu's FS	Strobeck
All	79	67	356	368	177	179	72.060	57	2349	58996	-1.54752^{NS}	-3.66490**	-3.33310**	-18.361	1.000
Lin.1	7	7	141	141	115	26	57.551	8	104	9870	-1.22421^{NS}	-1.21107^{NS}	-1.34621^{NS}	0.591	1.000
Lin.2	8	7	27	27	19	8	10.413	0	0	351	-0.91565^{NS}	-0.96132^{NS}	-1.05870^{NS}	-0.746	0.924
Lin.3	17	13	58	60	34	24	17.156	5	17	1540	-1.35840^{NS}	-1.54841^{NS}	-1.73059^{NS}	-1,430	0.925
Lin.4	47	40	152	152	96	56	34.415	14	210	11476	-2,03971*	-3,70043**	-3,68472**	-19,336	1.000

Supplementary table 4. Ka/Ks ratio and nucleotide substitution

Gene	Population	Na	Ns	Ka	Ks	Ka/Ks
C	1	4	12	0,00439	0,05242	0.084
	2	0	4	0,00000	0,01470	0.000
	3	0	3	0,00000	0,01470	0.000
	4	6	13	0,00192	0,01720	0.112
E	1	4	60	0,00123	0,05660	0.022
	2	2	8	0,00044	0,00789	0.056
	3	3	29	0,00031	0,01637	0.019
	4	18	74	0,00180	0,01862	0.097
prM	1	11	27	0,00936	0,07295	0.128
	2	1	1	0,00112	0,01290	0.087
	3	2	9	0,00066	0,00211	0.313
	4	5	28	0,00120	0,02594	0.046
NS1	1	7	45	0,00266	0,06469	0.041
	2	1	12	0,00052	0,02004	0.026
	3	1	16	0,00014	0,01504	0.010
	4	13	52	0,00135	0,01969	0.069
NS2A	1	11	26	0,00734	0,05352	0.137
	2	2	5	0,00101	0,00903	0.112
	3	3	15	0,00092	0,01733	0,053
	4	11	31	0,00144	0,02533	0.057
NS2B	1	4	16	0,00389	0,05396	0.072
	2	1	3	0,00085	0,00789	0.108
	3	0	6	0,00000	0,01776	0.000
	4	4	18	0,00058	0,01303	0.044
NS3	1	2	88	0,00054	0,06597	0,008
	2	5	17	0,00088	0,01310	0.067
	3	1	17	0,00034	0,00664	0.051
	4	7	100	0,00030	0,02192	0.013
NS4A	1	4	18	0,00465	0,06609	0.070
	2	3	6	0,00262	0,02299	0.114
	3	0	7	0,00000	0,01726	0,000
	4	2	20	0,00030	0,01543	0.019
NS4B	1	1	28	0,00052	0,04837	0.011
	2	1	4	0,00078	0,01149	0,068
	3	3	12	0,00107	0,01340	0.080
	4	7	32	0,00069	0,01393	0.050
NS5	1	20	121	0,00324	0,06714	0.048
	2	4	23	0,00048	0,01273	0,038
	3	13	47	0,00155	0,01470	0.105
	4	27	122	0,00112	0,02133	0,052

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Abstract: The Dengue National Control Program was established in Cambodia in 2000 and has reported between 10 000 to 40 000 dengue cases per year with a case fatality rate ranging from 0.7 to 1.7. In this study 39 DENV-2 and 57 DENV-3 viruses isolated from patients between 2000 and 2009 were fully sequenced. Five DENV2 and four DENV3 distinct lineages with different dynamics were identified. Each lineage was characterized by the presence of specific mutations with no evidence of recombination. In both DENV-2 and DENV-3 the lineages present prior to 2003 were replaced after that date by unrelated lineages. After 2003, DENV-2 lineages D2-3 and D2-4 cocirculated until 2007 when they were almost completely replaced by a lineage D2-5 which emerged from D2-3. Conversely, all DENV-3 lineages remained, diversified and cocirculated with novel lineages emerging. Years 2006 and 2007 were marked by a high prevalence of DENV-3 and 2007 with a large dengue outbreak and a high proportion of patients with severe disease. Selective sweeps in DENV-1 and DENV-2 were linked to immunological escape to a predominately DENV-3-driven immunological response. The complex dynamic of dengue in Cambodia in the last ten years has been associated with a combination of stochastic climatic events, cocirculation, coevolution, adaptation to different vector populations, and with the human population immunological landscape.

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INSTITUT PASTEUR
DU CAMBODGE

Phnom Penh, 4 March 2012.

Dear Editor-in-Chief,

We would like to submit a manuscript entitled "Complex dynamic of dengue virus serotypes 2 and 3 in Cambodia following series of climate disasters"

Dengue is the most rapidly spreading mosquito-borne viral disease with a 30-fold rise in the number of human cases reported in the last 50 years associated with a geographic expansion of the disease to new countries. In Cambodia, all the four DENV serotypes co-circulate each year although the predominant serotype has alternated mainly between DENV-2 and DENV-3 during the last decade. Therefore, separate in depth analyses of the lineage structure and dynamics of each serotype were undertaken over the period 2000-2009. We report in this study the first of this series of analyses: the characterization of the genetic diversity and lineage dynamic of the complete genome of DENV-2 and DENV-3 viruses isolated in Cambodia between 2000 and 2009. This manuscript shows that the complex dynamic of dengue in Cambodia in the last ten years has been associated with a combination of stochastic climatic events, cocirculation, coevolution, adaptation to different vector populations, and the human population immunological landscape. We believe that identifying and understanding these factors is important as it can potentially help to predict the risk of a major dengue epidemic in the transmission season following climatic disasters like, for instance, the flood that occurred in Thailand, Vietnam and Cambodia during the 2011 rainy season. This may thus help public health authorities to mitigate the epidemics and to save lives.

Yours sincerely,

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Highlights

► We reviewed the evolution dynamic of DENV-2 and DENV-3 during a 10 years period.

► We identified five DENV-2 and four DENV-3 distinct lineages with different evolutionary dynamics.

► The dynamic of dengue in Cambodia was associated with a combination of events.

► Stochastic events, coevolution, vector adaptation and human immune background could explain the pattern.

Complex dynamic of dengue virus serotypes 2 and 3 in Cambodia following series of climate disasters

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Running title: Comparative dynamic of dengue virus

Abstract

The Dengue National Control Program was established in Cambodia in 2000 and has reported between 10 000 to 40 000 dengue cases per year with a case fatality rate ranging from 0.7 to 1.7. In this study 39 DENV-2 and 57 DENV-3 viruses isolated from patients between 2000 and 2009 were fully sequenced. Five DENV2 and four DENV3 distinct lineages with different dynamics were identified. Each lineage was characterized by the presence of specific mutations with no evidence of recombination. In both DENV-2 and DENV-3 the lineages present prior to 2003 were replaced after that date by unrelated lineages. After 2003, DENV-2 lineages D2-3 and D2-4 cocirculated until 2007 when they were almost completely replaced by a lineage D2-5 which emerged from D2-3. Conversely, all DENV-3 lineages remained, diversified and cocirculated with novel lineages emerging. Years 2006 and 2007 were marked by a high prevalence of DENV-3 and 2007 with a large dengue outbreak and a high proportion of patients with severe disease. Selective sweeps in DENV-1 and DENV-2 were linked to immunological escape to a predominately DENV-3-driven immunological response. The complex dynamic of dengue in Cambodia in the last ten years has been associated with a combination of stochastic climatic events, cocirculation, coevolution, adaptation to different vector populations, and with the human population immunological landscape.

Keywords: Dengue virus; DENV-2; DENV-3; Diversity; Lineage dynamics; Genome sequence; Cambodia; Southeast Asia

Introduction

Dengue is the most rapidly spreading mosquito-borne viral disease with a 30-fold rise in the number of human cases reported in the last 50 years associated with a geographic expansion of the disease to new countries (WHO/TDR, 2009). Globally, about two-thirds of the world's population lives in area at risk for infection (more than 75% in the WHO South-East Asia and Western Pacific regions) and an estimated 50-100 million cases of dengue infection occur every year (WHO/TDR, 2009). Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. While most patients recover following a self-limiting non-severe clinical course, a small proportion progress to severe disease, mostly characterized by plasma leakage with or without hemorrhage (Duong et al., 2009; WHO/TDR, 2009).

Dengue virus (DENV) which comprises four genetically and antigenically distinct serotypes (DENV-1, -2, -3, and -4) belongs to the family *Flaviviridae*, genus *Flavivirus* (Calisher et al., 1989). DENV genome is a single stranded positive-sense RNA virus and has about 11 kb in length encoding a single open reading frame (Lindenbach and Rice, 2003). The translated protein is cleaved by host- and virus-derived proteases to produce structural proteins (capsid, premembrane/membrane, envelope; C, prM/M, E) and non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2003; Weaver and Vasilakis, 2009). The single open reading frame (ORF) is flanked by 5' untranslated region (UTR) capped with type I 7-methyl guanosine structure and by 3' UTR lacked of poly(A) region (Lindenbach and Rice, 2003). Nucleic acid sequencing has allowed the classification of each of the DENV serotype into genotypes (Rico-Hesse, 1990). Rico-Hesse defined these genotypes as clusters of DENV viruses having nucleotide sequence divergence not greater than 6% within a given genome region (in this case the E/NS1 junction). Various phylogenetic analyses based on partial E/NS1 or complete E nucleotide sequences indicated that (1) DENV-1 are organized into five genotypes (I to V), (2)

DENV-2 comprises 6 genotypes: South-East Asian/American, Asian I, Asian II, Cosmopolitan, American and sylvatic, (3) DENV-3 comprises of 4 genotypes (I to IV) and (4) DENV-4 are classed into 4 genotypes (I, II, III and sylvatic) (Holmes and Twiddy, 2003; Rico-Hesse, 2003; Vasilakis and Weaver, 2008; Weaver and Vasilakis, 2009).

Previous studies have shown that dengue genotypes are not fixed entities (Holmes et al., 1999; Tolou et al., 2001; Worobey et al., 1999) and evidence of selection pressure showing lineage turnover (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002; Zhang et al., 2005). Although, the mechanism of lineage replacement's occurrence is unclear, two main hypothesis have been proposed (Zhang et al., 2005): 1) a ladder-like phylogenetic trees showing a strong temporal topology. This event may be introduced by the elimination of deleterious mutation strains by purifying selection (Holmes, 2003) and/or a regular random population bottleneck perhaps due to decline in mosquito population and density during the annual dry season (Scott et al., 2000); 2) or a more dramatic change by entire clade replacement by a new clade of viruses. The virus evolutionary process remains unclear but the patterns observed may reflect the action of either dramatic population bottlenecks or natural selection such as clades with an improved fitness which out-compete previously circulating clades or lineages of viruses with mutations that allow them to evade cross-protective herd immunity (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002). In Cambodia, all the four DENV serotypes co-circulate each year although the predominant serotype has alternated mainly between DENV-2 and DENV-3 during the last decade. Therefore, separate in depth analyses of the lineage structure and dynamics of each serotype were undertaken over the period 2000-2009. We report in this study the first of this series of analyses: the characterization of the genetic diversity and lineage dynamic of the complete genome of DENV-2 and DENV-3 viruses isolated in Cambodia between 2000 and 2009.

MATERIAL AND METHODS

Virus strains. Dengue virus strains were isolated by the national dengue laboratory at Institut Pasteur in Cambodia established to support the 5 sentinel sites from the National Dengue Control Program (NDCP), Ministry of Health Cambodia, and by a dengue cohort study in Kampong Cham province (Vong et al., 2010). This study received approval from the National Ethics Committee for Health Research in Cambodia. Written informed consent was given by all patients (or their parents/guardians) included in the cohort study. Samples collection methods and diagnostic testing including serological, molecular and virological tests were previously described (Buchy et al., 2005; Huy et al., 2010). All strains were isolated in C6/36 mosquito cells (*Aedes albopictus* clone) and cultures were not passaged more than two times. After anonymization, the strains included in the study were selected to be representative of geographical origin, year of sampling and severity of the disease according to former WHO criteria (WHO, 1997).

DENV genome sequencing. Viral genomes were sequenced using the Broad Institute's capillary sequencing (Applied Biosystems) directed amplification viral sequencing pipeline (http://www.broadinstitute.org/annotation/viral/Dengue; (Vu et al., 2010). Briefly, viral RNA was extracted from low passage cell culture supernatants using the QIAmp viral RNA mini kit (Qiagen). The genome was reverse-transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen, California, USA), random hexamers (Roche Diagnostics GmbH, Mannheim, Germany) and a specific oligonucleotide targeting the 3` end of the target genome sequences (5` – AGA ACC TGT TGA TTC AAC AGC AC – 3`; nt 10700 – 10722). The cDNA obtained was then amplified using a high fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and a pool of specific primers to produce 14 overlapping amplicons of 1.5 to 2 kb in size for a physical coverage of 2-fold. Amplicons were then sequenced in the forward and reverse direction using

primer panels consisting of 96 specific primer pairs, tailed with M13 forward and reverse primer sequence, that produce 500–700 bp amplicons from the target viral genome. Amplicons were then sequenced in the forward and reverse direction using M13 primer. Total sequence coverage delivered post amplification and sequencing was about 8-fold. Resulting sequence reads were assembled *de novo* and annotated using the Broad Institute's AV454 algorithm (Henn, 2012) and an in-house annotation algorithm.

Sequence alignment and phylogenetic analysis. Multiple sequence alignment was conducted with Muscle (Edgar, 2004) available in Seaview version 4.2.5 package (Galtier et al., 1996; Gouy et al., 2010). Phylogenetic analyses were performed using maximum likelihood (ML) method for the complete coding region and for each gene separately for both the 39 DENV-2 and 57 DENV-3 isolates. Jmodeltest (Posada, 2008) was used to select the optimal evolution model by evaluating the selected parameters using the Akaike Information Criterion (AIC). A corrected version of the AIC (AICc) was used because the sample size (n) was small compared with the number of parameters (n/K<40). This approach suggested the following models: GTR+G4 for complete coding region, HKY+I+G for C; GTR+G for E, NS1 and NS2A; GTR+I+G for NS2B, NS3, NS4A, NS4B, NS5 and concatenated data set; and K80+I for PrM. Under the selected models, the parameters were optimized and ML analyses were performed with PhyML (version 2.4.4) (Guindon and Gascuel, 2003). The robustness of nodes was assessed with 1000 booststrap replicates for complete coding region, 100 bootstrap replicates for each gene and 500 bootstrap replicates for concatenated data set. Finally, trees were edited using FigTree v1.3.1 (BEAST software).

Genetic similarity and DNA polymorphism analysis. Genetic similarity and difference matrices were constructed from ClustalX2 alignments (Thompson et al., 1997) using BioEdit 7.0.9.0 (Hall, 1999). Phylogenetic analyses do not provide a comprehensive view of the relative dynamics and evolutionary patterns of the sequences analyzed. To assess the presence of specific selective

pressure and evolutionary patterns a DNA polymorphism analysis was conducted on each separate gene for the various lineages identified. DNA sequence polymorphism and all subsequent tests were investigated using several functions from the DnaSP5.00.02 package (Librado and Rozas, 2009). Haplotypes (alleles) were calculated according to Nei (Nei, 1987). Polymorphism was assessed using a the following parameters: nucleotide diversity, Pi (π), the average number of nucleotide differences per site between two sequences was calculated according to Nei (Nei, 1987), using the Jukes and Cantor (Jukes and Cantor, 1969) correction. Eta (η) is the total number of mutations, and S is the number of segregating (polymorphic) sites. Si is the number of singletons (one unique mutation at a given site) and Pa is the number parsimony informative sites (several mutations at a given site). Theta (θ) (Watterson's mutation parameter) was calculated for the whole sequence from η (Watterson, 1975). Na is the number of non-synonymous substitutions and Ns is the number of synonymous substitutions. Ka/Ks ratio was used to assess the presence of selective pressure. Ka (the number of non-synonymous substitutions per non- synonymous site), and Ks (the number of synonymous substitutions per synonymous site) for any pair of sequences were calculated according to Nei and Gojobori (Nei and Gojobori, 1986). Significant pairwise Ka/Ks biases were assessed by Fisher exact test with Bonferroni correction for multiple testing. Tajima's D test (Tajima, 1989) and Fu and Li's tests D* and F* (Fu and Li, 1993) were as used for testing the null hypothesis that all mutations are selectively neutral (Kimura, 1983). The Tajima's The D test is based on the differences between the number of segregating sites and the average number of nucleotide differences. The D* test statistic is based on the differences between hs, the number of singletons (mutations appearing only once among the sequences), and h, the total number of mutations. The F* test statistic is based on the differences between hs, the number of singletons (mutations appearing only once among the sequences), and k, the average number of nucleotide differences between pairs of sequences.

Multiple Correspondence Analysis (MCA). A very common question that arising when analyzing a population of sequences coming from the same species is to identify groups of individuals displaying the same mutations or, conversely, to identify groups of mutations characterizing some subpopulation (co-mutations). Although some aspects of this question are already addressed by traditional phylogeny reconstruction techniques, there is a more suitable statistical technique, widely used in econometrics and ecology, to fit this particular purpose: Multiple Correspondance Analysis (MCA). MCA is an exploratory statistical technique adapted to multivariate categorical data (Greenacre, 1984; Greenacre and Blasius, 2006; Lebart, 1984) . It can be viewed either as an extension of Correspondence Analysis (CA) to more than two variables or as an adaptation of Principal Component Analysis (PCA) when the variables are categorical instead of quantitative and the metrics is the chi-square rather than the euclidean distance (Tenehaus and Young, 1985). Its application to the analysis of co-mutations in multiple alignments has already been described in the context of protein analysis (Pazos et al., 2006). In this work, a 'site' is defined as a position in the multiple alignment, a 'state' denotes a particular nucleotide (A,C,G,T) and a 'trait' is a particular state at a particular site (e.g. trait 'A50' means state A at position 50). As a first step the multiple alignment was encoded into a boolean table (called an indicator matrix) where rows correspond to the sequences and columns to the traits. Entry will be 1 if the trait (column) is observed for this sequence (row) and 0 otherwise. The second step is to perform a standard Correspondence Analysis (CA) on this table using the ADE4 R package (Chessel et al., 2004; Thioulouse et al., 1997) . As with any principal component analysis, the purpose is to reduce the space dimension to the first eigenvectors (usually 2 or 3) and to interpret the plot of row points (sequences) or columns points (traits) in lower dimensional maps. Two row points (sequences) are close to each other when they tend to select the same traits (i.e. the same states at the same sites). In the same way, two columns points (traits) are close to each other when they tend to be observed on the same set of sequences. In a third step, row points (sequences) were clustered by k-means (R package cluster:pam) (Kaufman, 1990) into 4 and groups for DENV-2 and DENV-3, respectively, using the coordinates on the three

main axes. Finally, a Naive Bayes approach was used to identify which traits were specific of each group of sequences previously identified. A trait 't' (i.e. a state at a given site) is affected to a group of sequences i (i=1,2,3,4) according to the observed frequency of 't' in this group.

Lineage demography. Lineages history was inferred using the Bayesian skyline plot (BSP) method implemented in BEAST v 1.6 (Drummond and Rambaut, 2007). The analysis was conducted on the whole set of DENV-2 and DENV-3 sequences and on separate lineages. We use the GTR+ Γ4 substitution model (assuming a symmetric substitution matrix and time reversible) with different substitution rate for each codon position (Shapiro et al., 2006). In the same way, both strict and relaxed (uncorrelated lognormal) molecular clocks gave similar results. In the BSP model for population analysis, m=10 steps was used. MCMC analyses were run for 100. 10^6 states and posterior analysis were carried out with a 10% burn-in. The degree of uncertainty in each parameter estimate is provided by the 95% highest posterior density (HPD) values, while posterior probability values provide an assessment of the degree of support for each node on the tree.

Results

Clinical feature and distribution of samples. A total of 39 DENV-2 and 57 DENV-3 strains sampled from 2001 to 2008 and 2000 to 2008 respectively were sequenced and the full genome sequences used in this study were submitted to GenBank (Supplementary Tables 1 and 2). The average age of patients infected with DENV-2 was 11 years (standard deviation: 9.6, range: 1 to 48) and 10.6 years (standard deviation: 12.5, range: 0.4 to 72) for those infected with DENV-3. Details on disease severity and general demographical data are described in Supplementary Tables 1 and 2.

Lineage structure and clustering of sample sequences. Analyzing the 39 DENV-2 sequences from Cambodia by Multiple Correspondence Analysis (MCA) resulted in the identification of five independent lineages. The alignment had a total length of 10173 sites out of which 1411 (13.9%) were variable and 1123 (11%) were informative in the sense of parsimony (at least two states with an occurrence of at least 2). The parsimony informative sites were thus considered for MCA. The first three principal axes accounted for 63%, 10% and 9%, summing up to 82%, of the total inertia. The projection of sequence points on the first and second axis is shown on Supplementary Figure 1. Sequence points clustering by k-means yielded five lineages associated with a colour. Lineage D2-1 (black), lineage D2-2 (red), lineage D2-3 (green), lineage D2-4 (dark blue) and lineage D2-5 (light blue) contained 3, 4, 9, 9 and 14 sequences respectively (Supplementary Table 1). Traits (i.e. mutations) specific to each group of sequences, or lineages, were identified using a Naive Bayes approach. These traits can be seen as mutational features (SNPs) specific of each lineage. An overall 311 traits were selected: 124 for lineage D2-1, 106 for lineage D2-2, 1 for lineage D2-3, 41 for lineage D2-4 and 39 for lineage D2-5. These sites were evenly distributed along the genome (data not shown) and not related to particular proteins or genome segments, indicating the absence of recombination. In order to display the blocks of mutations characteristic of each lineage,

sequences (rows) and sites (columns) in the alignment were re-ordered according to the group they belong to (Supplementary Figure 2). For clarity, the majority state in each column was coloured in white, so that only minority states were depicted in colours. Lineages D2-1, D2-2, D2-4 and D2-5 were characterized by well-defined and completely different mutational markers (i.e. traits). These patterns were very homogeneous within each lineage but there was clearly no mutational relation between groups, suggesting that they evolved independently. A key element was the presence of only one mutational marker for lineage D2-3 which was equivalent to lack of mutational marker. This indicated that this lineage displayed the majority state on all sites but one and therefore that it did not evolve and also that lineage D2-3 was at the origin of other lineages (D2-4 and D2-5). The analysis of the 57 DENV-3 sequences through the same approach also led to the identification of several separate lineages. The alignment comprised 10170 sites out which 880 were variable (8.7%) and 526 were informative in the sense of parsimony (5.17%). As before for DENV-2 sequences, only the parsimony informative sites were considered for MCA. The first three principal axes accounted for 25%, 10% and 7%, and globally for to 42%, of the total inertia (Supplementary Figure 3). Sequence points clustering by k-means yielded four lineages associated with a colour comprising 54 sequences. Three sequences were not included in any lineage and behave like unique and independent sequences. Lineage D3-1 (black), lineage D3-2 (red), lineage D3-3 (green), and lineage D3-4 (blue) contained 7, 30, 10 and 7 sequences respectively (Supplementary Table 2). Traits specific to each group of sequences, or lineages, were identified using the same Naive Bayes approach as for DENV-2 (Supplementary Figure 4). An overall 241 traits were selected: 151 for lineage D3-1, 3 for lineage D3-2, 29 for lineage D3-3 and 58 for lineage D3-4. As per DENV-2, these sites were evenly distributed along the genome and not related to particular proteins or genome segments. The very low number of mutational markers in lineages D3-2 suggests that this lineage was at the origin of lineages D3-3 and D3-4. Two independent pairs of sequences (light blue; Fig. 1B), i.e. KHD3_HM631854_08_KCH / KHD3_GU131946_08_KCH and KHD3_GU131943_07_KCH / KHD3_GU131944_07_KCH, did not correspond to any of the

lineages identified. These sequences generated from viruses isolated from young patients originating from Kampong Cham province (central-east of Cambodia, at the border of Vietnam) were thus not considered in the Bayesian analysis of mutational markers.

Spatiotemporal distribution of traits. When considering DENV-2 sequences, the distribution at a threshold condition of $P_{best} \geq 0.7$ could only separate between lineages D2-1 and D2-2 on one hand and the three other lineages on the other hand. Lowering the threshold to $P_{best} \geq 0.6$ led to a good separation of lineages D2-1 and D2-2 while the other three lineages remained grouped together (Supplementary Figure 1). It is only at $P_{best} \geq 0.5$ that all lineages can be separated but with a major distance between lineages D2-1 (2001-2003) and D2-2 (2002) and the other three lineages, i.e. D2-3 (2003-2008), D2-4 (2003-2008) and D2-5 (2007-2008). These data indicate that two major gaps are present, the first one in 2002-2003 which led to the disappearance of lineages D2-1 and D2-2 and the second one in 2007 which led to the overwhelming presence of lineage D2-5. With respect to DENV-3 sequences, a similar pattern was observed in 2003 with the replacement of lineage D3-1 in 2003 by lineage D3-2 (Supplementary Figure 3). Following this replacement, lineage D3-2 extended and diversified into three distinct subclusters. Unlike what was seen in DENV-2, lineage D3-2 did not undergo a selective sweep in 2007 but instead remained present and diversified even more to yield lineages D3-3 and D3-4 from a sub-population represented by the strain KHD3_FJ639727_05_BTB isolated from a child in Battambang province (west Cambodia, at the border of Thailand). Furthermore two series of two strains isolated from children in Kampong Cham province, atypical with respect to mutational markers and MCOA, appeared in 2007 (KHD3_GU131943_07_KCH and KHD3_GU131944_07_KCH) and in 2008 (KHD3_HM631854_08_KCH and KHD3_GU131946_08_KCH). These isolates could represent other routes of diversification. An important limitation of this analysis is the overrepresentation of strains isolated from the large and populated province of Kampong Cham (KCH) in the geographical distribution of the samples and this may introduce a bias.

Coalescence analysis and demography. Coalescent analysis of the DENV-2 and DENV-3 genomes yielded similar rates of nucleotide substitution with 8.8 10^{-4} substitution/site/year and 8.7 10^{-4} substitution/site /year respectively, a value consistent with previous reports (Twiddy et al., 2003). The estimated year for the most recent common ancestor (MRCA) was 1979 for DENV-2 (Fig. 1A) and 1994 for DENV-3 (Fig. 1B). The Bayesian skyline plot (BSP) of DENV-2 showed a decrease of the effective population from 2005 to 2007 (Fig. 1A) whereas the DENV-3 BSP exhibited a progressive increase in relative genetic diversity starting during the year 2002 to reach a peak in 2007 (Fig. 1B). The same analysis restricted to the Asian 1 genotype of DENV-2 yielded a substitution rate of 8.7 10^{-4} substitution/site /year similar to that of overall DENV-2 sequences and a more recent common ancestor was observed at year 1996 (Fig. 1C). Unfortunately, data for the DENV-2 Asian-American genotype were insufficient to carry out the same analysis as with the Asian 1 genotype.

Phylogenetic analysis of the complete coding region. This analysis showed very different dynamics and tree topologies between DENV-2 and DENV-3. The distribution of DENV-2 isolates into five different lineages was confirmed by the phylogenetic analysis of the complete coding region (Fig. 1A). A Vietnamese DENV-2 isolate (accession number EU482640) was used as outgroup for rooting the tree. Lineages D2-1 and D2-2 on one hand and D2-3, D2-4 and D2-5 on the other hand made two phylogenetically unrelated clusters. Lineages D2-1 and D2-2, although belonging to the same genotype, i.e. Asian-American, make two separate subclusters. The lineages D2-3, D2-4 and D2-5 which belong to genotype Asian 1 were related subclusters. Lineage D2-3 was the original lineage from which lineages D2-4 and D2-5 emerged independently. The isolate KHD2_GQ868620_03_KCH represents the intermediate with lineage D2-4. The intermediate with lineage D2-5 is represented instead by a D2-3 subcluster of four isolates KHD2_GQ868121_03_SRP, KHD2_FJ639707_04_PHP, KHD2_FJ639708_05_SRP and

KHD2_GU131899_08_PHP. The distribution of DENV-3 isolates provided a different pattern (Fig. 1B). A Vietnamese DENV-3 isolate (accession number FJ547062) was used as out-group. The lineage D3-1 which was no longer detected after 2003 was genetically separated from all the other lineages. Lineages D3-2, D3-3 and D3-4 were all genetically related. Lineage D3-2 was diverse with three distinct subclusters characterized by bootstrap values of 100. Lineages D3-3 and D3-4 seemed to originate independently from the same population with isolate KHD3_FJ639727_05_BTB representing, along with isolates KHD3_FJ639724_03_PHP and KHD3_FJ639726_04_PHP, the intermediate between lineage D3-2 and lineages D3-3 and D3-4. Lineage D3-2 is also at the origin of two pairs of "atypic" sequences as defined by MCA and Bayesian analysis of mutational markers, i.e. KHD3_HM631854_08_KCH / KHD3_GU131946_08_KCH and KHD3_GU131943_07_KCH / KHD3_GU131944_07_KCH. These two pairs of sequences originated from two different subclusters of lineage D3-2 and were present on separate branches characterized by a long distance when compared to the other members of lineage D3-2.

Analysis of individual gene trees. Trees were constructed for each individual gene at both nucleotide and amino-acid level and the topologies were compared to those of the full length coding region described above. For clarity, individual genes confirmed the topology observed with full length genomes for both DENV-2 and DENV-3 are addressed separately.

Analysis of DENV-2 individual gene trees based on nucleotide sequences

These trees are shown in Supplementary Figure 5. The same tree topology was observed between the full length genomes and the individual genes with the exception of NS2A, NS3 and NS4B. For these three genes, the sequences were highly conserved and almost no or very little nucleotide diversity was observed indicating a possible negative selective pressure on these loci.

Analysis of DENV-2 individual gene trees based on amino-acid sequences

The distribution of the isolates based on amino-acid similarity for each individual protein provided slightly different information. With respect to DENV-2 (Supplementary Figure 5), the same distribution as with DNA phylogeny is observed only with E and NS5. Lineage D2-1 and D2-2 were consistently separated from the other three lineages in all cases except for NS2B. Lineages D2-3, D2-4 and D2-5 were closely associated in the same cluster for NS3, NS4A, NS4B and C suggesting a potential negative selective pressure. NS1 displayed a specific topology in which lineages D2-3 and D2-5 were mixed in the same cluster and clearly separated from lineage D2-4. A different topology was observed with NS2A and prM in which isolates from lineage D2-3 were spread between lineages D2-4 and D2-5. NS2B displayed a clear separation, associated with a significant distance, between lineages D2-1, D2-2, D2-4 on one hand and lineages D2-3 and D2-5 on the other hand, suggesting differential selective pressure on these clusters. The higher diversity of lineage D2-3 was confirmed by the proteins prM, NS2A and NS2B. This also confirmed the ancestor status of lineage D2-3 for lineages D2-4 and D2-5. A recurrent feature with DENV-2 was the separation from the Vietnamese isolate used as out-group.

Analysis of DENV-3 individual gene trees based on nucleotide sequences

When considering DENV-3 nucleotide sequences of individual genes (Supplementary Figure 6), different tree topologies were observed depending on the gene considered. A topology similar to that of the full length sequence was observed with the genes E, NS3 and NS5. Slightly altered topologies were also observed in which isolates from either lineage D3-2 and D3-3 (NS2A) or D3-2, D3-3 and D3-4 (prM) were mixed. Another slight variation of topology corresponded to the distribution of the D3-2 intermediate isolates (as defined by the full-length topology) KHD3_FJ639724_03_PHP and KHD3_FJ639726_04_PHP with lineages D3-3 and D3-4. However, an important change of topology was observed with NS1 and NS2B where the discrimination

between lineage D3-1 and the other three lineages was no longer present and the subcluster 2 of lineage D3-2 made a separate group.

Analysis of DENV-3 individual gene trees based on amino-acid sequences

Unlike what was observed for DENV-2, Vietnamese isolate was in DENV-3 consistently associated with the Cambodian isolates in most of the cases genes, i.e. C, prM, NS1, NS2A, NS2B, NS3, NS4A and NS4B (Supplementary Figure 6). Only E and NS5 proteins displayed a separate out-group as in DENV-2. Another feature was the non-resolved tree topology, or star-shape topology when seen under a radial view, for 6 genes out of 9, i.e. C, NS2A, NS2B, NS4A, NS4B and prM. In several instances, i.e. NS1, NS2A, NS2B and NS4B, lineage D3-1 was associated in the same cluster as other lineages. These data suggest a strong negative selective pressure. Other specific features were displayed by DENV-3 isolates. The protein E associated lineage D3-2 and D3-3 while separating lineages D3-1 and D3-4. The proteins NS1 and NS3 displayed a different feature in which clustering was not strictly associated to lineages but rather to specific strains within the lineages. NS1 associated lineage D3-1 with part of lineage D3-2 and one individual isolate from lineage D3-4 while isolating the second part of lineage D3-2 on one hand and lineages D3-3 and D3-4 on the other hand. NS3 also separated lineage D3-2 into two clusters. However, in this case it was lineage D3-3 and three individual isolates from lineage D3-4 which were associated with part of lineage D3-2 whereas the remaining parts of lineages D3-2 and D3-4 and lineage D3-1 clustered separately.

Relative distribution of non-synonymous mutations. Non synonymous mutations associated to the different lineages are displayed in Supplementary Table 3. Although lineage D2-3 was at the origin of both lineages D2-4 and D2-5, several non-synonymous mutations were found to associate lineages D2-3 and D2-5 whereas no mutation associating lineages D2-3 and D2-4 could be found. These non-synonymous mutations common to D2-3 and D2-5 were G228E in E, H129Y in NS1,

T65A in NS2A and V112I in NS2B proteins. Other non-synonymous mutations associated lineage D2-5 with part of lineage D2-3 such as I120V in NS2A and A152V in prM proteins. Other mutations were lineage specific such as K160M in E protein for D2-5 or Q286E and I27T in NS5 protein for D2-5 and D2-4, respectively. Mutation V637A in NS5 protein associated lineage D2-4 with two isolates from lineage D2-1, KHD2_FJ639702_03_BTB and KHD2_FJ639703_03_BTB. Interestingly, these two isolates did not carry in many instances the amino acid characteristic of lineage D2-1 but instead that found in lineages D2-3, D2-4 or D2-5 (data not shown). With respect to DENV-3, non-synonymous mutations were found to be associated to individual lineages or subparts of lineages. Lineage D3-1 was characterized by mutations R173K, Y482F, T649N and I789V in NS5. Lineage D3-4 was associated to mutations R217K in NS2A and T188N in NS5. Other mutations were linked to combination of lineages clustering together such as the mutations K630E in NS5 linking D3-4 and D3-5, R436K in NS5 associating D3-3 and D3-4, S17N and T293A in NS1 characterizing D3-3 and D3-4.

DNA polymorphism and selective pressure. Data are shown in Supplementary Tables 4 and 5. A common trait shared by DENV-2 and DENV-3 isolates is the higher diversity of lineage D2-3 and lineage D3-2, respectively. These lineages were both characterized by a lack of mutational markers and they both replaced the initial lineage after 2003 and gave rise to the other lineages. However, lineage D3-2 from DENV-3 was the only one to show significant neutrality tests whereas lineage 3 from DENV-2 displayed non-significant neutrality tests. The only lineage in DENV-2 showing significant neutrality tests was lineage D2-5. This indicates either a positive selection or an expanding population. Another common trait of all lineages in both DENV-2 and DENV-3 was the recurrent higher number of synonymous substitutions. This feature was confirmed by the Ka/Ks analysis which displayed a very low Ka/Ks ratio for all lineages and all genes in both DENV-2 and DENV-3 (Supplementary Tables 6 and 7). This suggests the presence of a strong negative selective pressure. Combining these data with the occurrence of significant neutrality tests for DENV-2

lineage D2-3 and DENV-3 lineage D3-2, indicates that these two lineages were likely to be expanding, an hypothesis also supported by their higher diversity. No event of recombination could be detected in either DENV-2 or DENV-3 lineages.

Discussion

The data reported here on the genetic diversity of both DENV-2 and DENV-3 isolates in Cambodia over ten years demonstrate the existence of multiple lineages and the occurrence of differing population dynamics for DENV-2 and DENV-3. A feature is the replacement after 2003 in both DENV-2 and DENV-3 of the lineages present before that date by unrelated lineages. Clade replacements have been commonly reported (Duong et al., 2009; McElroy et al., 2011; OhAinle et al., 2011; Raghwani et al., 2011; Vu et al., 2010). As already shown for lineage 1 from DENV-1, D3-1 completely disappeared after 2003. All isolates from D3-1 share the same specific mutational markers, indicative of a true lineage. Similarly, DENV-2 lineages D2-1 and D2-2, which belonged to the Asian-American genotype, also disappeared after 2003 to be were replaced by novel and unrelated lineages from the Asian 1 genotype. This synchronized replacement of all lineages present prior to 2003 by unrelated lineages in both DENV-1 (Duong et al., 2009), DENV-2 and DENV-3 (Fig. 1) suggests the occurrence of an event unrelated to the virus itself or to specific virus lineage-host interactions. An extinction and replacement event of DENV-1 virus lineages has been documented in Myanmar in 2001 that was associated with stochastic events (Myat Thu et al., 2005). Similarly, Duong et al. (2011) proposed stochastic events as the cause of the DENV-1 decline in Cambodia. The synchronized replacement of multiple serotype lineage as in Cambodia, suggests that a factor such as a change in the vector population is associated with the abrupt clade replacement events. A plausible explanation is the occurrence of major climatic events. Considering that a mosquito population displays a very limited range of spread and displays a limited life span (Harrington et al., 2005; Honorio et al., 2009; Huber et al., 2004; Melo et al., 2007), the simultaneity of the replacement throughout the country for all the serotypes suggest a country-wide phenomenon with a major impact. The floods, which occurred in 2000, 2001 and 2002 and the subsequent drought of 2002 were among the ten worst natural disasters to have occurred in Cambodia between 1900 and 2010 (EM-DAT, 2011; MAO, 2005). This succession of three

devastating floods followed by the worst drought over 110 years may have had a profound effect on the mosquito populations across Cambodia. The novel lineages present after 2003 may have arisen from small mosquito populations - which survived the floods and droughts or from mosquito populations imported from neighboring countries through trade routes, movement of goods across borders - and perhaps movements of infected people. In addition, the DENV-2 Asian-American genotype was still found in the neighboring Vietnam until 2006 (Vu et al., 2010) which would also support the resurgence of DENV-2 in Cambodia from small remaining pockets of infected vector populations. However a recent analysis of the DENV-2 dynamics in Vietnam showed that the Asian 1 genotype also expanded from 2003 onward although the Asian-American genotype was still present (Vu et al., 2010). The expansion of the Asian 1 genotype in Vietnam was associated with a higher viraemia in infected individuals, a trait potentially associated with improved transmissibility to mosquitoes (Vu et al., 2010). In Cambodia, this improved fitness and higher capacity for transmission is most likely to have provided a selective advantage over the Asian-American strains.

Following this initial 2003 replacement, different dynamics took place in DENV-2 and DENV-3. With respect to DENV-2, lineages D2-4 diverged from D2-3 soon after replacement and both lineages cocirculated until 2008. However, lineage D2-5 evolved also from D2-3 in 2007 and comprised the majority of DENV-2 isolates in 2008, outcompeting lineages D2-3 and D2-4. Interestingly, very limited signs of strict lineage specific selective pressure were detected at the protein level. Two mutations only, both involving charged amino acids, are strictly specific to lineage D2-5, K160M in E protein and Q286E in NS5 protein, whereas only one mutation, I127T in NS5 protein, is characteristic of lineage D2-4. Similarly, DENV-1 lineages from Myanmar were shown to display some limited signs of selective pressure and some specific amino-acid replacements with potential functional significance only in NS5 (Myat Thu et al., 2005). However, in DENV-1 lineages from Cambodia, several potentially important amino acid substitutions were detected, especially a mutation from proline to serine at position 119 in the NS3 protein (Duong et

al., 2011). Although it would be rather speculative to discuss the role of the D2-5 and D2-4 lineage-specific mutations, they nevertheless involve significant changes most likely to have a functional impact. In particular, the D2-5-specific mutation in the envelop protein corresponds to a loss of a positively charged amino-acid and is located in the functional domain I. Positively charged residues have been reported to play a role in heparan-sulfate binding at the cell surface (Modis et al., 2004; Perera and Kuhn, 2008). The other lineage specific sites, Q286E for D2-5 and I127T for D2-4, are both located in the methyltransferase domain of NS5. With respect to DENV-3, lineage-specific amino acids were found mostly for lineage D3-1 in the NS5 protein. The only lineage present after 2003 with specific amino-acid substitutions is D3-4 which is characterized by only two mutations, a conservative one in NS3 (R217K) and the other one in the methyltransferase domain of NS5 protein (T188N). The limited number of mutations is well in line with the observed strong negative selective pressure shown by Ka/Ks data and tree topologies. Unlike DENV-2, DENV-3 isolates diversified after 2006 to generate several lineages, all subjected to a similar selective pressure. This diversification seemed to be a continuous process as exemplified by the "atypic" isolates identified by MCA which are most likely to be emerging lineages. The presence of diversified lineages characterized by mutational markers on one hand and homogenous proteins on the other suggests that lineages are cocirculating and diversifying probably due to different populations or species of vectors while being exposed to the same negative selective pressure. This differs from what was observed in DENV-2 in this work and was previously reported in DENV-1 (Duong et al., 2011). In both cases, a selective sweep occurred in 2007 leading to a single remaining lineage in DENV-1 (Duong et al., 2011) and to the D2-5 lineage in DENV-2 overwhelmingly present in 2008.

Dengue virus dynamics must also be considered at the serotype level. Dengue virus pattern of transmission is characterized by the replacement of serotypes over time, each serotype going through a period of increasing prevalence prior to apeak prevalence and then a decrease to a low prevalence, with another serotype replacing it. Zhang et al. (Zhang et al., 2005) observed that in

Thailand the genetic diversity within DENV-1 peaked at the time of high prevalence and that clade replacement was associated with periods of low prevalence. These results do not correlate with what was observed in Cambodia in 2003. If DENV-1 and DENV-3 were at low prevalence at that time, DENV-2 was at a peak of prevalence. Inversely, the conclusion of Zhang et al. corresponds to what was observed in Cambodia in 2007. Between 2003 and 2007, the prevalence of DENV-2 decreased while that of DENV-3 increased to reach a peak in 2007. During this period, DENV-1 prevalence fluctuated to reach its lowest level in 2007. In 2006-2007, the peak of DENV-3 prevalence was at its highest. The 2006-2007 period was associated with a very important outbreak characterized by 54.2% of patients with severe disease amongst all cases reported. This major outbreak and the high prevalence of DENV-3 in 2006-2007 may have been associated with a human immunological response at the population level (Huy et al., 2010). This would in turn impose a strong selective pressure which may explain the homogeneity found at the protein level in DENV-3 isolates. Escape relating to this DENV-3-driven immunological response could explain the occurrence of a selective sweep in DENV-1 and DENV-2 in 2007 with the emergence in both cases of a single lineage with specific mutational markers at both DNA and protein level whereas several cocirculating DENV-3 lineages were emerging at the same time. A variety of epidemiological models have been proposed to explain these complex serotype dynamics (Adams et al., 2006; Wearing and Rohani, 2006). They have shown that clade replacement events are linked to interserotypic immune responses invoking a degree of immunological cross-protection.

The data reported here demonstrate that the dynamic of dengue infection is a complex process integrating different evolutionary mechanisms and levels of interaction. The dengue lineages currently circulating in Cambodia may result from a combination of unpredictable and stochastic major climatic events, discrete coevolution and important host immunological response triggering immunological escapes. It is also a complex combination of purifying and host-driven selection

generating contradictory signs in the viral populations. Addressing evolution and spread of dengue virus through a comprehensive approach with the human population immune landscape, environmental factors and the vector component is the key in order to better understand the complex dynamic of the most important human arboviral disease worldwide. Being able to understand and potentially predict the risk of a major dengue epidemic in the transmission season following climatic disasters like the flood that occurred in Thailand, Vietnam and Cambodia during the 2011 rainy season may help public health authorities to mitigate the epidemics.

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Figures legend

Figure 1. Comparative dynamics of DENV-2, DENV-3 from 2000 to 2009 in Cambodia

A. Phylogenetic tree and Bayesian skyline plot of DENV-2 lineages.

B. Phylogenetic tree and Bayesian skyline plot of DENV-3 lineages.

C. Same plot as in a) but restricted to the Asian 1 genotype of DENV-2.

Maximum Clade Credibility Trees were built using BEAST for 39 complete coding region of DENV-2, 32 of Asian 1 genotype and 57 of DENV-3 isolates. The posterior value is presented at each node after 100 million run using Beast. For the Bayesian skyline, the step is m=10 and the x-axis is in units of years (same scale as in -a-). The y-axis (log scale) represents the relative genetic diversity, which is equal to the product $N_e.t$ of the effective population size by the generation length. The thick line is the median estimate and upper and lower blue lines show the 95% HPD (highest posteriori density) limits. Lineages are represented by the following colours: 1. for DENV-2: lineage D2-1 (black), lineage D2-2 (red), lineage D2-3 (green), lineage D2-4 (dark blue) and lineage D2-5 (light blue) and 2. For DENV-3: lineage D3-1 (black), lineage D3-2 (red), lineage D3-3 (green), lineage D3-4 (dark blue) and the "atypic" isolates shown in light blue

Supplementary Figure 1. Multiple correspondence analysis of co-mutations in the multiple alignment of DENV-2 sequences

Projection of the 39 sequence points on the first, second and third main axes. The first line represents axis 1/axis 2 and axis 1/axis 3. The second line represents axis 2/axis 1 and axis 2/axis 3. The third line represents axis 3/axis1 and axis 3/axis 2. Clusters of points (k-means) are the same as

those described in Figure 1 and are represented by the following colours: lineage D2-1 (black), lineage D2-2 (red), lineage D2-3 (green), lineage D2-4 (dark blue) and lineage D2-5 (light blue).

Supplementary Figure 2. 'Re-ordered alignment' of DENV-2 specific mutational traits

Sequences (rows) and sites (columns) have been reordered according to the group they belong to (indicated by colours; within each group the order is arbitrary). Lineages are represented by the following colours: lineage D2-1 (black), lineage D2-2 (red), lineage D2-3 (green), lineage D2-4 (dark blue) and lineage D2-5 (light blue). For clarity, the majority state (nucleotide) in a column is in white (and represented by a dot) and only minority states are coloured.

Supplementary Figure 3. Multiple correspondence analysis of co-mutations in the multiple alignment of DENV-3 sequences

Projection of the 57 sequence points on the first, second and third main axes. Projection of the 39 sequence points on the first, second and third main axes. The first line represents axis 1/axis 2 and axis 1/axis 3. The second line represents axis 2/axis 1 and axis 2/axis 3. The third line represents axis 3/axis1 and axis 3/axis 2. Clusters of points (k-means) are represented by the following colours: lineage D3-1 (black), lineage D3-2 (red), lineage D3-3 (green) and lineage D3-4 (dark blue).

Supplementary Figure 4. 'Re-ordered alignment' of DENV-3 specific mutational traits

Sequences (rows) and sites (columns) have been reordered according to the group they belong to (indicated by colours; within each group the order is arbitrary). For clarity, the majority state (nucleotide) in a column is in white (and represented by a dot) and only minority states are

coloured. Lineages are represented by the following colours: lineage D3-1 (black), lineage D3-2 (red), lineage D3-3 (green) and lineage D3-4 (dark blue). The four "atypic" isolates have been omitted.

Supplementary Figure 5. Distribution of DENV-2 individual nucleotides (gene) and amino-acid (protein) sequences

For nucleotide sequences, trees were built using the Maximum Likelihood method with the following evolutionary models: HKY+I+G for C; GTR+G for E, NS1 and NS2A; GTR+I+G for NS2B, NS3, NS4A, NS4B, NS5, and K80+I for PrM. lineage D2-1 (black), lineage D2-2 (red), lineage D2-3 (green), lineage D2-4 (dark blue) and lineage D2-5 (light blue).

For proteins, trees were built using the Maximum Likelihood method.

5A: Gene C (nucleotide sequence) and protein C (amino-acid sequence)

5B: Gene E (nucleotide sequence) and protein E (amino-acid sequence)

5C: Gene prM (nucleotide sequence) and protein prM (amino-acid sequence)

5D: Gene NS1 (nucleotide sequence) and protein NS1 (amino-acid sequence)

5E: Gene NS2A (nucleotide sequence) and protein NS2A (amino-acid sequence)

5F: Gene NS2B (nucleotide sequence) and protein NS2AB (amino-acid sequence)

5G: Gene NS3 (nucleotide sequence) and protein NS3 (amino-acid sequence)

5H: Gene NS4A (nucleotide sequence) and protein NS4A (amino-acid sequence)

5I: Gene NS4B (nucleotide sequence) and protein NS4B (amino-acid sequence)

5J: Gene NS5 (nucleotide sequence) and protein NS5 (amino-acid sequence)

Supplementary Figure 6. Distribution of DENV-3 individual nucleotides (gene) and amino-acid (protein) sequences

For nucleotide sequences, trees were built using the Maximum Likelihood method with the following evolutionary models: HKY+I+G for C; GTR+G for E, NS1 and NS2A; GTR+I+G for NS2B, NS3, NS4A, NS4B, NS5, and K80+I for PrM. Lineages are represented by the following colours: lineage D3-1 (black), lineage D3-2 (red), lineage D3-3 (green) and lineage D3-4 (dark blue). The "atypic" isolates are shown in light blue.

For proteins, trees were built using the Maximum Likelihood method.

6A: Gene C (nucleotide sequence) and protein C (amino-acid sequence)

6B: Gene E (nucleotide sequence) and protein E (amino-acid sequence)

6C: Gene prM (nucleotide sequence) and protein prM (amino-acid sequence)

6D: Gene NS1 (nucleotide sequence) and protein NS1 (amino-acid sequence)

6E: Gene NS2A (nucleotide sequence) and protein NS2A (amino-acid sequence)

6F: Gene NS2B (nucleotide sequence) and protein NS2B (amino-acid sequence)

6G: Gene NS3 (nucleotide sequence) and protein NS3 (amino-acid sequence)

6H: Gene NS4A (nucleotide sequence) and protein NS4A (amino-acid sequence)

6I: Gene NS4B (nucleotide sequence) and protein NS4B (amino-acid sequence)

6J: Gene NS5 (nucleotide sequence) and protein NS5 (amino-acid sequence)

Figure 1. Comparative dynamics of DENV-2, DENV-3 from 2000 to 2009 in Cambodia.

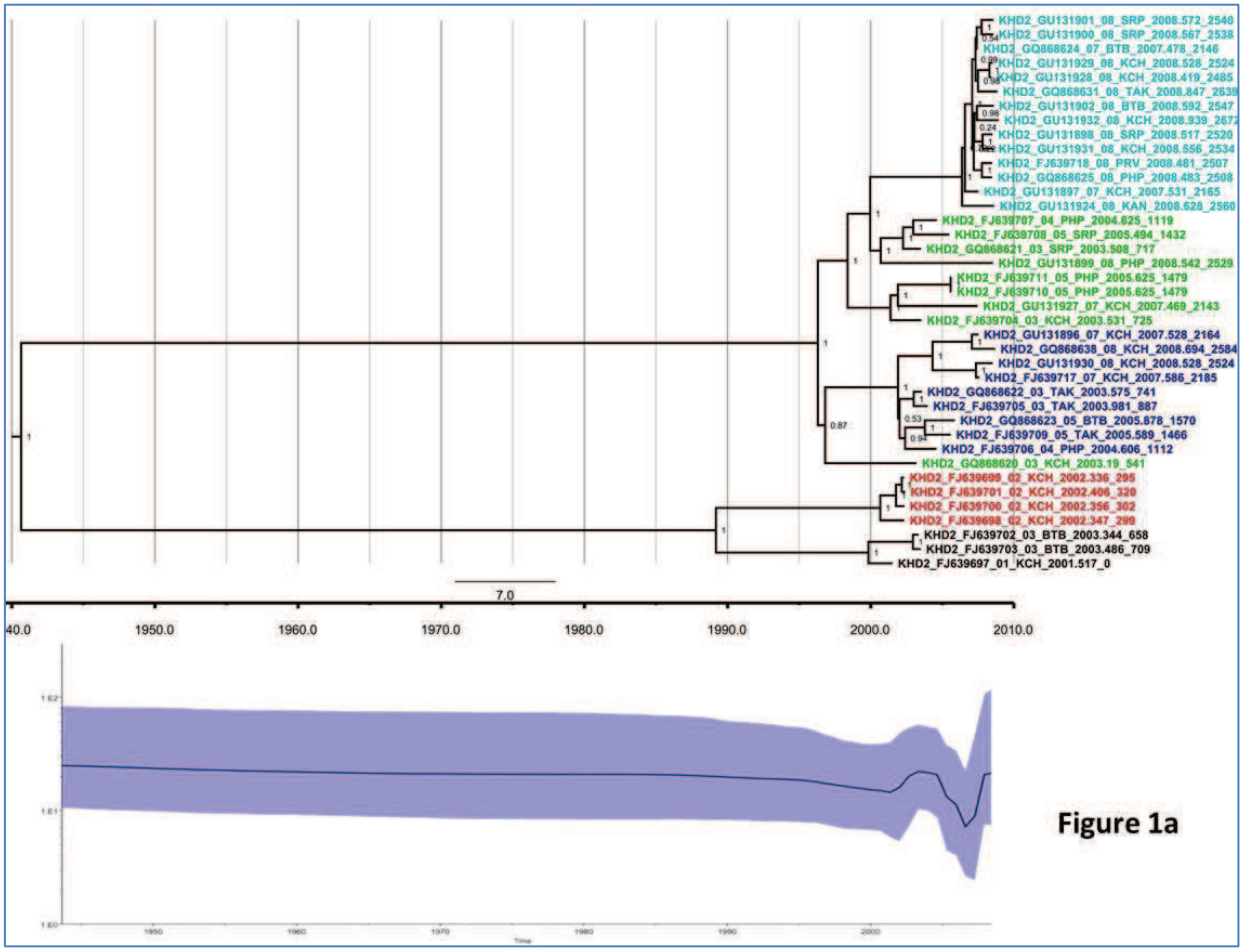


Figure 1a

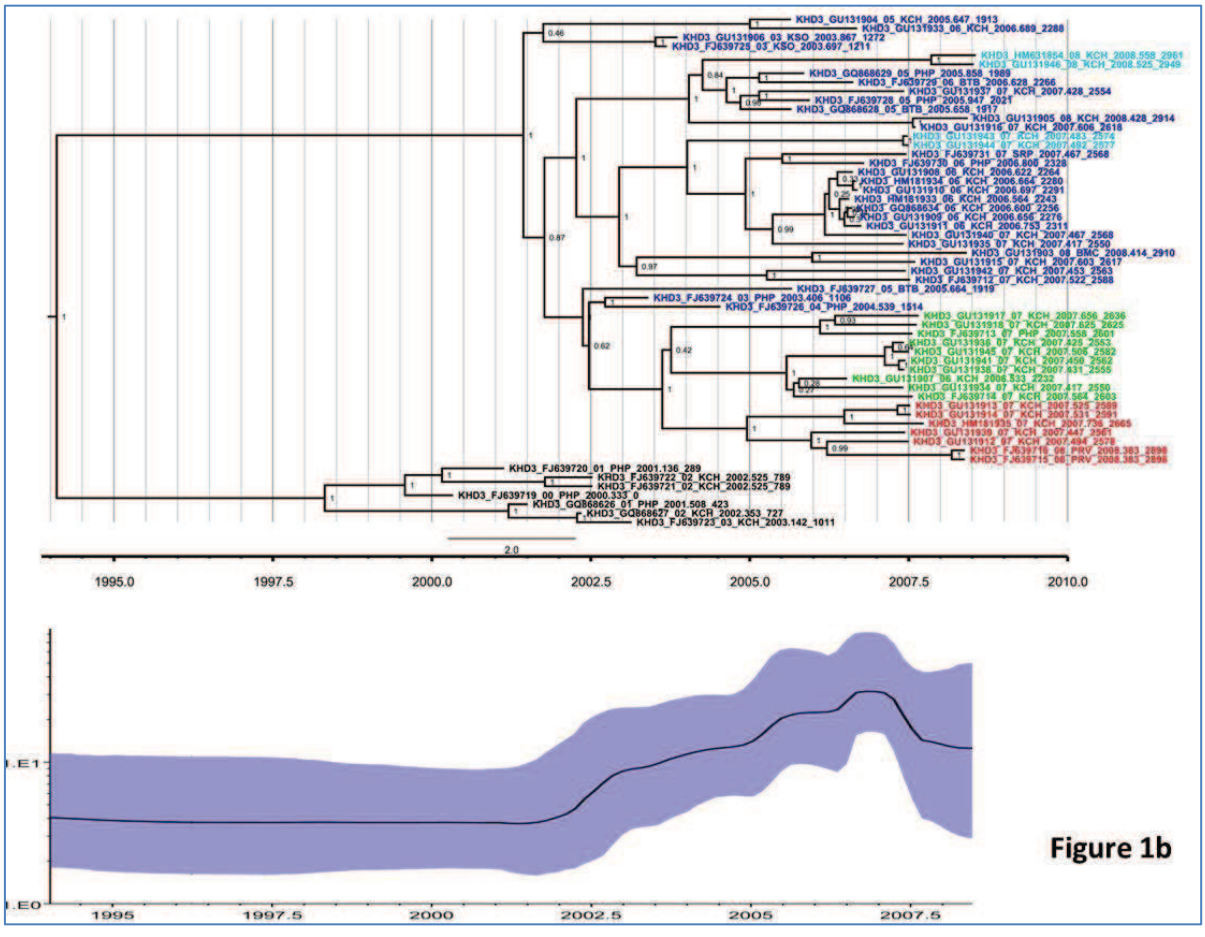
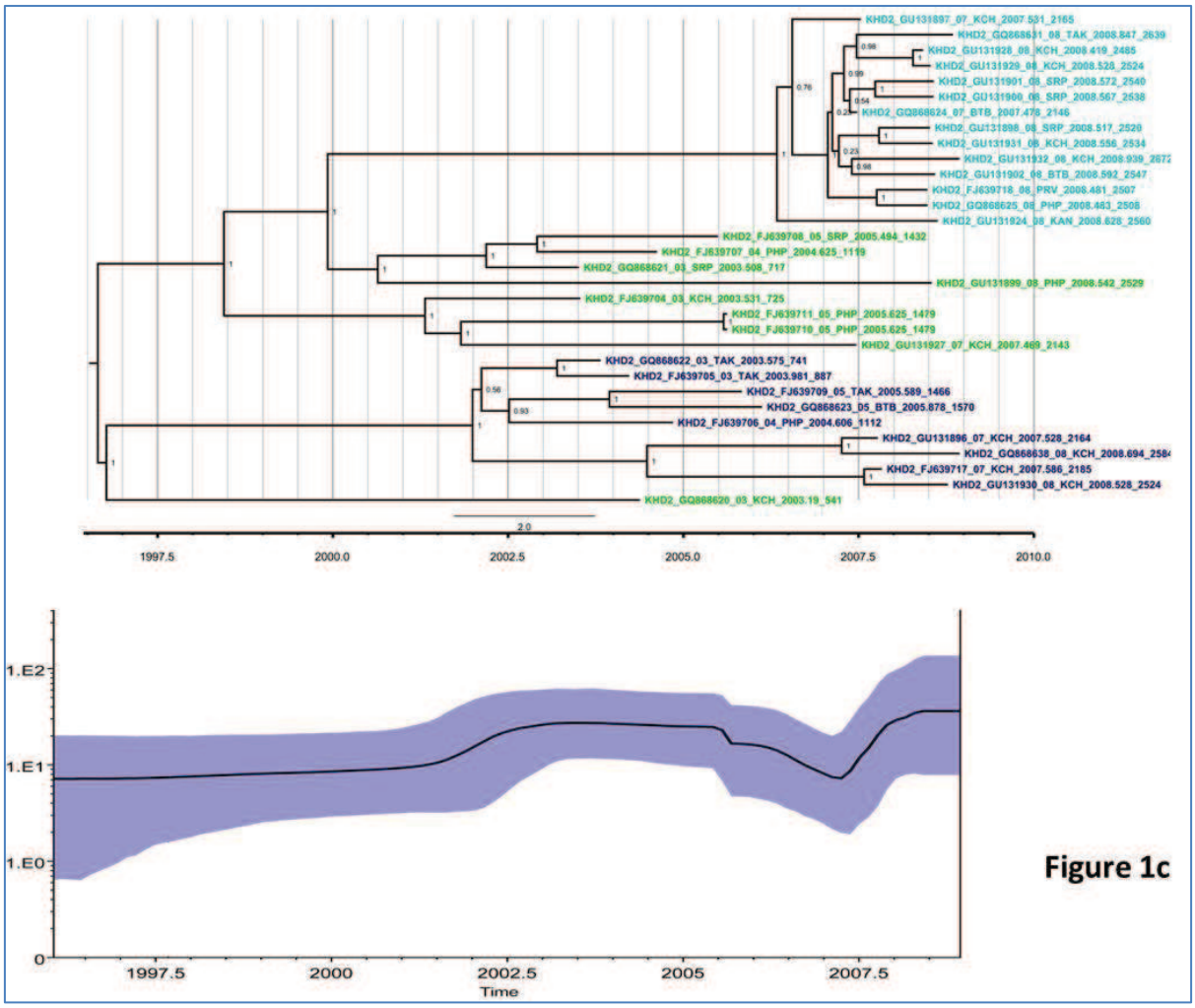


Figure 1b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KHD2_GU131901_08_SRP_2008.572_2540
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KHD2_GU131931_08_KCH_2008.556_2534
KHD2_GU131932_08_KCH_2008.939_2672
KHD2_GU131902_08_BTB_2008.592_2547
KHD2_FJ639718_08_PRV_2008.481_2507
KHD2_GQ868625_08_PHP_2008.483_2508
KHD2_GU131824_08_KAN_2008.628_2560
KHD2_FJ639708_05_SRP_2005.494_1432
KHD2_FJ639707_04_PHP_2004.625_1119
KHD2_GQ868621_03_SRP_2003.508_717
KHD2_GU131899_08_PHP_2008.542_2529
KHD2_FJ639704_03_KCH_2003.531_725
KHD2_FJ639711_05_PHP_2005.625_1479
KHD2_FJ639710_05_PHP_2005.625_1479
KHD2_GU131927_07_KCH_2007.469_2143
KHD2_GQ868622_03_TAK_2003.575_741
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KHD2_FJ639717_07_KCH_2007.586_2185
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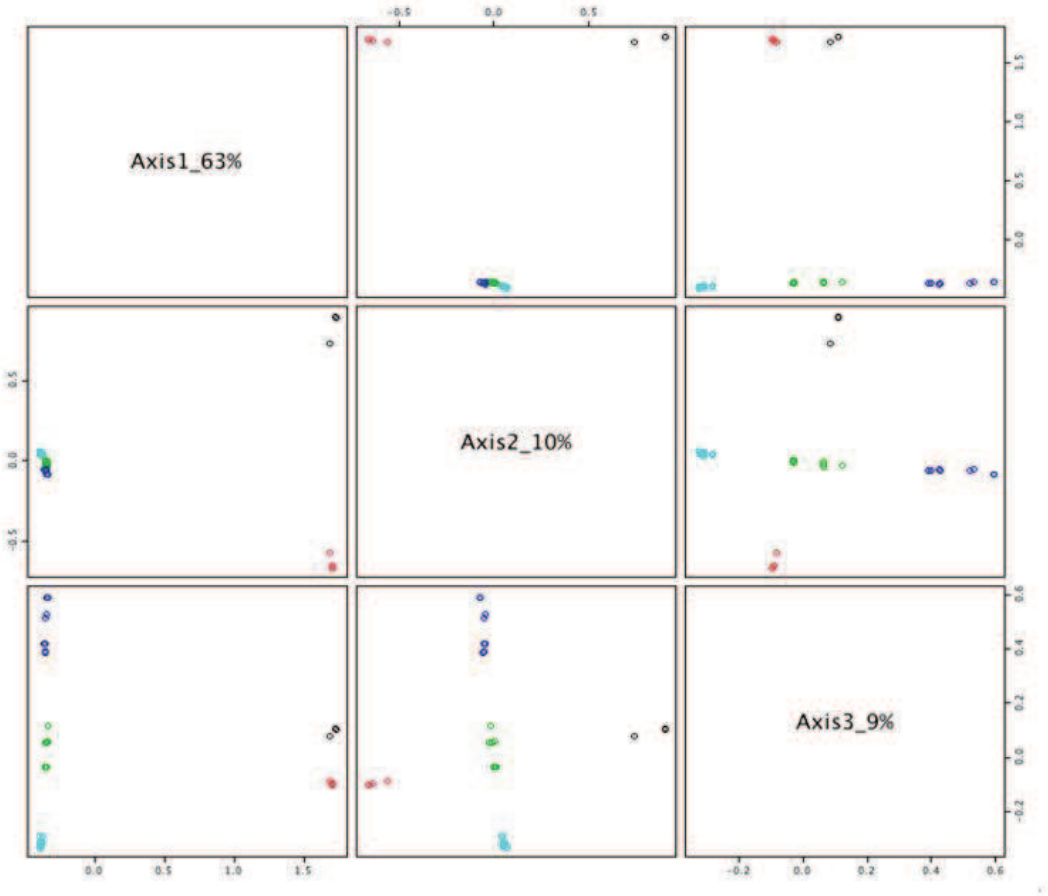
KHD2_GQ868626_03_KCH_2003.19_541
2.0
1997.5
2000.0
2002.5
2005.0
2007.5
2010.0

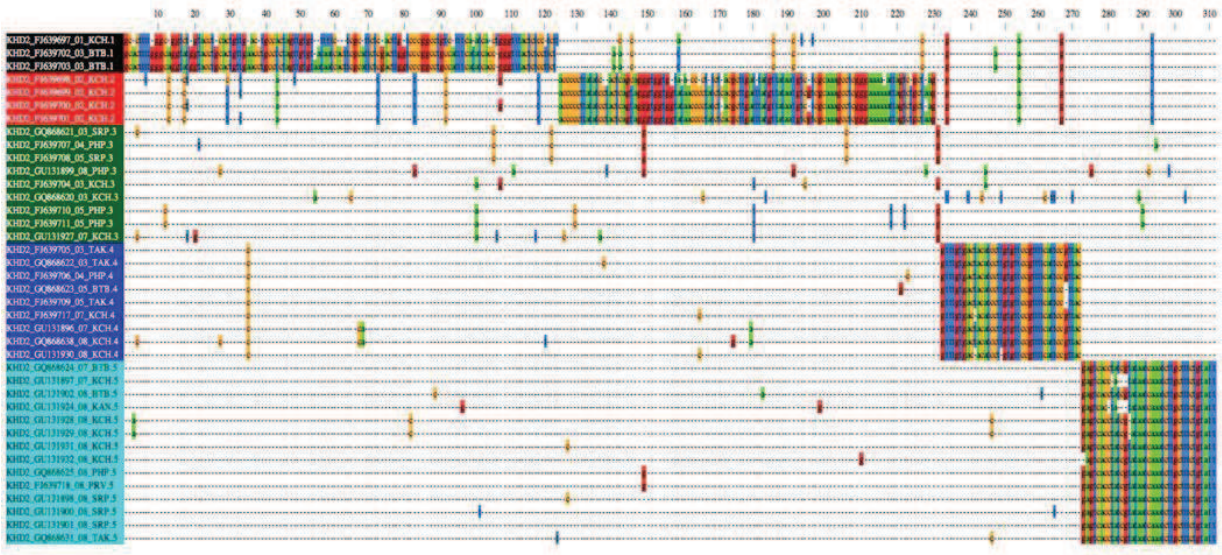
1.E2
1.E1
1.E0
0
1997.5
2000
2002.5
2005
2007.5
Time

Figure 1c

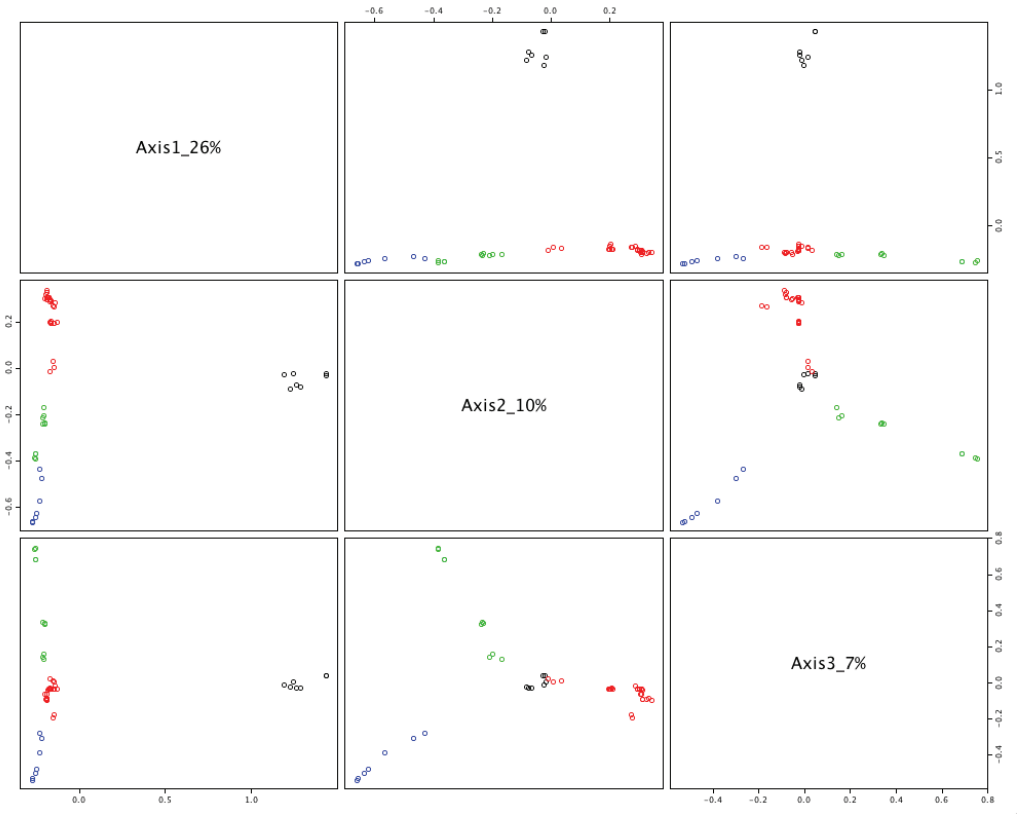
Supplementary Figure 1. Multiple correspondence analysis of co-mutations in the multiple alignment of DENV-2 sequences



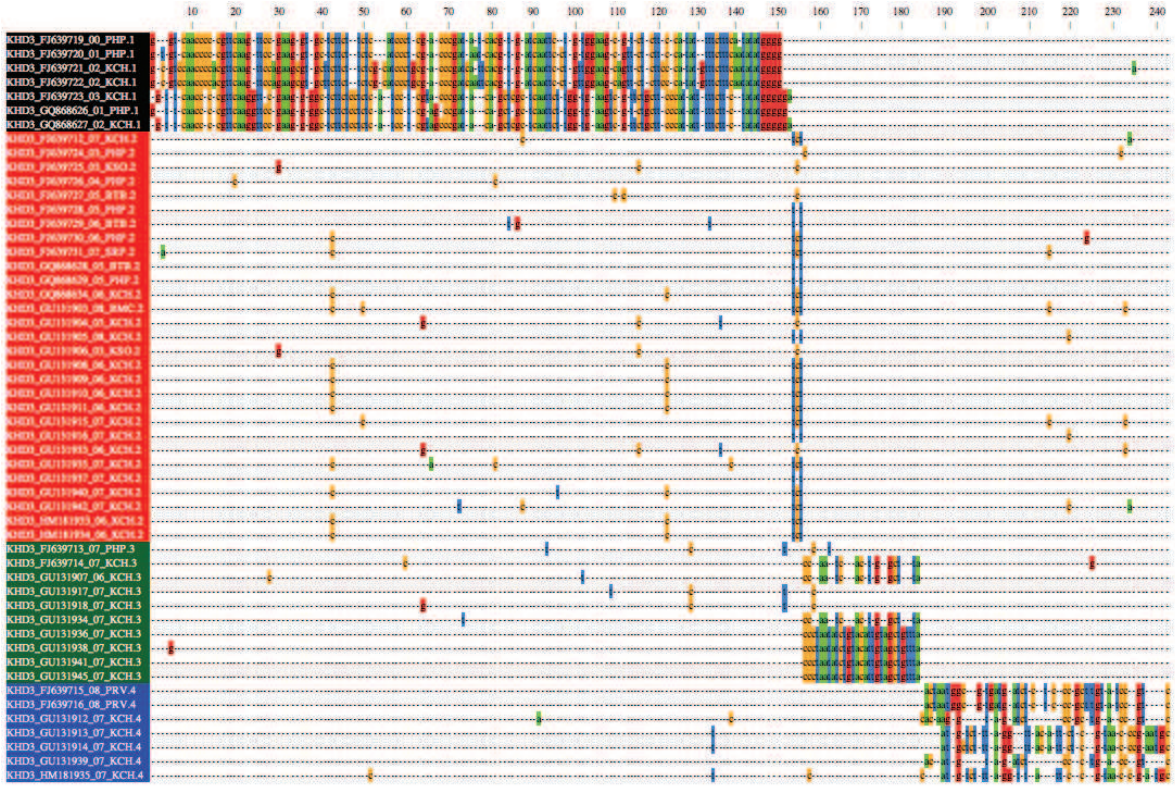
Supplementary Figure 2. 'Re-ordered alignment' of DENV-2 specific mutational traits



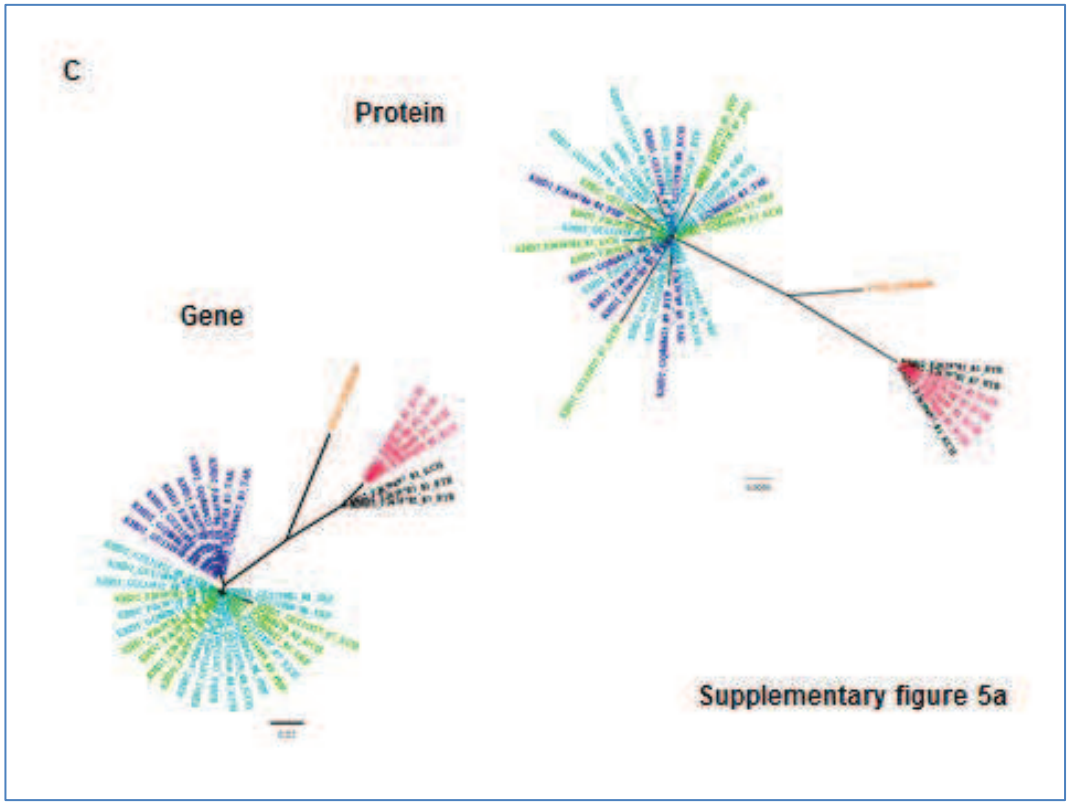
Supplementary Figure 3. Multiple correspondence analysis of co-mutations in the multiple alignment of DENV-3 sequences

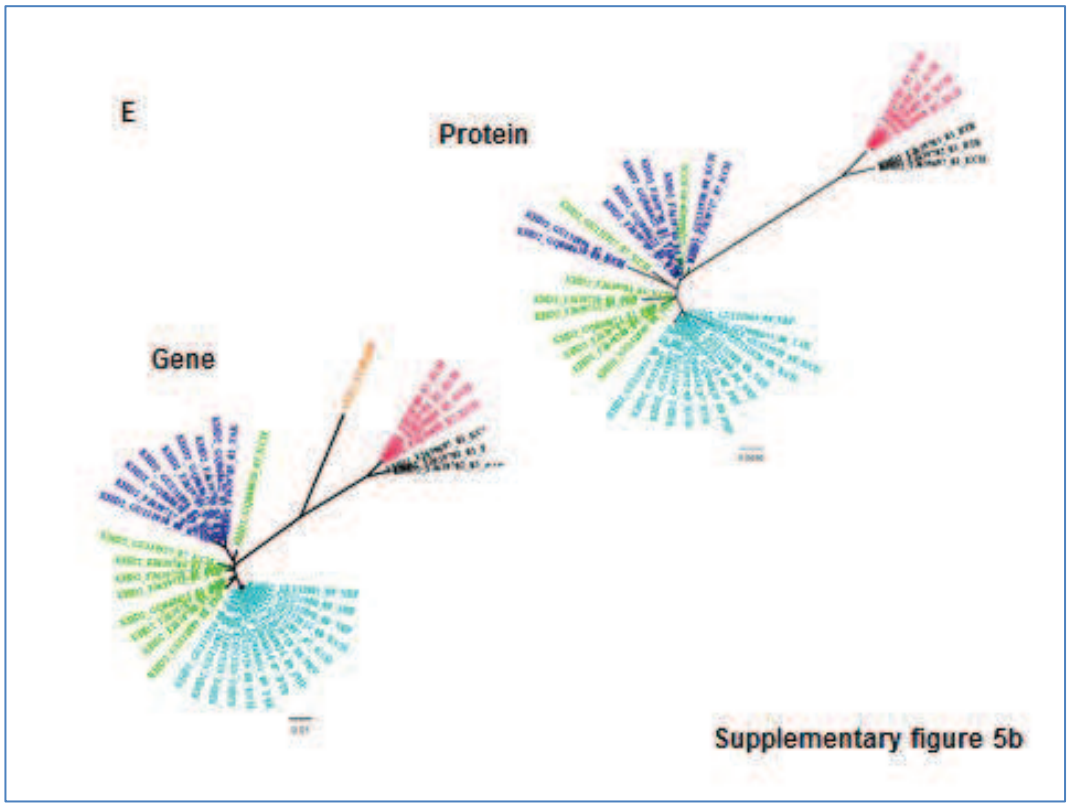


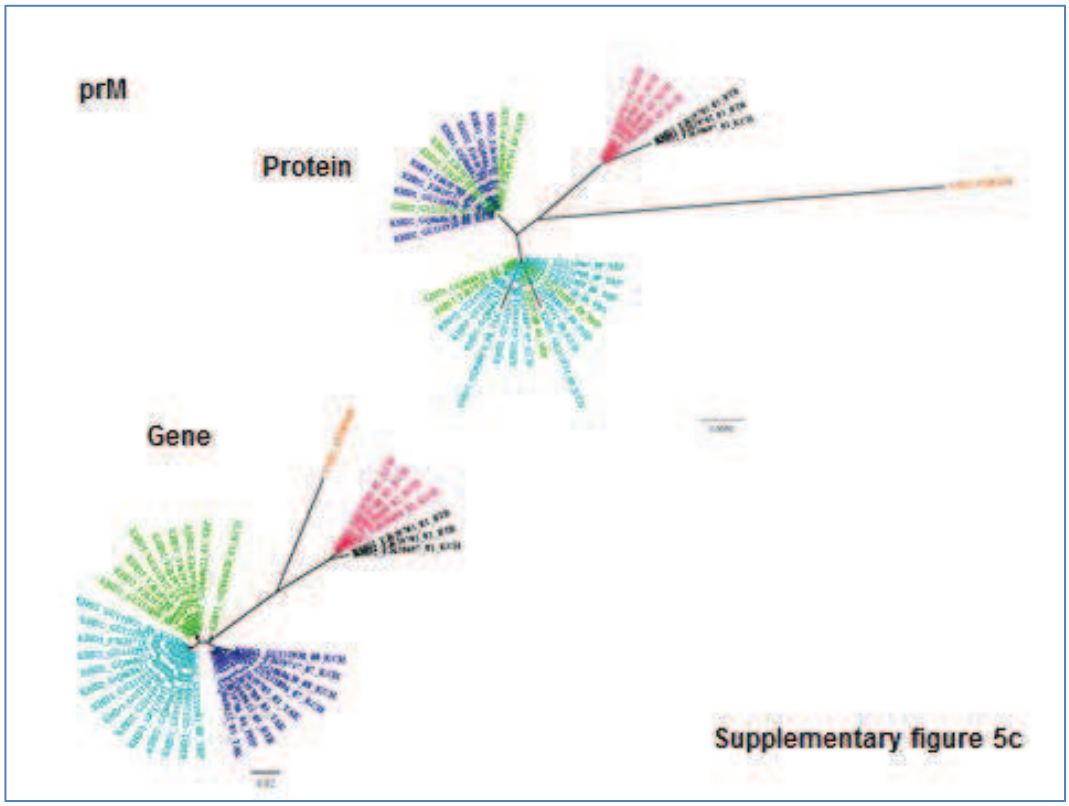
Supplementary Figure 4. 'Re-ordered alignment' of DENV-3 specific mutational traits

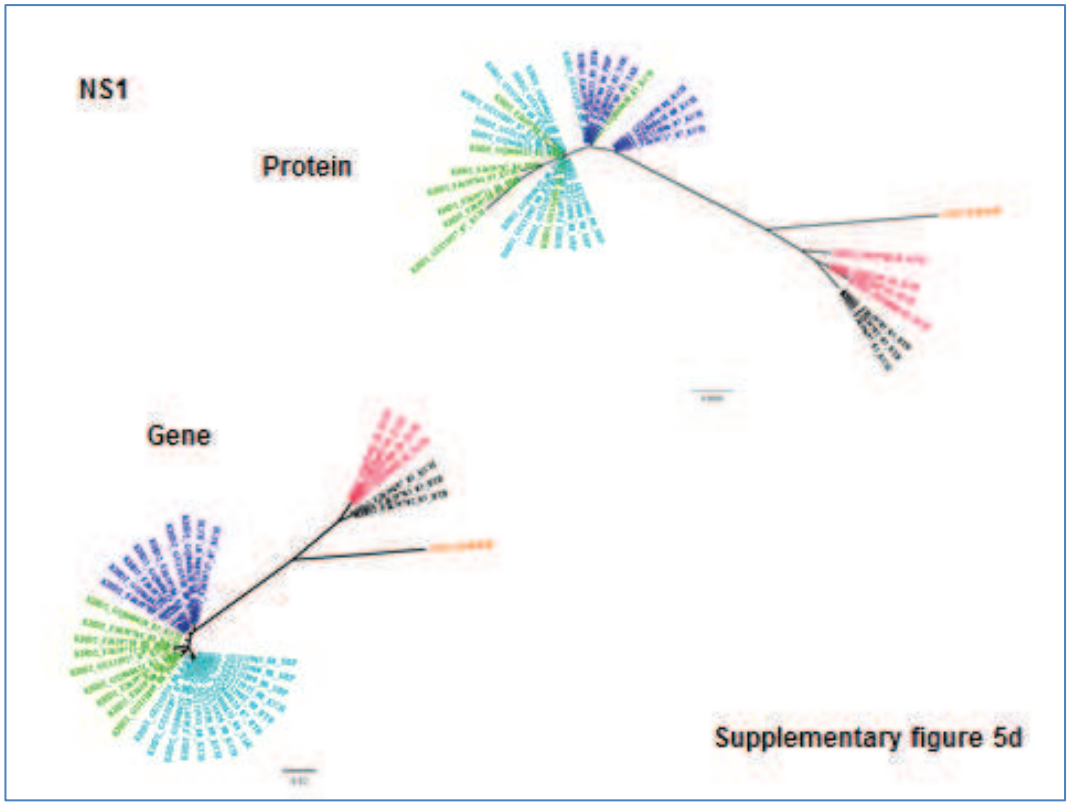


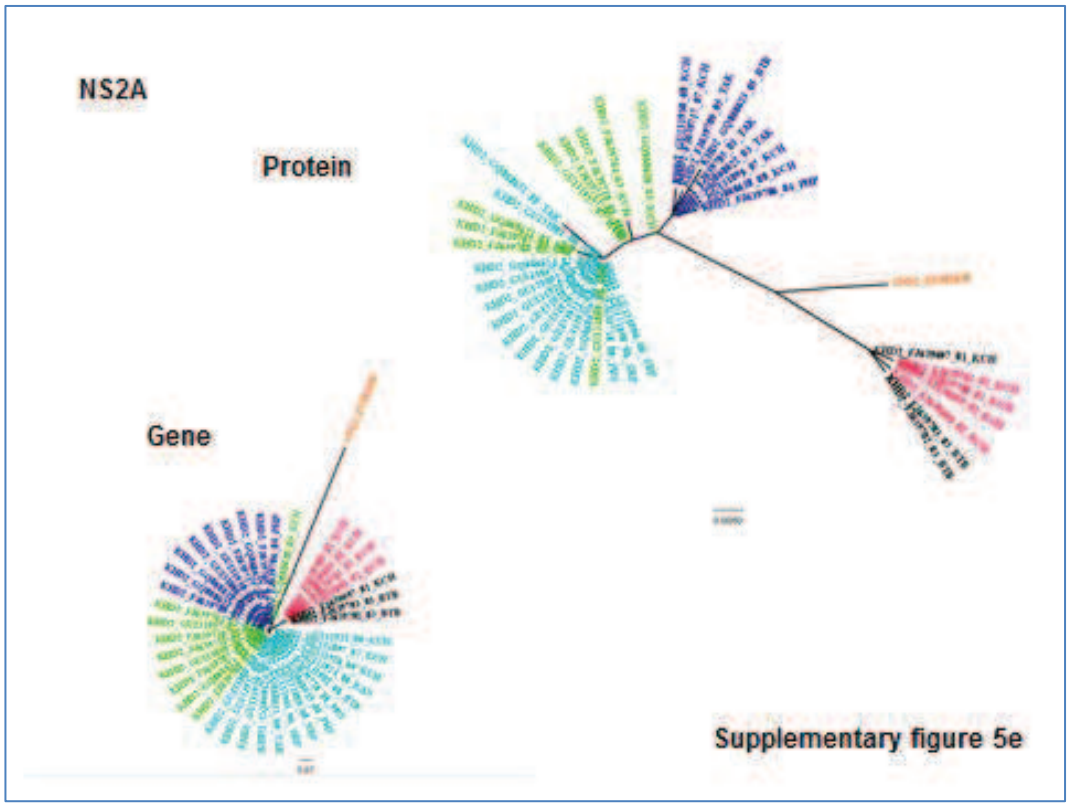
Supplementary Figure 5. Distribution of DENV-2 individual genes and proteins sequences



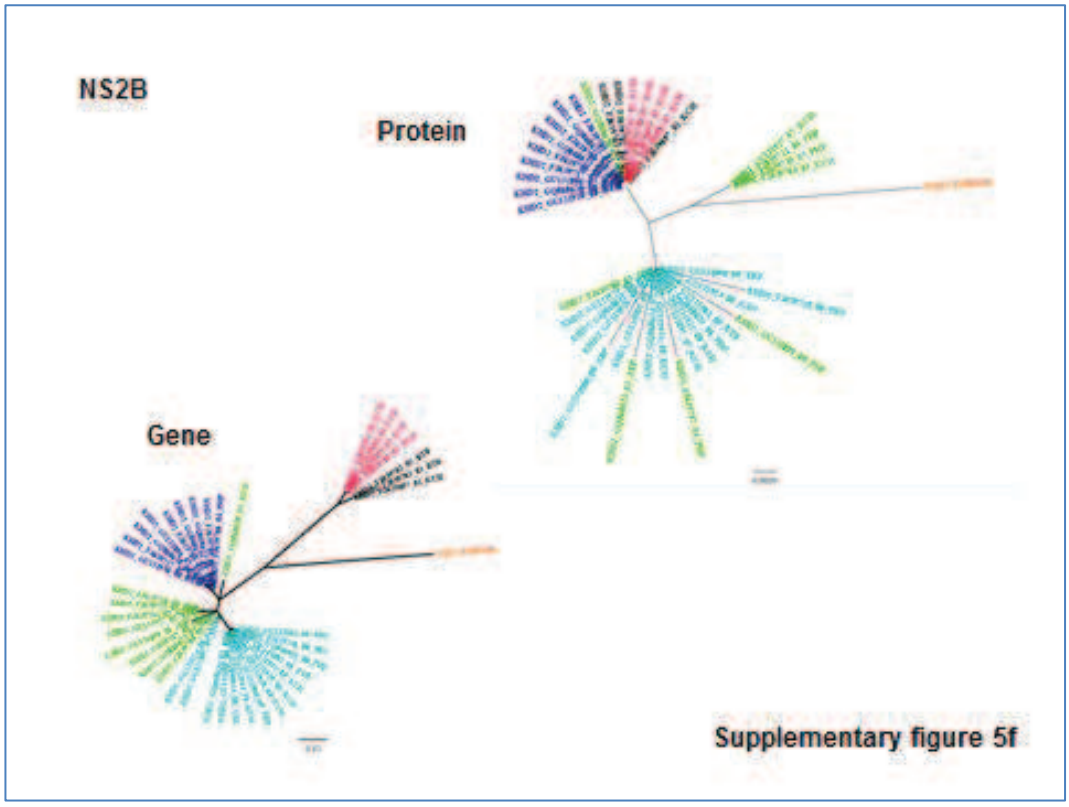



prM
Protein
Gene
Supplementary figure 5c

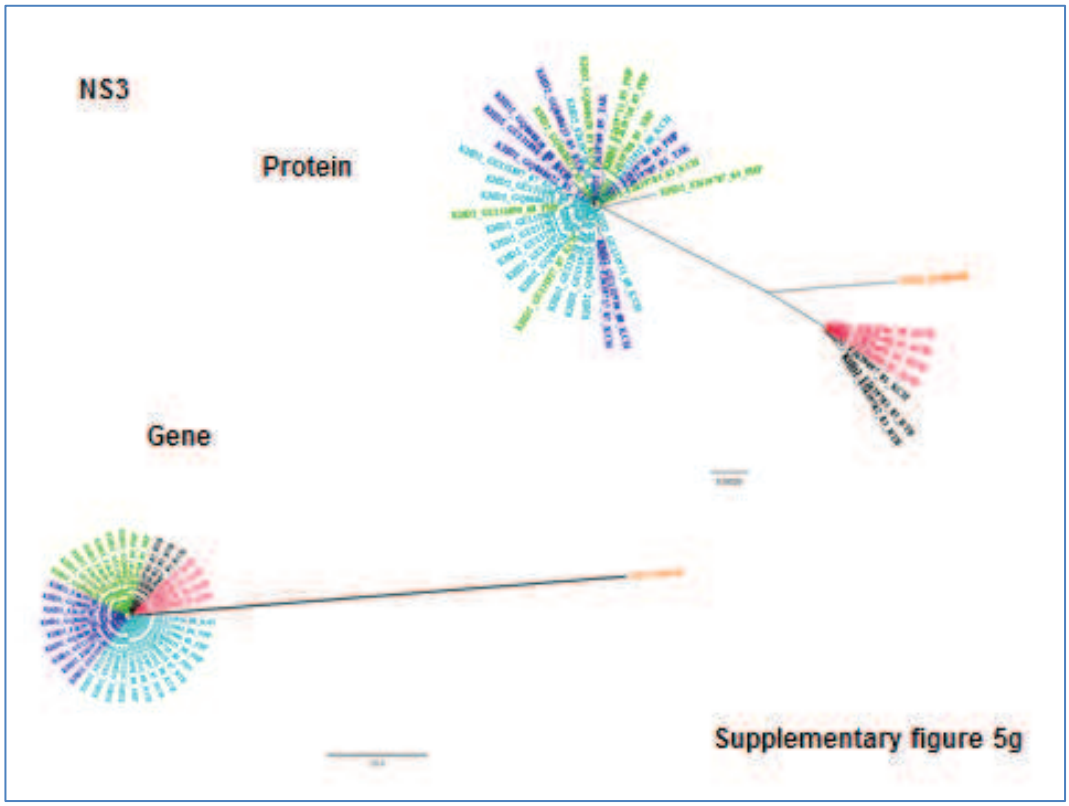

NS1
Protein
Gene
Supplementary figure 5d



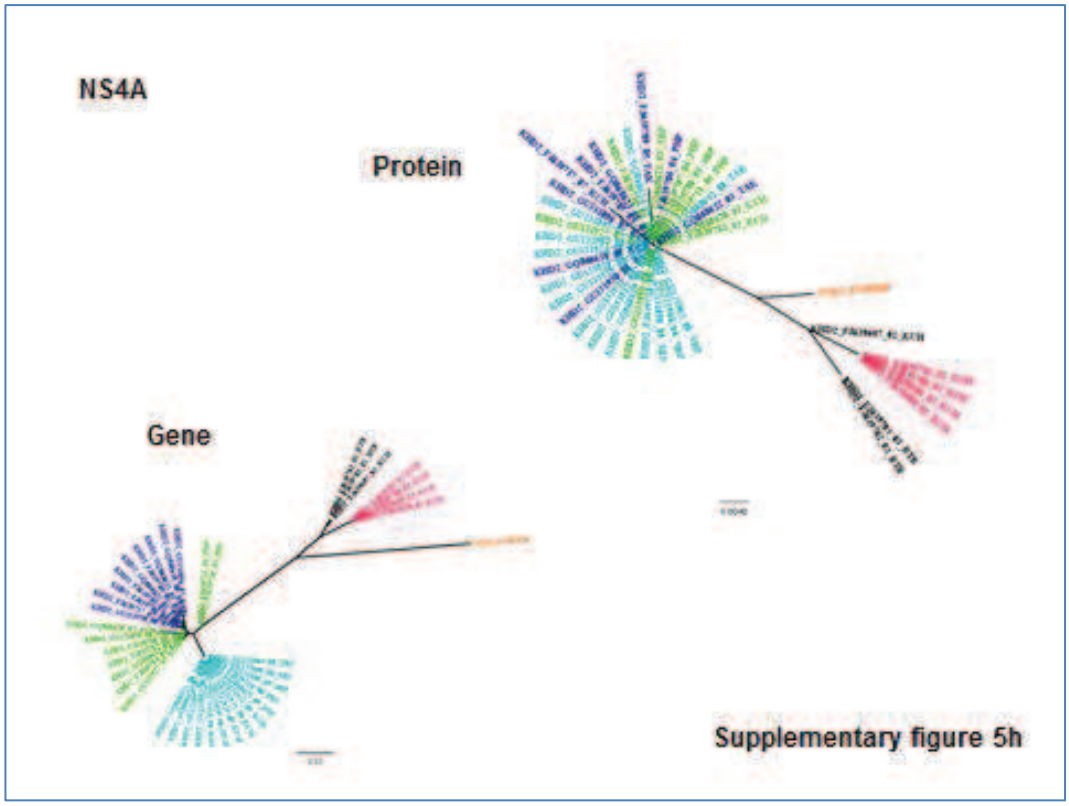
Supplementary figure 5e



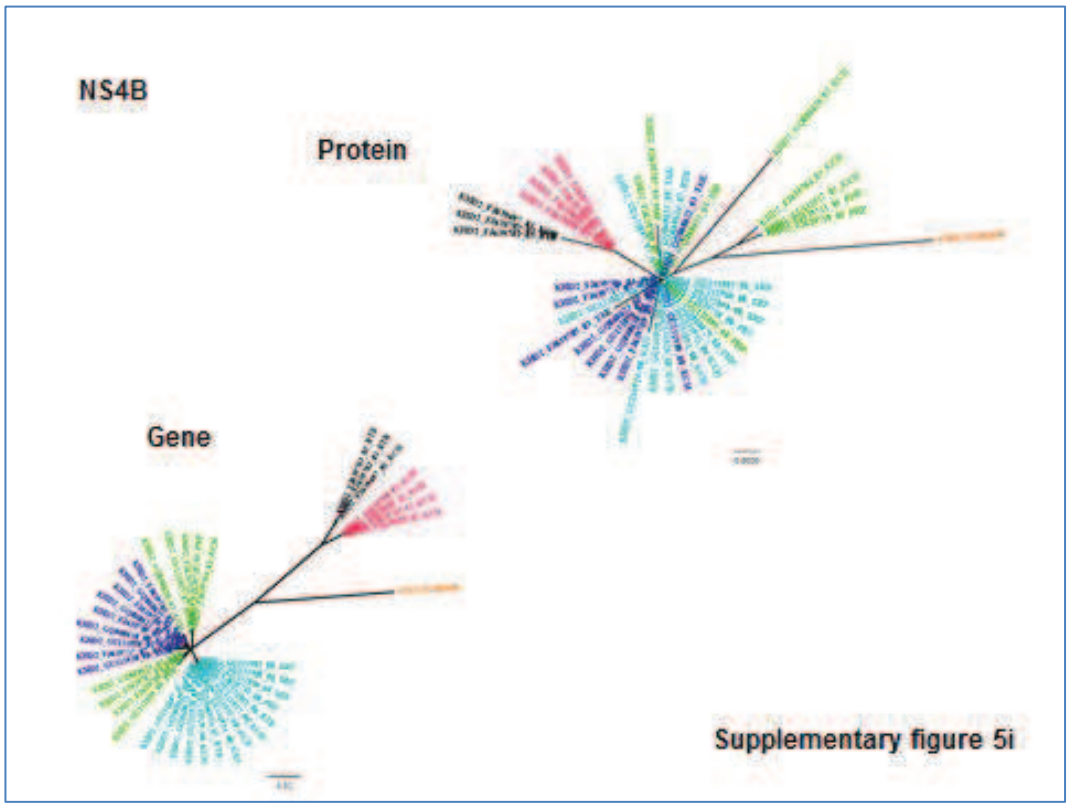
Supplementary figure 5f



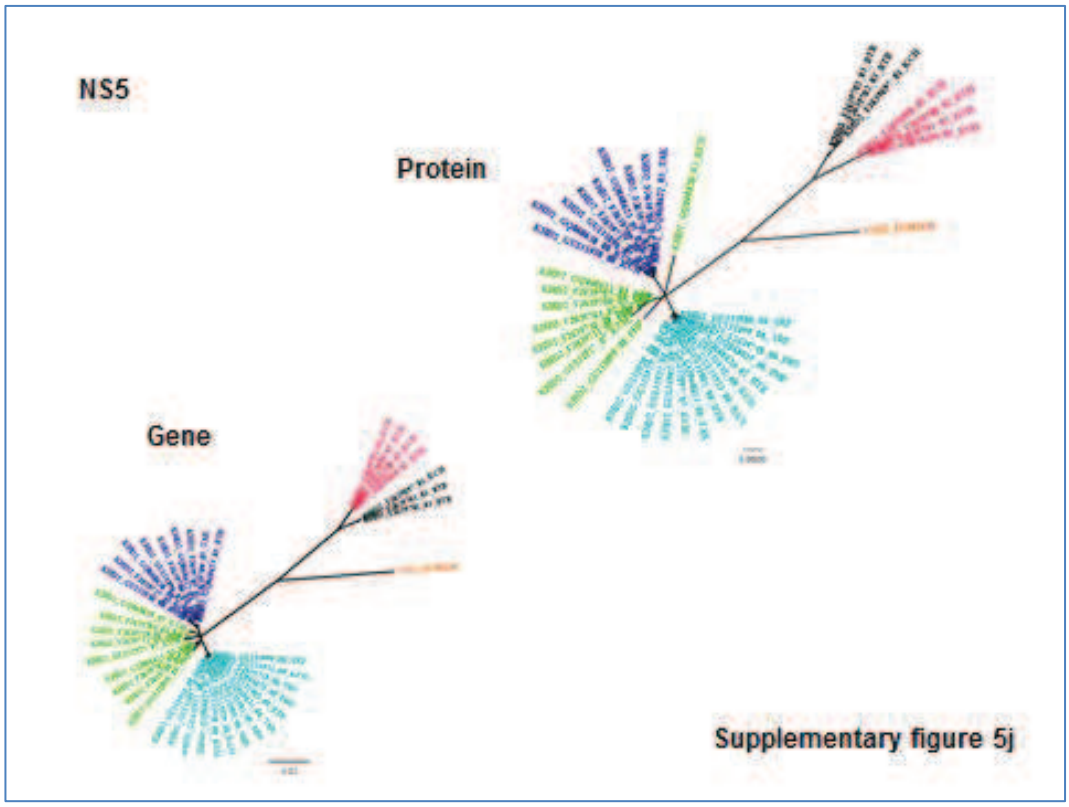
Supplementary figure 5g



Supplementary figure 5h

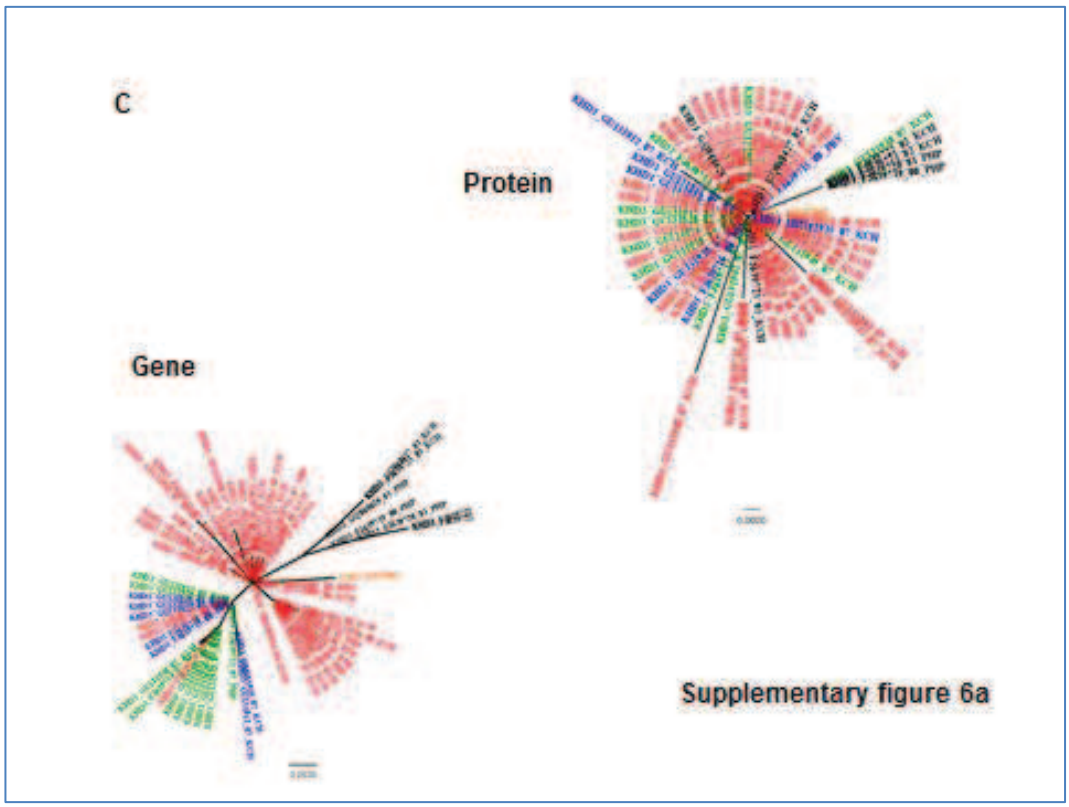


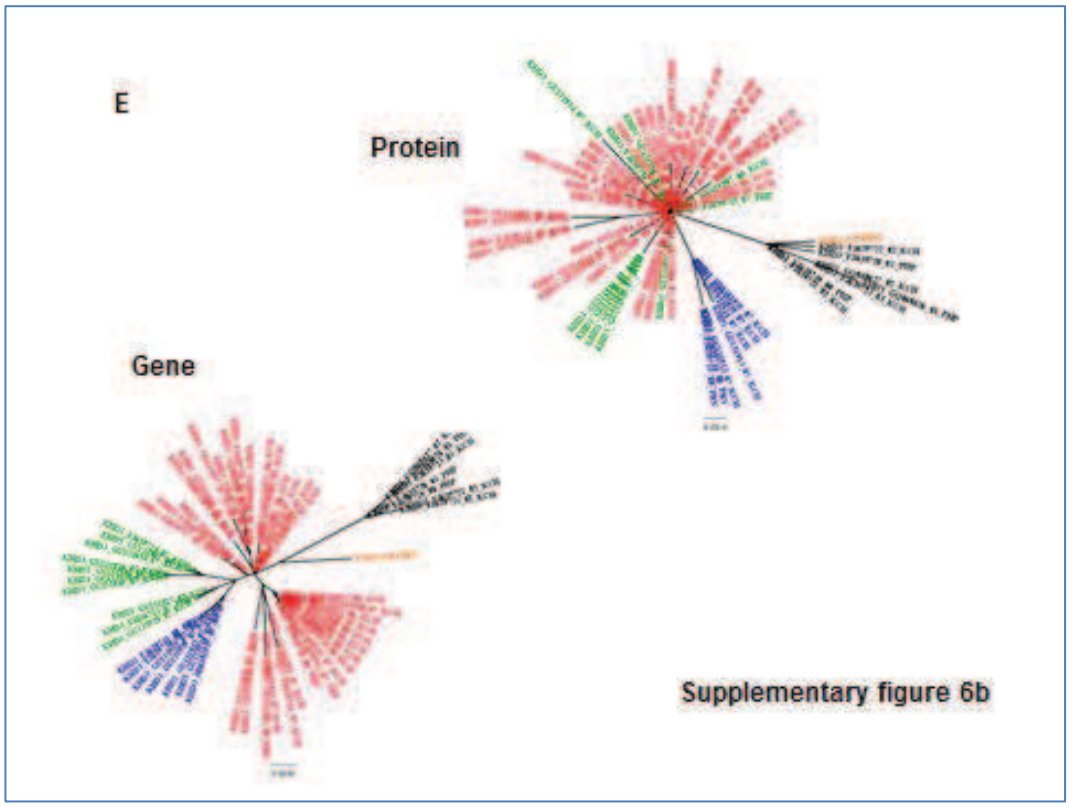
Supplementary figure 5i

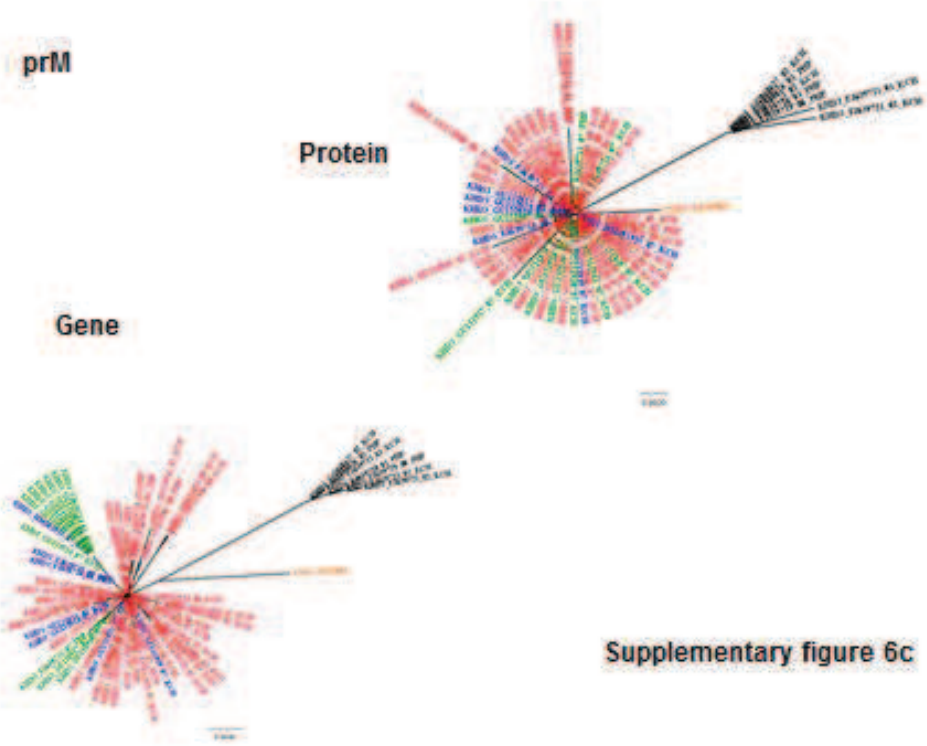


Supplementary figure 5j

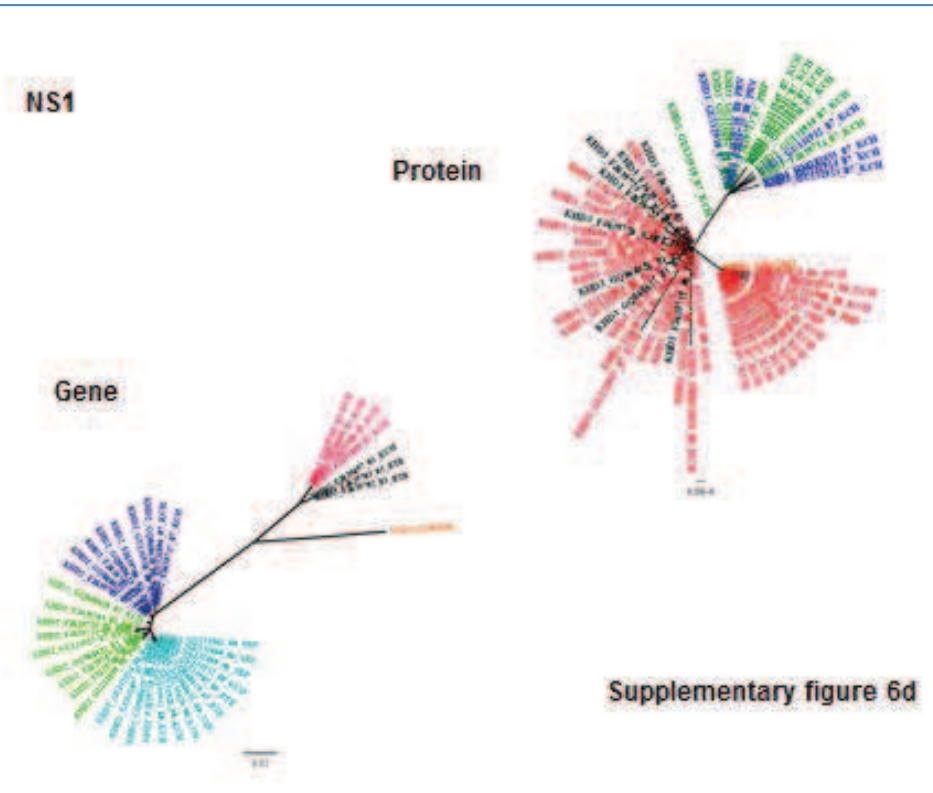
Supplementary Figure 6. Distribution of DENV-3 individual genes and proteins sequences



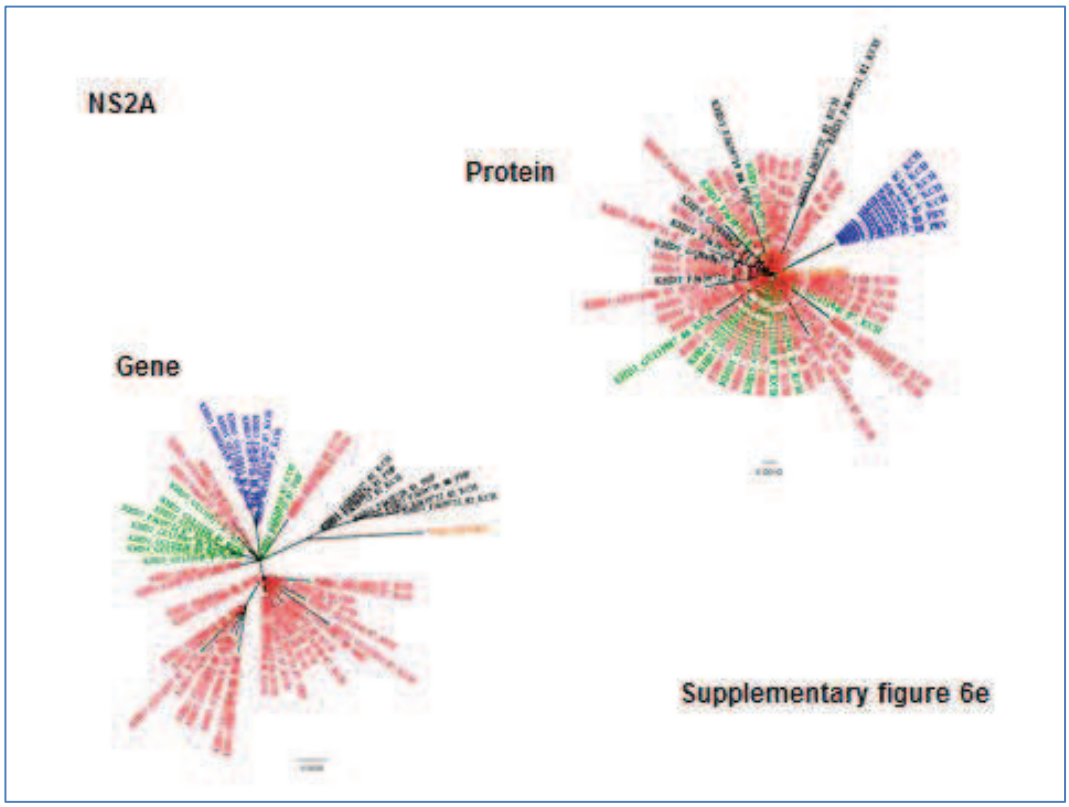




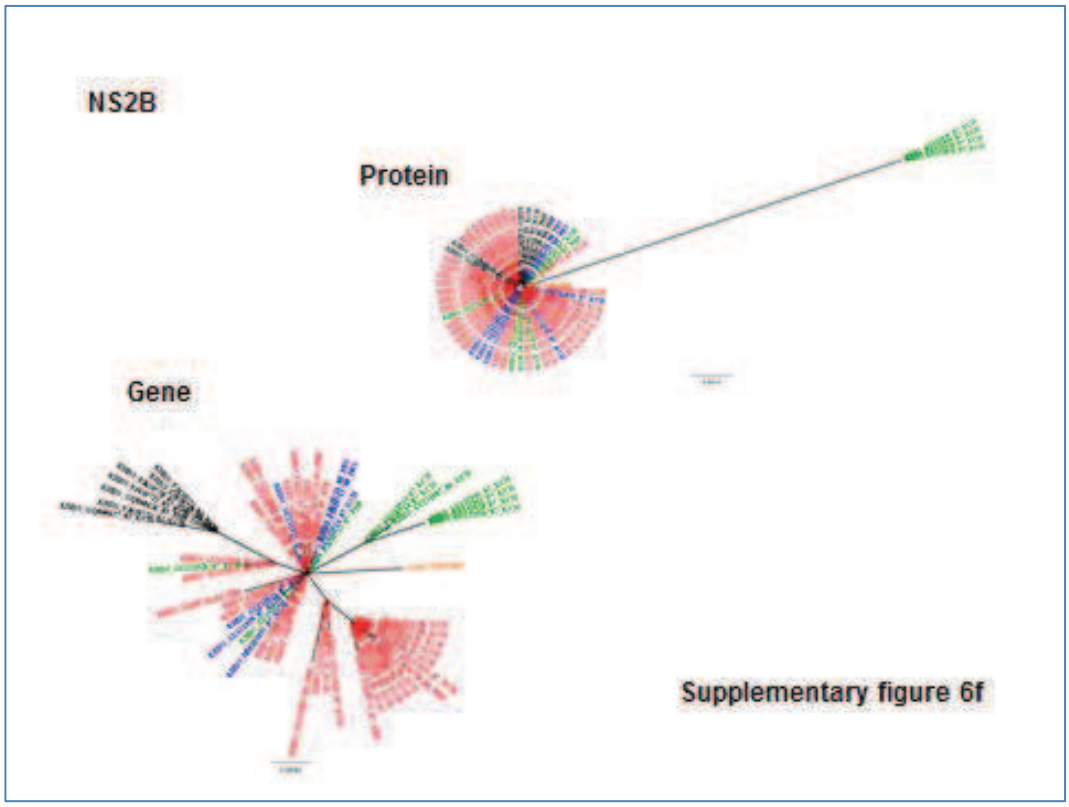
Supplementary figure 6c



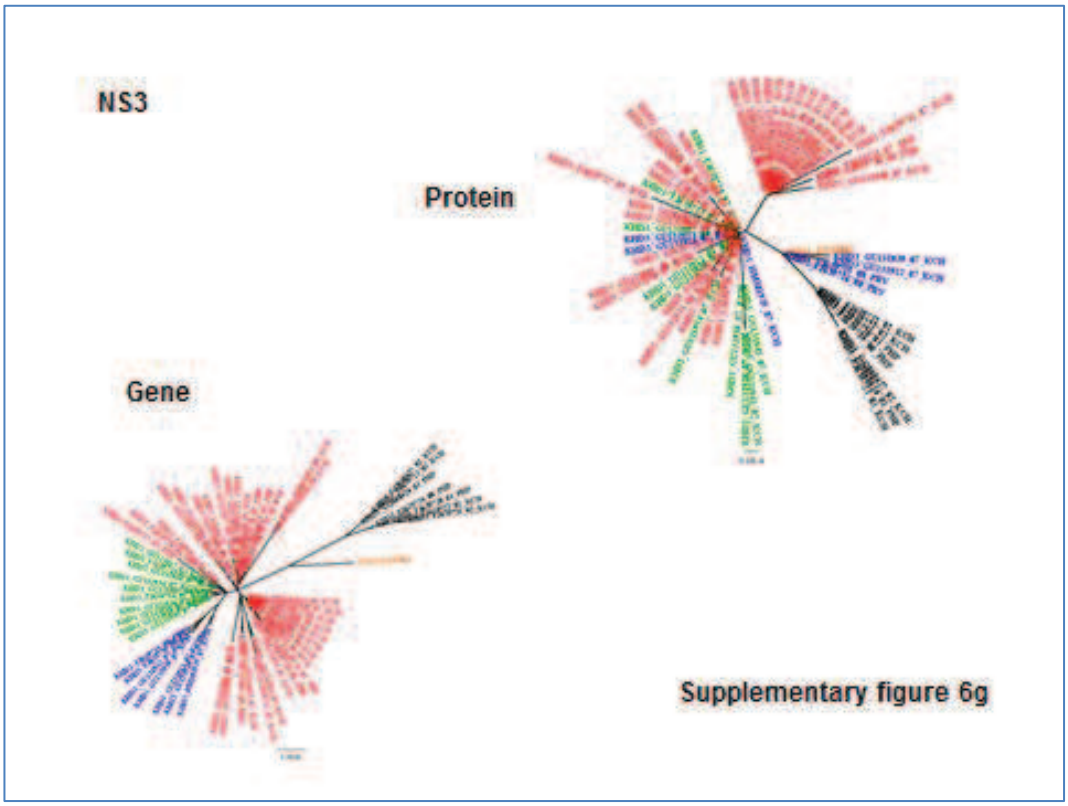
Supplementary figure 6d



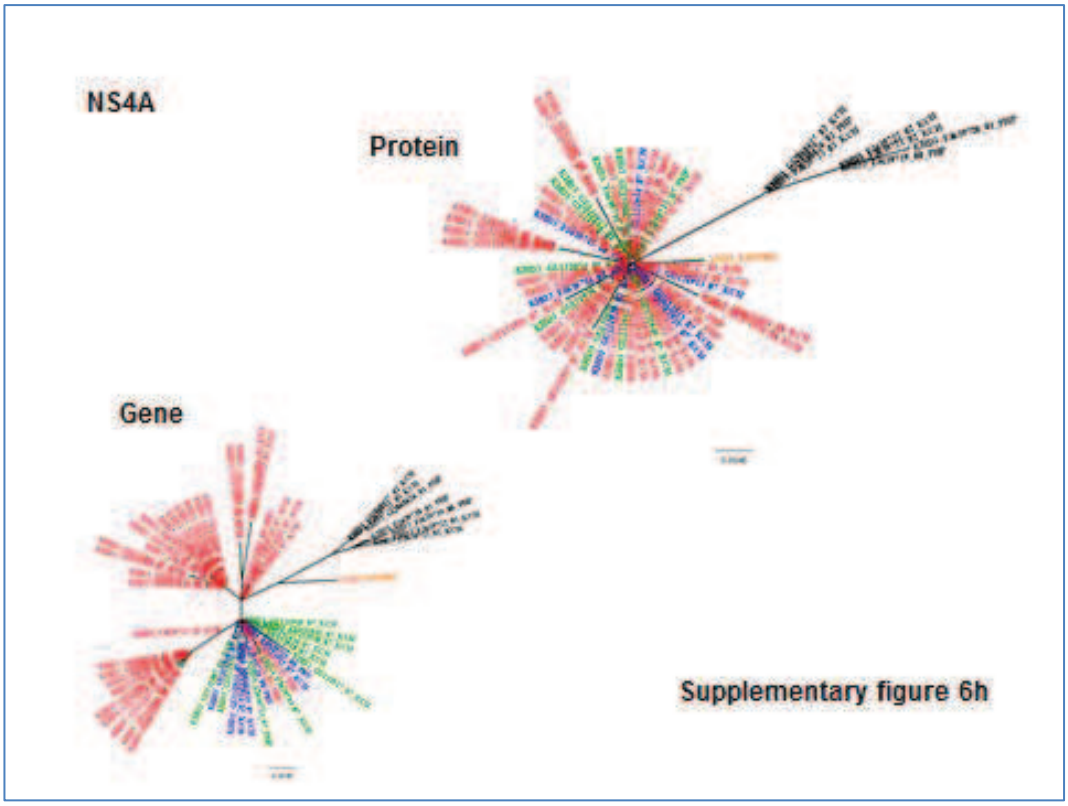
Supplementary figure 6e



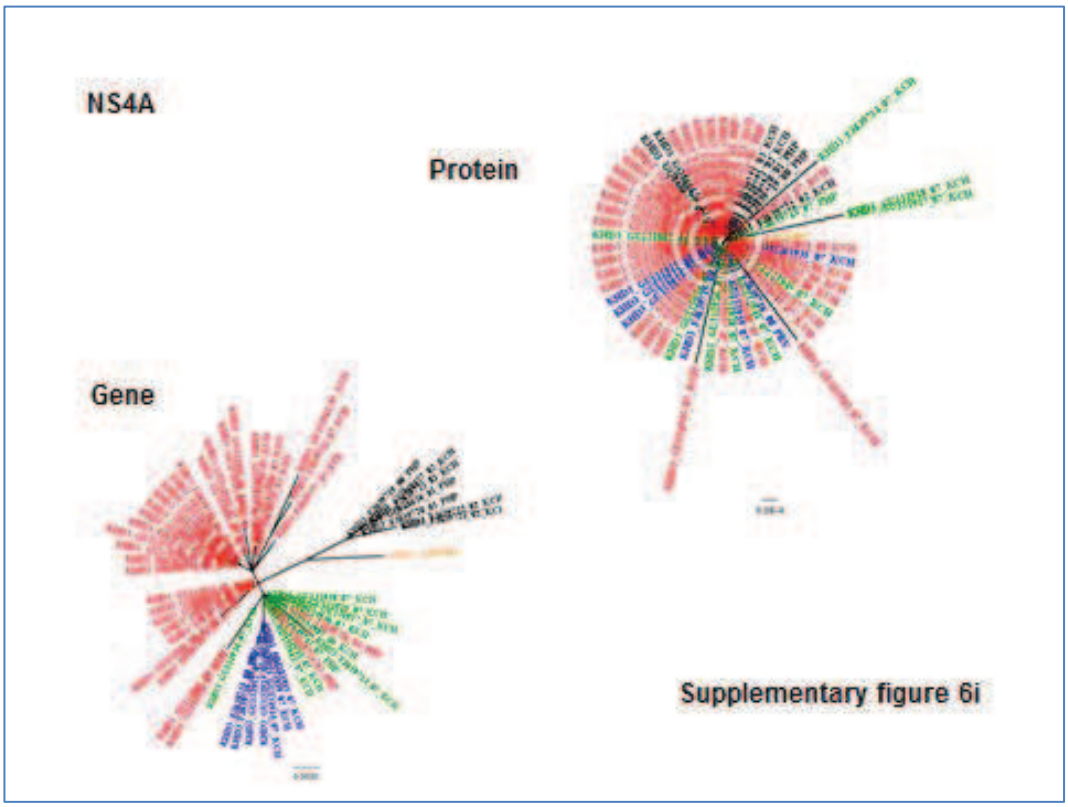
Supplementary figure 6f



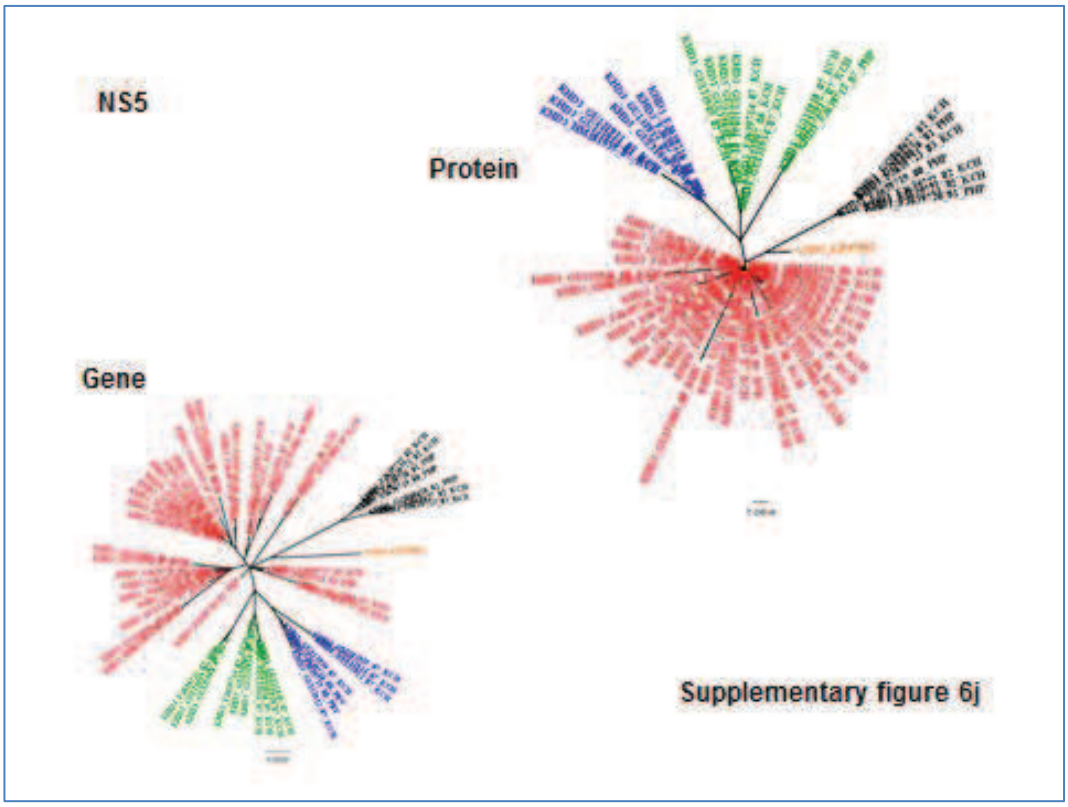
Supplementary figure 6g



Supplementary figure 6h



Supplementary figure 6i



Supplementary figure 6j

Supplementary table 1: GenBank accession number, demographic and geographic distribution of 39 DENV-2 isolates from Cambodia

Sequence ID used in this study	Lineage	Accession No.	Age (year)	Gender	Province	Year of sampling	Serotype	DOF (day)	WHO severity classification
KHD2_FJ639697_01_KCH	D2-1	FJ639697	10	MALE	Kampong Cham	2001	DENV-2	5	DF
KHD2_FJ639702_03_BTB	D2-1	FJ639702	12	MALE	Battambang	2003	DENV-2	2	DF
KHD2_FJ639703_03_BTB	D2-1	FJ639703	4	FEMALE	Battambang	2003	DENV-2	3	DHF
KHD2_FJ639698_02_KCH	D2-2	FJ639698	5	MALE	Kampong Cham	2002	DENV-2	1	DHF
KHD2_FJ639699_02_KCH	D2-2	FJ639699	9	MALE	Kampong Cham	2002	DENV-2	3	DHF
KHD2_FJ639700_02_KCH	D2-2	FJ639700	10	FEMALE	Kampong Cham	2002	DENV-2	3	DF
KHD2_FJ639701_02_KCH	D2-2	FJ639701	10	MALE	Kampong Cham	2002	DENV-2	1	DF
KHD2_FJ639704_03_KCH	D2-3	FJ639704	10	FEMALE	Kampong Cham	2003	DENV-2	2	DHF
KHD2_GQ868620_03_KCH	D2-3	GQ868620	7	MALE	Kampong Cham	2003	DENV-2	5	DHF
KHD2_GQ868621_03_SRP	D2-3	GQ868621	31	MALE	Siem Reap	2003	DENV-2	3	DHF
KHD2_FJ639707_04_PHP	D2-3	FJ639707	29	MALE	Phnom Penh	2004	DENV-2	5	DHF
KHD2_FJ639708_05_SRP	D2-3	FJ639708	3	MALE	Siem Reap	2005	DENV-2	1	DF
KHD2_FJ639710_05_PHP	D2-3	FJ639710	38	MALE	Phnom Penh	2005	DENV-2	1	DF
KHD2_FJ639711_05_PHP	D2-3	FJ639711	48	FEMALE	Phnom Penh	2005	DENV-2	2	DHF
KHD2_GU131927_07_KCH	D2-3	GU131927	14	MALE	Kampong Cham	2007	DENV-2	2	unknown
KHD2_GU131899_08_PHP	D2-3	GU131899	14	MALE	Phnom Penh	2008	DENV-2	4	DHF
KHD2_GQ868622_03_TAK	D2-4	GQ868622	13	MALE	Takeo	2003	DENV-2	3	DHF
KHD2_FJ639705_03_TAK	D2-4	FJ639705	8	MALE	Takeo	2003	DENV-2	3	DF
KHD2_FJ639706_04_PHP	D2-4	FJ639706	13	FEMALE	Phnom Penh	2004	DENV-2	4	DHF
KHD2_FJ639709_05_TAK	D2-4	FJ639709	6	FEMALE	Takeo	2005	DENV-2	3	DF
KHD2_GQ868623_05_BTB	D2-4	GQ868623	4	FEMALE	Battambang	2005	DENV-2	5	DHF
KHD2_FJ639717_07_KCH	D2-4	FJ639717	5	FEMALE	Kampong Cham	2007	DENV-2	2	DHF
KHD2_GU131896_07_KCH	D2-4	GU131896	10	MALE	Kampong Cham	2007	DENV-2	5	DHF
KHD2_GQ868638_08_KCH	D2-4	GQ868638	10	FEMALE	Kampong Cham	2008	DENV-2	4	DHF
KHD2_GU131930_08_KCH	D2-4	GU131930	4	FEMALE	Kampong Cham	2008	DENV-2	2	unknown
KHD2_GQ868624_07_BTB	D2-5	GQ868624	11	FEMALE	Battambang	2007	DENV-2	2	DHF
KHD2_GU131897_07_KCH	D2-5	GU131897	7	MALE	Kampong Cham	2007	DENV-2	3	unknown
KHD2_GQ868625_08_PHP	D2-5	GQ868625	6	MALE	Phnom Penh	2008	DENV-2	3	DHF
KHD2_GQ868631_08_TAK	D2-5	GQ868631	1	MALE	Takeo	2008	DENV-2	4	DHF
KHD2_GU131898_08_SRP	D2-5	GU131898	6	MALE	Siem Reap	2008	DENV-2	3	DHF

KHD2_FJ639718_08_PRV	D2-5	FJ639718	10	MALE	PRV	2008	DENV-2	3	DHF
KHD2_GU131900_08_SRP	D2-5	GU131900	3	FEMALE	Siem Reap	2008	DENV-2	3	DHF
KHD2_GU131901_08_SRP	D2-5	GU131901	9	FEMALE	Siem Reap	2008	DENV-2	4	DHF
KHD2_GU131902_08_BTB	D2-5	GU131902	6	FEMALE	Battambang	2008	DENV-2	2	DF
KHD2_GU131924_08_KAN	D2-5	GU131924	5	MALE	Kandal	2008	DENV-2	5	DHF
KHD2_GU131928_08_KCH	D2-5	GU131928	8	FEMALE	Kampong Cham	2008	DENV-2	2	unknown
KHD2_GU131929_08_KCH	D2-5	GU131929	15	MALE	Kampong Cham	2008	DENV-2	1	unknown
KHD2_GU131931_08_KCH	D2-5	GU131931	13	FEMALE	Kampong Cham	2008	DENV-2	1	unknown
KHD2_GU131932_08_KCH	D2-5	GU131932	7	MALE	Kampong Cham	2008	DENV-2	2	unknown

DOF: Duration after onset of fever
DENV-2: Dengue virus serotype 2
DF: Dengue fever
DHF: Dengue hemorrhage fever
DSS: Dengue shock syndrome

Supplementary table 2: GenBank accession number, demographic geographic distribution of 57 DENV-3 isolates from Cambodia

Sequence ID used in this study	Lineage	Accession No.	Age (year)	Gender	PROVINCE	Year of sampling	Serotype	DOF (days)	WHO severity classification
KHD3_FJ639719_00_PHP	D3-1	FJ639719	26	Female	PHNOM PENH	2000	DENV-3	3	DF
KHD3_FJ639720_01_PHP	D3-1	FJ639720	6	Female	PHNOM PENH	2001	DENV-3	4	DF
KHD3_GQ868626_01_PHP	D3-1	GQ868626	33	Female	PHNOM PENH	2001	DENV-3	3	DF
KHD3_FJ639721_02_KCH	D3-1	FJ639721	1	Female	KAMPONG CHAM	2002	DENV-3	2	DHF
KHD3_FJ639722_02_KCH	D3-1	FJ639722	8	Female	KAMPONG CHAM	2002	DENV-3	3	DF
KHD3_GQ868627_02_KCH	D3-1	GQ868627	12	Female	KAMPONG CHAM	2002	DENV-3	5	DF
KHD3_FJ639723_03_KCH	D3-1	FJ639723	4	Male	KAMPONG CHAM	2003	DENV-3	3	DHF
KHD3_FJ639724_03_PHP	D3-2	FJ639724	22	Female	PHNOM PENH	2003	DENV-3	5	DHF
KHD3_FJ639725_03_KSO	D3-2	FJ639725	53	Male	KAMPONG SOM	2003	DENV-3	3	DHF
KHD3_GU131906_03_KSO	D3-2	GU131906	20	Female	KAMPONG SOM	2003	DENV-3	4	DF
KHD3_FJ639726_04_PHP	D3-2	FJ639726	72	Male	PHNOM PENH	2004	DENV-3	1	DHF
KHD3_FJ639727_05_BTB	D3-2	FJ639727	5	Female	BATTAMBONG	2005	DENV-3	4	DSS
KHD3_FJ639728_05_PHP	D3-2	FJ639728	30	Male	PHNOM PENH	2005	DENV-3	5	DF
KHD3_GQ868628_05_BTB	D3-2	GQ868628	10	Female	BATTAMBONG	2005	DENV-3	4	DSS
KHD3_GQ868629_05_PHP	D3-2	GQ868629	12	Female	PHNOM PENH	2005	DENV-3	6	DHF
KHD3_GU131904_05_KCH	D3-2	GU131904	6	Female	KAMPONG CHAM	2005	DENV-3	5	DHF
KHD3_FJ639729_06_BTB	D3-2	FJ639729	13	Female	BATTAMBONG	2006	DENV-3	4	DHF
KHD3_FJ639730_06_PHP	D3-2	FJ639730	10	Male	PHNOM PENH	2006	DENV-3	5	DF
KHD3_GQ868634_06_KCH	D3-2	GQ868634	10	Female	KAMPONG CHAM	2006	DENV-3	2	DHF
KHD3_GU131908_06_KCH	D3-2	GU131908	14	Male	KAMPONG CHAM	2006	DENV-3	N/A	UD
KHD3_GU131909_06_KCH	D3-2	GU131909	4	Female	KAMPONG CHAM	2006	DENV-3	3	DF
KHD3_GU131910_06_KCH	D3-2	GU131910	6	Male	KAMPONG CHAM	2006	DENV-3	5	UD
KHD3_GU131911_06_KCH	D3-2	GU131911	6	Female	KAMPONG CHAM	2006	DENV-3	2	DF
KHD3_GU131933_06_KCH	D3-2	GU131933	11	Male	KAMPONG CHAM	2006	DENV-3	4	unknown
KHD3_HM181933_06_KCH	D3-2	HM181933	9	Female	KAMPONG CHAM	2006	DENV-3	2	DHF
KHD3_HM181934_06_KCH	D3-2	HM181934	3	Male	KAMPONG CHAM	2006	DENV-3	3	DF
KHD3_FJ639712_07_KCH	D3-2	FJ639712	9	Male	KAMPONG CHAM	2007	DENV-3	3	DF
KHD3_FJ639731_07_SRP	D3-2	FJ639731	12	Female	SIEM REAP	2007	DENV-3	3	DF
KHD3_GU131915_07_KCH	D3-2	GU131915	5	Female	KAMPONG CHAM	2007	DENV-3	5	DSS
KHD3_GU131916_07_KCH	D3-2	GU131916	4	Male	KAMPONG CHAM	2007	DENV-3	4	unknown
KHD3_GU131935_07_KCH	D3-2	GU131935	6	Female	KAMPONG CHAM	2007	DENV-3	2	unknown

KHD3_GU131937_07_KCH	D3-2	GU131937	1	Female	KAMPONG CHAM	2007	DENV-3	1	unknown
KHD3_GU131940_07_KCH	D3-2	GU131940	4	Female	KAMPONG CHAM	2007	DENV-3	1	unknown
KHD3_GU131942_07_KCH	D3-2	GU131942	3	Female	KAMPONG CHAM	2007	DENV-3	N/A	unknown
KHD3_GU131903_08_BMC	D3-2	GU131903	4	Male	BANTEAY MEAN CHHEY	2008	DENV-3	4	DHF
KHD3_GU131905_08_KCH	D3-2	GU131905	8	Female	KAMPONG CHAM	2008	DENV-3	1	DF
KHD3_GU131907_06_KCH	D3-3	GU131907	13	Male	KAMPONG CHAM	2006	DENV-3	4	DHF
KHD3_FJ639713_07_PHP	D3-3	FJ639713	30	Female	PHNOM PENH	2007	DENV-3	2	DF
KHD3_FJ639714_07_KCH	D3-3	FJ639714	8	Male	KAMPONG CHAM	2007	DENV-3	3	DHF
KHD3_GU131917_07_KCH	D3-3	GU131917	4	Male	KAMPONG CHAM	2007	DENV-3	5	DF
KHD3_GU131918_07_KCH	D3-3	GU131918	3	Male	KAMPONG CHAM	2007	DENV-3	4	DF
KHD3_GU131934_07_KCH	D3-3	GU131934	5	Female	KAMPONG CHAM	2007	DENV-3	4	unknown
KHD3_GU131936_07_KCH	D3-3	GU131936	5	Female	KAMPONG CHAM	2007	DENV-3	2	unknown
KHD3_GU131938_07_KCH	D3-3	GU131938	6	Female	KAMPONG CHAM	2007	DENV-3	N/A	unknown
KHD3_GU131941_07_KCH	D3-3	GU131941	0.4	Male	KAMPONG CHAM	2007	DENV-3	3	unknown
KHD3_GU131945_07_KCH	D3-3	GU131945	2	Female	KAMPONG CHAM	2007	DENV-3	1	unknown
KHD3_GU131912_07_KCH	D3-4	GU131912	3	Female	KAMPONG CHAM	2007	DENV-3	4	DF
KHD3_GU131913_07_KCH	D3-4	GU131913	7	Female	KAMPONG CHAM	2007	DENV-3	2	DHF
KHD3_GU131914_07_KCH	D3-4	GU131914	11	Female	KAMPONG CHAM	2007	DENV-3	4	unknown
KHD3_GU131939_07_KCH	D3-4	GU131939	3	Male	KAMPONG CHAM	2007	DENV-3	1	unknown
KHD3_HM181935_07_KCH	D3-4	HM181935	3	Female	KAMPONG CHAM	2007	DENV-3	3	DF
KHD3_FJ639715_08_PRV	D3-4	FJ639715	18	Female	PREY VENG	2008	DENV-3	2	DF
KHD3_FJ639716_08_PRV	D3-4	FJ639716	3	Female	PREY VENG	2008	DENV-3	2	DF
KHD3_GU131943_07_KCH	Atypic	GU131943	6	Female	KAMPONG CHAM	2007	DENV-3	1	unknown
KHD3_GU131944_07_KCH	Atypic	GU131944	3	Male	KAMPONG CHAM	2007	DENV-3	2	unknown
KHD3_GU131946_08_KCH	Atypic	GU131946	2	Male	KAMPONG CHAM	2008	DENV-3	2	unknown
KHD3_HM631854_08 KCH	Atypic	HM631854	7	Female	KAMPONG CHAM	2008	DENV-3	2	unknown

DOF: Duration after onset of fever
N/A: Not available
DENV-3: Dengue virus serotype 3
DF: Dengue fever
DHF: Dengue hemorrhage fever
DSS: Dengue shock syndrome
UD: Unable to determine

Supplementary table 3. Lineage-specific non-synonymous mutations in DENV-2 and DENV-3

Protein	DENV-2 mutations	DENV-2 lineage s	DENV-3 mutations	DENV-3 lineages
C	None	None	None	None
E	G228E	D2-3 + D2-5	None	None
	K160M	D2-5	None	None
prM	None	None	None	None
NS1	H129Y	D2-3 + D2-5	S17N	D3-3 + D3-4
	T293A	D3-3 +D3-4		
NS2A	I120V	D2-5 + part of D2-3	R217K	D3-4
	T65A	D2-3 + D2-5		
NS2B	V112I	D2-3 et D2-5	None	None
NS3	None	None	None	None
NS4A	None	None	None	None
NS4b	None	None	None	None
NS5	I27T	D2-4	T188N	D3-4
	Q286E	D2-5	R436K	D3-3 + D3-4
	V637A	D2-4 + 2 D2-2 isolates	K630E	D3-4 + D3-5
			T649N	D3-1
			I789V	D3-1
			R173K	D3-1
			Y482F	D3-1

Supplementary table 4. Polymorphism analysis and neutrality tests for the different DENV-2 lineages

Gene C

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	16	40	40	14	26	13	27	0.0236	9.461	-0.55102 ^{NS}	-0.81967 ^{NS}	-0.86273 ^{NS}
Pop1	3	2	1	1	1	0	0	1	0.0020	0.667	NA	NA	NA
Pop2	4	1	0	0	0	0	0	0	0.0000	0.000	NA	NA	NA
Pop3	9	5	8	8	1	2	8	8	0.0057	2.944	-1.54052 ^{NS}	-1.59647 ^{NS}	-1.76646 ^{NS}
Pop4	9	3	4	4	4	0	2	2	0.0026	1.472	-1.60974 ^{NS}	-1.79883 ^{NS}	-1.94803 ^{NS}
Pop5	14	6	6	6	4	2	2	4	0.0032	1.887	-1.49893 ^{NS}	-1.24773 ^{NS}	-1.49993 ^{NS}

Gene E

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	33	201	205	34	167	26	176	0.0330	48.487	0.04169 ^{NS}	0.57401 ^{NS}	0.45707 ^{NS}
Pop1	3	3	5	5	5	0	0	5	0.0022	3.333	NA	NA	NA
Pop2	4	3	4	4	3	1	0	4	0.0015	2.182	0.06501 ^{NS}	-0.06501 ^{NS}	-0.06004 ^{NS}
Pop3	9	7	50	50	34	16	6	44	0.0102	18.397	-0.91056 ^{NS}	-1.04922 ^{NS}	-1.14046 ^{NS}
Pop4	9	7	26	26	7	19	5	21	0.0063	9.566	-0.09248 ^{NS}	0.55626 ^{NS}	0.44776 ^{NS}
Pop5	14	13	23	23	16	7	4	19	0.0029	7.232	-1.75241	-1.66052	-1.93313

Gene prM

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	22	65	67	11	54	6	61	0.0333	15.847	0.17325 ^{NS}	0.63316 ^{NS}	0.63316 ^{NS}
Pop1	3	1	0	0	0	0	0	0	0.0000	0	NA	NA	NA
Pop2	4	2	3	3	3	0	0	3	0.0030	1.636	-0.75445 ^{NS}	-0.75445^{NS}	-0.67466 ^{NS}
Pop3	9	7	14	14	11	3	1	13	0.0076	5.151	-1.27970 ^{NS}	-1.36983 ^{NS}	-1.50855 ^{NS}
Pop4	9	5	9	9	1	8	0	9	0.0080	3.311	0.95701 ^{NS}	1.06477 ^{NS}	1.16002 ^{NS}
Pop5	14	7	10	10	9	1	2	8	0.0031	3.144	-1.97509*	-2.35691*	-2.57929*

Gene NS1

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	34	142	147	29	113	23	124	0.0334	34.769	0.04909 ^{NS}	0.21173 ^{NS}	0.18317 ^{NS}
Pop1	3	2	2	2	2	0	0	2	0.0013	1.333	NA	NA	NA
Pop2	4	3	4	4	4	0	3	1	0.0019	2.182	-0.78012 ^{NS}	-0.78012 ^{NS}	-0.72052 ^{NS}
Pop3	9	8	38	38	26	12	6	32	0.0110	13.982	-0.85765 ^{NS}	-1.05643 ^{NS}	-1.13168 ^{NS}
Pop4	9	8	18	18	10	8	1	17	0.0058	6.623	-0.33658 ^{NS}	-0.33658 ^{NS}	-0.54334 ^{NS}
Pop5	14	13	21	21	17	4	4	17	0.0034	6.603	-1.91940*	-2.17215 ^{NS}	-2.41210 ^{NS}

Gene NS2A

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	26	128	131	33	95	19	109	0.0430	30.895	-0.33886 ^{NS}	-0.14117 ^{NS}	-0.25179 ^{NS}
Pop1	3	2	4	4	4	0	1	3	0.0041	2.667	NA	NA	NA
Pop2	4	2	6	6	6	0	2	4	0.0046	3.273	-0.80861 ^{NS}	-0.80861 ^{NS}	-0.77723 ^{NS}
Pop3	9	7	26	27	18	8	4	23	0.0128	9.934	-0.79074 ^{NS}	-1.11612 ^{NS}	-1.16344 ^{NS}
Pop4	9	8	13	13	9	4	5	8	0.0054	4.783	-1.22450 ^{NS}	-1.01879 ^{NS}	-1.19513 ^{NS}
Pop5	14	7	13	13	11	2	2	11	0.0032	4.088	-1.95516*	-2.21625 ^{NS}	-2.45722*

Gene NS2B

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	26	60	62	19	41	7	55	0.0338	14.665	-0.36883	-0.63908	-0.64725
Pop1	3	2	1	1	1	0	0	1	0.0017	0.667	NA	NA	NA
Pop2	4	3	2	2	2	0	0	2	0.0026	1.091	-070990 ^{NS}	-0.70990 ^{NS}	-0.60427 ^{NS}
Pop3	9	8	20	20	17	3	5	15	0.0135	7.359	-1.39066 ^{NS}	-1.65097^{NS}	-1.77947^{NS}
Pop4	9	5	4	4	2	2	0	4	0.0036	1.472	-0.22884 ^{NS}	-0.26418 ^{NS}	-0.28409 ^{NS}
Pop5	14	8	11	11	6	5	2	9	0.0056	3.459	-1.46211 ^{NS}	-0.88155 ^{NS}	-1.18688 ^{NS}

Gene NS3

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	34	239	245	49	190	18	224	0.0297	57.949	-0.19192 ^{NS}	0.22703 ^{NS}	0.22703 ^{NS}
Pop1	3	3	12	12	12	0	1	11	0.0043	8.000	NA	NA	NA
Pop2	4	4	8	8	7	1	0	8	0.0023	4.364	-0.44637 ^{NS}	-0.44637 ^{NS}	-0.43935 ^{NS}
Pop3	9	8	61	61	47	14	6	55	0.0094	22.444	-1.44098^{NS}	-1.14083 ^{NS}	-1.51326 ^{NS}
Pop4	9	8	38	39	19	19	3	36	0.0065	14.349	-0.78975 ^{NS}	-0.38689 ^{NS}	-0.54386 ^{NS}
Pop5	14	11	17	17	12	5	1	16	0.0016	5.346	-1.78462*	-1.65774 ^{NS}	-1.93908 ^{NS}

Gene NS4A

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	20	52	53	9	43	7	46	0.0346	12.536	0.18647 ^{NS}	0.57531 ^{NS}	0.52265 ^{NS}
Pop1	3	2	4	4	4	0	1	3	0.0070	2.667	NA	NA	NA
Pop2	4	2	1	1	1	0	0	1	0.0013	0.545	-0.61237^{NS}	-0.61237^{NS}	-0.47871^{NS}
Pop3	9	6	13	13	8	5	0	13	0.0098	4.783	-1.05826 ^{NS}	-0.73727 ^{NS}	-0.91129 ^{NS}
Pop4	9	9	10	10	6	4	2	8	0.0076	3.679	-1.00013 ^{NS}	-0.66259 ^{NS}	-0.83097 ^{NS}
Pop5	14	1	0	0	0	0	0	0	0.0000	0.000	NA	NA	NA

Gene NS4B

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	31	115	124	25	90	8	118	0.0352	29.329	-0.44291^{NS}	0.07443^{NS}	-0.13019^{NS}
Pop1	3	3	7	7	7	0	0	7	0.0064	4.667	NA	NA	NA
Pop2	4	3	2	2	1	1	0	2	0.0016	1.091	0.59158^{NS}	0.59158^{NS}	0.50356^{NS}
Pop3	9	8	33	33	20	13	3	26	0.0147	12.142	-0.56557^{NS}	-0.74684^{NS}	-0.78875^{NS}
Pop4	9	9	15	15	8	7	1	14	0.0066	5.519	-0.59953^{NS}	-0.44255^{NS}	-0.53775^{NS}
Pop5	14	8	11	11	10	1	1	10	0.0023	3.459	-2.01307*	-2.43156*	-2.65415*

Gene NS5

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	39	36	362	371	63	299	51	314	0.0319	87.750	-0.06704^{NS}	0.46870^{NS}	0.32921^{NS}
Pop1	3	2	13	13	13	0	3	10	0.0032	8.667	NA	NA	NA
Pop2	4	4	6	6	4	2	2	4	0.0012	3.273	0.17969^{NS}	0.17969^{NS}	0.17272^{NS}
Pop3	9	8	85	85	54	31	11	74	0.0097	31.275	-0.82305^{NS}	-0.88328^{NS}	-0.97610^{NS}
Pop4	9	9	49	50	27	22	7	43	0.0057	18.397	-0.82588^{NS}	-0.57602^{NS}	-0.71489^{NS}
Pop5	14	13	35	35	26	9	7	28	0.0024	11.006	-1.78966*	-1.93907^{NS}	-2.17908^{NS}

Supplementary table 5. Polymorphism analysis and neutrality tests for the different DENV-3 lineages

Gene C

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	21	22	24	8	14	6	18	0.0072	5.204	-1.68605^{NS}	-1.57311^{NS}	-1.91949^{NS}
Pop1	7	5	6	7	0	6	1	6	0.0095	2.857	0.69027^{NS}	1.04417^{NS}	1.05357^{NS}
Pop2	33	13	14	14	9	5	4	10	0.0045	3.450	-1.82485*	-2.33425^{NS}	-2.55229*
Pop3	10	4	3	3	2	1	1	2	0.0025	1.060	-0.65748^{NS}	-0.80490^{NS}	-0.85947^{NS}
Pop4	7	3	3	3	3	0	1	2	0.0025	1.225	-1.35841^{NS}	-1.42725^{NS}	-1.52246^{NS}

Gene E

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	45	136	139	54	82	21	118	0.0104	48.487	-1.73093^{NS}	-1.83205^{NS}	-2.14740^{NS}
Pop1	7	7	14	14	6	8	4	10	0.0040	5.714	0.18346^{NS}	0.15907^{NS}	0.18092^{NS}
Pop2	33	23	77	79	38	39	10	69	0.0065	19.465	-1.89503*	-1.81114^{NS}	-2.18003^{NS}
Pop3	10	9	28	28	16	12	3	25	0.0061	9.898	-0.39113^{NS}	-0.72324^{NS}	-0.72260^{NS}
Pop4	7	6	20	21	14	6	3	18	0.0050	8.571	-0.78156^{NS}	-0.81418^{NS}	-0.88973^{NS}

Gene prM

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	31	45	45	16	29	8	37	0.0095	9.758	-1.74438^{NS}	-1.21379^{NS}	-1.68424^{NS}
Pop1	7	6	5	5	3	2	2	3	0.0039	2.041	-0.33025^{NS}	-0.37037^{NS}	-0.39280^{NS}
Pop2	33	17	23	23	12	11	3	20	0.0045	5.667	-2.10727*	-1.76988^{NS}	-2.21647^{NS}
Pop3	10	5	6	6	4	2	1	5	0.0035	2.121	-0.75600^{NS}	-0.93878^{NS}	-1.00285^{NS}
Pop4	7	5	5	5	3	2	0	5	0.0037	2.041	-0.56143^{NS}	-0.37037^{NS}	-0.44892^{NS}

Gene NS1

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	46	97	97	44	53	23	74	0.0093	21.035	-1.86886*	-2.28425^{NS}	-2.54382*
Pop1	7	5	9	9	2	7	1	8	0.0042	3.673	1.02361^{NS}	0.81778^{NS}	0.94592^{NS}
Pop2	33	28	70	70	42	28	18	52	0.0072	17.248	-2.08521*	-2.59403*	-2.6901*
Pop3	10	7	10	10	6	4	4	6	0.0031	3.535	-0.33629^{NS}	-0.76777^{NS}	-0.74352^{NS}
Pop4	7	6	21	21	14	7	5	16	0.0070	8.571	-0.75029^{NS}	-0.65134^{NS}	-0.74273^{NS}

Gene NS2A

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	37	60	61	28	32	10	51	0.0083	13.228	-2.02342*	-2.23301^{NS}	-2.57510*
Pop1	7	5	8	8	5	3	3	5	0.0047	3.265	-0.35022^{NS}	-0.47334^{NS}	-0.48748^{NS}
Pop2	33	22	43	43	23	20	6	37	0.0071	10.595	-2.03671*	-2.01885^{NS}	-2.39487^{NS}
Pop3	10	6	6	6	4	2	1	5	0.0025	2.121	-0.97273^{NS}	-0.93878^{NS}	-1.06255^{NS}
Pop4	7	5	6	6	4	2	1	5	0.0032	2.449	-0.73384^{NS}	-0.58502^{NS}	-0.67369^{NS}

Gene NS2B

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	26	27	28	14	13	1	27	0.0078	6.072	-1.61513*	-2.61201*	-2.68262*
Pop1	7	6	4	4	2	2	0	4	0.0039	1.633	-0.31870^{NS}	-0.06863^{NS}	-0.13477^{NS}
Pop2	33	13	13	14	9	4	0	14	0.0047	3.450	-1.53549^{NS}	-2.76313*	-2.78792*
Pop3	10	5	7	7	3	4	1	6	0.0067	2.474	0.21500^{NS}	-0.12602^{NS}	-0.04729^{NS}
Pop4	7	4	5	5	4	1	0	5	0.0041	2.041	-1.02379^{NS}	-0.96867^{NS}	-1.06618^{NS}

Gene NS3

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	46	163	166	68	95	17	149	0.0088	35.997	-1.93959*	-2.04104^{NS}	-2.39795^{NS}
Pop1	7	7	20	20	6	14	1	19	0.0049	8.163	0.67374^{NS}	0.60105^{NS}	0.68057^{NS}
Pop2	33	24	104	105	57	47	10	95	0.0057	25.872	-2.21548**	-2.29101^{NS}	-2.68607*
Pop3	10	8	17	17	11	6	3	14	0.0027	6.009	-0.76376^{NS}	-0.99529^{NS}	-1.05670^{NS}
Pop4	7	7	32	32	20	12	4	28	0.0064	13.061	-0.54789^{NS}	-0.51746^{NS}	-0.57938^{NS}

Gene NS4A

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	27	38	38	14	24	9	29	0.0113	8.240	-1.59750^{NS}	-1.29499^{NS}	-1.67763^{NS}
Pop1	7	6	8	8	5	3	2	6	0.0080	3.265	-0.35022^{NS}	-0.47334^{NS}	-0.48748^{NS}
Pop2	33	15	19	19	5	14	4	15	0.0082	4.682	-1.13415^{NS}	-0.05793^{NS}	-0.47775^{NS}
Pop3	10	6	9	9	8	1	0	9	0.0051	3.181	-1.68719^{NS}	-1.81276^{NS}	-2.00290^{NS}
Pop4	7	3	4	4	3	1	1	3	0.0035	1.633	-0.87642^{NS}	-0.78927^{NS}	-0.87602^{NS}

Gene NS4B

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	36	57	58	21	36	4	54	0.0083	12.577	-1.76216^{NS}	-1.47715^{NS}	-1.89336^{NS}
Pop1	7	5	6	6	6	0	0	6	0.0042	2.449	1.24188^{NS}	1.46255*	1.53986^{NS}
Pop2	33	21	37	37	18	19	1	36	0.0059	9.117	-1.88489*	-1.64320^{NS}	-2.03725^{NS}
Pop3	10	5	8	8	7	1	2	6	0.0024	2.828	-1.63600^{NS}	-1.73270^{NS}	-1.91991^{NS}
Pop4	7	6	11	11	8	3	1	10	0.0052	4.490	-0.81948^{NS}	-0.82346^{NS}	-0.90255^{NS}

Gene NS5

	N	Hp	S	η	Si	Pa	Na	Ns	π	θ	Tajima's D	D*	F*
All	57	49	226	235	84	142	35	197	0.0093	50.960	-1.81083*	-1.58527^{NS}	-2.00509^{NS}
Pop1	7	7	18	18	6	12	2	16	0.0031	7.347	0.78606^{NS}	0.48465^{NS}	0.60840^{NS}
Pop2	33	27	152	155	77	75	22	155	0.0066	38.191	-2.02904*	-1.94442^{NS}	-2.34151^{NS}
Pop3	10	9	37	37	17	20	8	29	0.0048	13.079	-0.00456*	-0.27360^{NS}	-0.23303^{NS}
Pop4	7	6	48	48	18	30	7	41	0.0065	19.592	-0.63783^{NS}	-0.52316^{NS}	-0.60747^{NS}

Supplementary table 6. Ka/Ks ratio and nucleotide substitution for DENV-2 isolates

Gene	Population	Na	Ns	Ka	Ks	Ka/Ks
C	1	0	1	0.00000	0.00812	0.000
	2	0	0	0.00000	0.00000	0.000
	3	8	8	0.00411	0.01096	0.375
	4	2	2	0.00173	0.00548	0.315
	5	2	4	0.00111	0.01006	0.110
E	1	0	5	0.00000	0.00991	0.000
	2	0	4	0.00000	0.00643	0.000
	3	6	44	0.00180	0.03949	0.045
	4	5	21	0.00170	0.02226	0.076
	5	4	19	0.00069	0.01021	0.068
prM	1	0	0	0.00000	0.00000	0.000
	2	0	3	0.00000	0.01301	0.000
	3	1	13	0.00146	0.02782	0.053
	4	0	9	0.00000	0.03492	0.000
	5	2	8	0.00075	0.01081	0.070
NS1	1	0	2	0.00000	0.00577	0.000
	2	3	1	0.00182	0.00216	0.844
	3	6	32	0.00236	0.04280	0.055
	4	1	17	0.00067	0.02461	0.027
	5	4	17	0.00069	0.01308	0.053
NS2A	1	1	3	0.00137	0.01210	0.113
	2	2	4	0.00206	0.01208	0.170
	3	4	23	0.00307	0.04291	0.072
	4	5	8	0.00262	0.01396	0.187
	5	2	11	0.00058	0.01109	0.053

NS2B	1	0	1	0.00000	0.00703	0.000
	2	0	2	0.00000	0.01056	0.000
	3	5	15	0.00494	0.04085	0.121
	4	0	4	0.00000	0.01446	0.000
	5	2	9	0.00097	0.01989	0.049
NS3	1	1	11	0.00047	0.01716	0.027
	2	0	8	0.00000	0.00971	0.000
	3	6	55	0.00105	0.03805	0.028
	4	3	36	0.00070	0.02632	0.027
	5	1	16	0.00010	0.00676	0.015
NS4A	1	1	3	0.00233	0.02146	0.109
	2	0	1	0.00000	0.00532	0.000
	3	0	13	0.00000	0.03974	0.000
	4	2	8	0.00156	0.02584	0.060
	5	0	0	0.00000	0.00000	0.000
NS4B	1	0	7	0.00000	0.02607	0.000
	2	0	2	0.00000	0.00646	0,000
	3	3	26	0.00456	0.04685	0.097
	4	1	14	0.00040	0.02574	0.016
	5	1	10	0.00026	0.00856	0.030
NS5	1	3	10	0.00095	0.01119	0.085
	2	2	4	0.00048	0.00390	0.122
	3	11	74	0.00156	0.03941	0.040
	4	7	43	0.00074	0.02353	0.031
	5	7	28	0.00059	0.00876	0.067

Supplementary table 7. Ka/Ks ratio and nucleotide substitution for DENV-3 isolates

Gene	Lineage	Na	Ns	Ka	Ks	Ka/Ks
C	1	0	6	0.00218	0.03428	0.064
	2	4	10	0.00136	0.01490	0.091
	3	1	2	0.00076	0.00843	0.090
	4	2	2	0.00109	0.00721	0.151
E	1	4	10	0.00126	0.01318	0.095
	2	10	69	0.00092	0.02532	0.037
	3	3	25	0.00082	0.02412	0.034
	4	3	18	0.00101	0.01841	0.055
prM						
	1	2	3	0.00152	0.01135	0.134
	2	3	20	0.00063	0.01705	0.037
	3	1	5	0.00053	0.01310	0.041
	4	0	5	0.00000	0.01549	0.000
NS1	1	1	8	0.00035	0.01757	0.020
	2	18	52	0.00205	0.02555	0.080
	3	4	6	0.00150	0.00870	0.173
	4	5	16	0.00197	0.02514	0.079
NS2A	1	3	5	0.00211	0.01292	0.163
	2	6	37	0.00084	0.02792	0.030
	3	1	5	0.00040	0.00922	0.044
	4	1	5	0.00057	0.01179	0.049
NS2B	1	0	4	0.00000	0.01620	0.000
	2	0	14	0.00000	0.01949	0.000
	3	1	6	0.00181	0.02215	0.082
	4	0	5	0.00000	0.01724	0.000

NS3	1	1	19	0.00040	0.02023	0.020
	2	10	95	0.00086	0.02227	0.039
	3	3	14	0.00076	0.00920	0.083
	4	4	28	0.00100	0.02449	0.041
NS4A	1	2	6	0.00297	0.02429	0.122
	2	4	15	0.00178	0.02886	0.062
	3	0	9	0.00000	0.02160	0.000
	4	1	3	0.00099	0.01150	0.086
NS4B	1	0	6	0.00000	0.01640	0.000
	2	1	36	0.00011	0.02314	0,005
	3	2	6	0.00102	0.00641	0.159
	4	1	10	0.00053	0.01893	0.028
NS5	1	2	16	0.00054	0.01232	0.044
	2	22	155	0.00082	0.02752	0.032
	3	8	29	0.00142	0.01714	0.083
	4	7	41	0.00113	0.02577	0.044

Recent molecular evolution and epidemiology of dengue virus in Cambodia

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23 **ABSTRACT**

INTRODUCTION

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. While most patients recover following a self-limiting non-severe clinical course, a small proportion progress to severe disease, largely characterized by plasma leakage with or without hemorrhage (Duong et al., 2009; WHO, 2009). It is estimated that there are between 50 and 100 million cases of dengue each year, of which up to 500 000 are severe life-threatening infections, affecting more than 100 countries with epidemics especially common in Southeast Asia (Gubler, 2002; WHO, 2009).

Dengue virus (DENV) is comprised of four genetically and antigenically distinct serotypes (denoted DENV-1, -2, -3, and -4) classified within the family *Flaviviridae*, genus *Flavivirus* (Calisher et al., 1989).The DENV genome is a single stranded positive-sense RNA of approximately 11 kb in length encoding a single open reading frame (Lindenbach and Rice, 2003). The translated protein is cleaved by host and virus derived proteases to produce 3 structural proteins (capsid, premembrane/membrane, envelope; C, prM/M, E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach and Rice, 2003; Weaver and Vasilakis, 2009). The single open reading frame (ORF) is flanked by 5' untranslated region (UTR) capped with type I 7-methyl guanosine structure and by 3' UTR lacked of poly(A) region (Lindenbach and Rice, 2003).

Gene sequence data has enabled the classification of each DENV serotype into a number of genotypes, initially based on genetic variation within the E/NS1 junction region (Rico-Hesse, 1990), but more recently incorporating complete E gene or even complete genome sequence data (Holmes and Twiddy, 2003; Klungthong et al., 2004; Lanciotti et al., 1997; Rico-Hesse, 2003; Weaver and Vasilakis, 2009). Indeed, a variety of phylogenetic analyses have indicated that (i) DENV-1 can be grouped into five genotypes (denoted I to V), (i) DENV-2 comprises 6 genotypes: SE Asian/American, Asian I, Asian II, Cosmopolitan, American and Sylvatic, (iii) DENV-3 comprises 4 genotypes (I to IV), and (iv) DENV-4 can be classified into 4 genotypes (I, II, III and Sylvatic) (Holmes and Twiddy, 2003). Importantly, these genotypes have different geographic distributions. For example, in South-East Asia, genotype I of DENV-1 (Schreiber et al., 2009; Zhang et al., 2005), genotype Asian/American and Asian 1 of

DENV-2 (Vu et al., 2010), genotype I and II of DENV-3 (Zhang et al., 2005) and genotype I and III of DENV-4 (Klungthong et al., 2004; Lanciotti et al., 1997) were the most common strains observed in recent circulation.

Previous studies have shown that dengue genotypes are not fixed entities, as the genetic diversity within viral populations changes through time (Holmes et al., 1999; Tolou et al., 2001; Worobey et al., 1999). In particular, a process of 'lineage replacement' has been observed on a regular basis, such that new lineages are created whereas others die out, and sometimes involving entire clades of viruses (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002; Zhang et al., 2005). Currently, two hypotheses have been put forward to explain the mechanism of lineage replacement: either that they reflect the selective elimination or favoring of specific strains in the population, such as those better adapted to local host or vector populations are able to proliferate, including the evasion of cross-protective herd immunity (Sittisombut et al., 1997; Vu et al., 2010; Wittke et al., 2002), or purely stochastic processes such as caused by the regular random bottlenecks in the size of the mosquito population that occur during the annual dry season (Scott et al., 2000).

In Cambodia, dengue is considered an endemic disease with an epidemic seasonality during the rainy season from May to November. Dengue virus was first isolated in Cambodia in 1963 (Hahn and Chastel, 1970) and dengue fever has been reported through passive surveillance since 1980. In 2000 surveillance was enhanced to include laboratory diagnosis for a sample of patients with suspected dengue and, in 2001, with the introduction of active sentinel surveillance implemented by the National Dengue Control Program (NDCP). The long-term trend was characterized by a cyclical pattern of epidemics at intervals of about 3–4 years (Huy et al., 2010). Since virological testing started in 2000, all four dengue virus serotypes have been observed to be in circulation each year, with DENV-2 and DENV-3 being predominant (Figure 1). Notably, the predominant circulating serotype changed from DENV-3 to DENV-2 in 2002 and then switched back to DENV-3 four years later (Huy et al., 2010).

Although it is 50 years since DENV was first isolated in Cambodia, relatively little is known about the molecular evolution and epidemiology of the virus in this country. In particular, which virus genotypes contribute most to the genetic diversity of the virus, and how they

have changed through time, is unclear. Using DENV strains from the collection of NDCP, we present the first in-depth study the recent evolution of the 4 DENV serotypes in Cambodia sampled over a period of 13 years.

MATERIAL AND METHODS

Virus strains

Dengue virus strains were obtained from patients recruited by the NDCP at 5 sentinel sites since the establishment of virological surveillance in 2000 (and since 1998 for some DENV-4 strains) and by a dengue cohort study in Kampong Cham province (Vong et al., 2010). The cohort study was approved by the National Ethics Committee for Health Research and a written informed consent was provided by the patients (or their parents/guardians) enrolled in the study. Sample collection methods and diagnostic testing including serological, molecular and virological tests were described elsewhere (Buchy et al., 2005; Huy et al., 2010). All strains were isolated after inoculation of the patient's sera onto C6/36 mosquito cells (*Aedesalbopictus* clone) and cultures were passaged a maximum of two times. The strains included in the study were selected randomly by geographical origin, year of sampling, and severity of the disease according to former WHO criteria (WHO, 2009), retaining patient anonymity in all cases.

DENV genome sequencing of DENV-1, DENV-2 and DENV-3

Cambodian viral genomes were sequenced using the Broad Institute's capillary sequencing (Applied Biosystems) directed amplification viral sequencing pipeline (http://www.broadinstitute.org/annotation/viral/Dengue; (Vu et al., 2010)). Briefly, viral RNA was extracted from low passage cell culture supernatants using the QIAmp viral RNA mini kit (Qiagen). The genome was reverse-transcribed to cDNA with SuperScript III reverse transcriptase (Invitrogen, California, USA), random hexamers (Roche Diagnostics GmbH, Mannheim, Germany) and a specific oligonucleotide targeting the 3` end of the target genome sequences (5` – AGA ACC TGT TGA TTC AAC AGC AC – 3`; nt 10700 – 10722). The cDNA obtained was then amplified using a high fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and a pool of specific primers to produce 14 overlapping amplicons of 1.5 to 2 kb in size for a physical coverage of 2X. Amplicons were then sequenced in the forward and reverse direction using primer panels consisting of 96 specific primer pairs, tailed with M13 forward and reverse primer sequence, that produce 500–700 bp amplicons from the target viral genome. Amplicons were then sequenced in the forward

and reverse direction using M13 primer. Total coverage delivered post amplification and sequencing was about 8-fold. Resulting sequence reads were assembled *de novo* and annotated using the Broad Institute's in-house viral assembly and annotation algorithms.

This sequencing effort was part of the Broad Institute's Genome Resources in Dengue Consortium (GRID) project. Of all 301 samples selected, 82/101 DENV-1, 39/78 DENV-2 and 57/98 DENV-3 were sequenced and the full genome sequences used in this study are available in GenBank (Supplementary Table 1). Unfortunately, none of our DENV-4 strains (0/24) were able to be fully sequenced using this approach, so that the E gene in isolation was used in the analysis of this virus (see below).

Sequencing and analysis of E gene of DENV-2 and DENV-4.

In brief, RNA was extracted using the QIAmp viral RNA mini kit (Qiagen) from low passage cell culture supernatants as template. Next, the nucleotide sequence of the E gene was amplified in 3 overlapping amplimers of between 502 to 772 nt for DENV-2 and 855 to 1191 nt for DENV-4 in length. Amplification was done by RT-PCR using QIAGEN OneStep RT-PCR (Qiagen). PCR primers and reaction conditions used in the RT-PCR amplification of DENV-2 and DENV-4 are detailed in Supplementary Table 2 and 3 in the supplemental material. DNA sequencing was performed in an ABI 3730XL Analyzer (96 capillary type) using the ABI Prism BigDyeTM Terminator Cycle Sequencing Kit according to the manufacturer's instructions (Macrogen, Seoul, South Korea) using specific primers. The resulting sequence reads were assembled using the CLC Main Workbench 5.5 package (CLC bio A/S, Aarhus, Denmark). All E gene sequences of 100 DENV-2 and 64 DENV-4 newly determined have been deposited in GenBank and assigned accession numbers (Supplementary Table 4)

Evolutionary analysis

Multiple sequence alignment was conducted using the MUSCLE program (Edgar, 2004) available in the Seaview package (Galtier et al., 1996; Gouy et al., 2010). Phylogenetic analysis was conducted on complete coding region of the genome sequences of DENV-1 (82) and DENV-3 (57) combined with reference sequences collected from GenBank. To place DENV-2 in Cambodia in a broader phylogenetic context and to complete the missing DENV-4 sequences, a phylogenetic analysis of the E gene from a total of 139 DENV-2 and 64 DENV-4

strains was performed together with references sequences available in GenBank, covering the major genotypes of each DENV serotype. This compilation process resulted in data sets of the following sizes: (i) 143 and 166 complete coding region of genome sequences of DENV-1 and DENV-3, respectively, and (ii) 231 and 125 E gene sequences of DENV-2 and DENV-4, respectively. To determine the best fit model of nucleotide substitution, we used the MODELTEST program (Posada and Crandall, 1998). Phylogenetic analysis was performed using the maximum likelihood (ML) approach available within the PHYML package (Guindon and Gascuel, 2003) and incorporating the GTR+Γ_4 model of nucleotide substitution. A bootstrap resampling process (1,000 replications) was performed to assess the robustness of individual nodes on each phylogeny utilizing the ML substitution model.

To determine the comparative evolutionary dynamics of all 4 DENV serotypes in Cambodia, we used the BEAST package (Drummond and Rambaut, 2007) to estimate the rate of nucleotide substitution per site, the Time to the Most Recent Common Ancestor (TMRCA), and the Maximum Clade Credibility (MCC) tree of complete coding region (Drummond and Rambaut, 2007) and E gene data sets, utilizing information on the exact day of sample collection. This analysis utilized the GTR model of nucleotide substitution as well as a different substitution rate for each codon position. The invariant-sites parameter (I) was excluded since it tended to over-fit to the data. For the analysis of the E gene sequences the simpler HKY85+Γ_4 substitution model was used as we failed to obtain a high enough effective sample size under the GTR+ Γ4 model. Due to the complex population dynamics of DENV, with marked changes in population frequency over the sampling period, a Bayesian Skyline Plot (BSP) was used as a coalescent prior. This provides a piecewise graphical depiction of changes of relative genetic diversity through time ($N_e\tau$), where N_e is the effective population size and τ is the host-to-host generation time. We also utilized both strict and relaxed (uncorrelated lognormal) molecular clocks. The degree of uncertainty in each parameter estimate is provided by the 95% highest posterior density (HPD) values, while posterior probability values provide an assessment of the degree of support for each node on the tree. All Markov Chain Monte Carlo chains were run for sufficient time at 100 million steps to ensure statistical convergence, with 10% removed as burn-in.

Recombination analysis

We used the Recombinant Detection Program version 3 (RDP 3.0b42) (Martin et al., 2010) to screen for recombination in our data. This program implements several methods for the identification of recombinant sequences and recombination breakpoints. Accordingly, we used the RDP (Martin and Rybicki, 2000), LARD (Holmes et al., 1999), Bootscanning (Salminen et al., 1995), Maxchi (Smith, 1992), Chimaera (Posada and Crandall, 2001), GeneConv (Padidam et al., 1999), 3Seq (Boni et al., 2007) and Sis-scan (Gibbs et al., 2000) methods, using the default settings in each case. We only considered recombination events that were identified by at least three methods. Statistical significance was set at the $p<0.05$ level, after considering Bonferroni correction for multiple comparisons as implemented in RDP.

Analysis of selection pressures

To determine the nature of selection pressures acting at individual codon sites of the complete coding sequences of DENV-1, DENV-2 and DENV-3 and of the E gene of DENV-2 and DENV-4, we computed the mean ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions per site (d_N/d_S) using the Single Likelihood Ancestor Counting (SLAC) method available through the HYPHY package accessible at http://www.hyphy.org (Pond et al., 2005) and assuming the GTR model of nucleotide substitution and an input neighbor-joining tree. Sites were considered to be under positive selection if high statistical significance ($p<0.1$) was observed.

RESULTS

Since the establishment of national surveillance of dengue in 1980, a total of 194,726 clinically diagnosed dengue cases have been reported to the NDCP (Huy et al., 2010) and DENV serotypes were identified in of these 4039 cases since the establishment of virological surveillance in 2000 (IPC's own data). The pattern of DENV serotype evolution is marked by a shift in predominance serotype between DENV-2 (2002-2005) and DENV-3 (2005-2007), and by a low prevalence of DENV-1 and DENV-4 with small peaks every 2 or 3 years coinciding with the period of transition among the dominant serotypes (Figure 1).

DENV-1

DENV-1 sampled between 2000 to 2009 were aligned with representative DENV-1 isolates of three genotypes sequences available in GenBank. Phylogenetic analysis revealed that all Cambodian DENV-1 genome fell within genotype 1 which commonly circulates in Southeast Asia (Figure 2). A marked temporal pattern was also observed among the Cambodian DENV-1 strains, with four distinct lineages with different sampling periods observed within genotype 1. In addition, these data present clear evidence for lineage replacement. Specifically, lineages 1, 2 and 3 were all detected since 2000-2002 and disappeared in 2003, 2004 and 2007, respectively. Lineage 4 which appeared in 2003 was the only one remaining at the end of the sampling period in 2009 (Figure 2). The only apparent exception was the isolate KHD1_GQ868636_08_KOP which was isolated in 2008 from a southern province near Vietnam. The sequence of this isolate was associated with a cluster of known Vietnamese strains, suggesting that it is a recent important from Viet Nam. In addition, another isolate "D1HM181952_KH_07KCH" clustered with Thai strains isolated in 2001, indicative of importation from Thailand. In addition, lineage 2 viruses seem to cluster with those viruses sampled from other countries in Asia (Thailand, Singapore, Myanmar and China).

Bayesian molecular clock analysis suggested that the TMRCA of the Cambodian DENV-1 existed between 1979 and 1987 (Table 1). The nucleotide substitution rate of all 82 DENV-1 was estimated at 6.9×10^{-4} substitution/site/year (s/s/y; 95% HPD: $6.0 \times 10^{-4} – 7.8 \times 10^{-4}$), and hence broadly similar to those rates estimated previously (Twiddy et al., 2003). Due to

the small number of sequences in lineages 1-3, our analysis of evolutionary dynamics was performed on combined sequences (n=33) of lineages 1-3 and on lineage 4 (n=49) in isolation. The substitution dynamics was 7.7 x 10^{-4} s/s/y (95% HPD: 6.5 x 10^{-4} – 9 x 10^{-4}) with a TMRCA at year 1983-1989 for lineage 1-3 group and 6.7 x 10^{-4} s/s/y (95% HPD: 5.6 x 10^{-4} – 7.8 x 10^{-4}) with a more recent TMRCA in 1996 (95% HPD: 1994 - 1998) for lineage 4 isolates (Table1).

DENV-2

Due to the limited number of complete genome sequences of DENV-2, our analysis of this serotype was conducted on coding sequences of E gene only (n=139). Accordingly, phylogenetic analysis revealed that DENV-2 in Cambodia belonged to the Asian/American genotype (n=30) and more notably to the Asian I (n=109) genotype. This genotype division was confirmed by the analysis of the 39 complete coding region sequences (data not shown). Once again, the temporal pattern of ML tree showed clear evidence of lineage replacement. Specifically, the strains belonging to the Asian/American genotype were isolated from 2000 to 2004 and not thereafter (Figure 3). In contrast, Asian I strains were first sampled in 2002 and became quickly the only genotype of DENV-2 in circulationsince 2005. High bootstrap values allowed us to divide the Asian 1 genotype into 4 lineages: lineage 1: 2003-2008, lineage 2: 2003-2008, lineage 3: 2002-2008 and lineage 4: 2007-2009 (Figure 3). Among lineage 3 viruses, two Cambodian strains isolated in 2006 (D2Q0605081_KH06_KRT) and 2008 (D2GU131899_KH08_PHP) clustered among the Vietnamese viruses. Our phylogenetic analysis of the Asian 1 genotype revealed a large cluster of Cambodian and Vietnamese viruses while Thai viruses fall basal of this cluster. This confirms previous studies in which Thailand was suggested to be the source of the Asian 1 genotype (Vu et al. 2010).

Similar molecular clock rates and dates were obtained using the 139 E gene sequences of DENV-2 and the 39 complete genome sequences (data not shown). The rate of evolutionary change of Asian/American and Asian viruses were 13.1 x 10^{-4} s/s/y (95% HPD: 7.2 x 10^{-4} – 19.2 x 10^{-4}) and 9.7 x 10^{-4} s/s/y (95% HPD: 7.3 x 10^{-4} - 10.9 x 10^{-4}), respectively (Table 1). Accordingly, the TMRCA of the Asian/American viruses was 1988 (95% HPDs: 1980 to 1996)

while that of the dominant Asian I viruses was also 1988 (95% HPD: 1982-1993), such that these viruses appear to have co-circulated for a substantial time period.

DENV-3

All our 57 DENV-3 strains isolated from 2000 to 2008 fell into genotype II (Figure 4), and clustered with a large group of viruses including recent Vietnamese strains from 2006 to 2008 as well as those from Thailand detected during 1990s. There is also an important temporal and spatial division among the Cambodian genotype II viruses. Seven Cambodian strains assigned as lineage 1 (sampled between 2000 and 2003) clustered closely with Thai viruses that were isolated in 1998. The other 50 Cambodian sequences were assigned as lineage 2 (sampled only from 2003 onwards) and were positioned closer to Vietnamese viruses (2006-2008). The majority of DENV-3 strains used in this study were isolated from outbreaks in 2006 and 2007, particularly from Kampong Cham province located in the Eastern part of Cambodia. The nucleotide substitution rate for the 57 DENV-3 was estimated 8.7×10^{-4} (95% HPD: 7.4×10^{-4} - 10×10^{-4}), which gave a TMRCA of around 1994 (95% HPD: 1992-1995) (Table 1).

DENV-4

We were not able to generate complete genome of Cambodian DENV-4 strains; hence, all analyses of this serotype were performed using E gene sequences of strains isolated from 1998 to 2010. The phylogenetic analysis of 125 E gene sequences shows that DENV-4 viruses can be placed into 4 distinct genotypes, with all Cambodian DENV-4 (n=64) falling into two lineages within the large genotype I (Figure 5). While there is no clear temporal division in these data, the phylogenetic tree shows that the 2 Cambodian lineages are separated by a clear spatial clustering: lineage 1 (n=35; sampled between 1998 and 2009) consisted of viruses closely related to Vietnamese isolates (1998-2006), while lineage 2 (n=29) sampled between 2003 and 2009) was closely related to Thai viruses isolated between 2000 and 2002. Despite these likely differing geographic origins, there was no marked difference in geographical distribution between these 2 lineages in Cambodia. However, lineage 2 seems

to compose of more recent strains (2007-2010), although there was co-circulation with lineage 1 viruses. Interestingly, three strains within the 2 lineages did not cluster with other Cambodian viruses: 1 strain of lineage 1 isolated in 2008 (D3S1006107_KH08_PHP) grouped closely with some Vietnamese viruses while 2 strains of lineage 2 isolated in 2003 (D4N0604280_KH03_PHP and D4N0521088_KH03_SRP) were located closely to a Thai virus isolated in 1991, again indicative of international gene flow (Figure 5).

The estimate rate of evolutionary change in the Cambodian DENV-4 was 11.3×10^{-4} s/s/y (95% HPD: $8.5 \times 10^{-4} – 14.2 \times 10^{-4}$), such that the common ancestor of these viruses existed between 1986 and 1995 (average: 1990; Table 1). An equivalent analysis on each lineage revealed that the mean age of TMRCA of lineage 2 (11.5 years, 95% HPD: 8.3-15.3) was on average younger compared to lineage 1 (14.2 years, 95% HPD: 12.2-16.5), although with overlapping distributions (Table 1).

Population Dynamics of DENV in Cambodia

We also sought to understand the changing patterns of relative genetic diversity in the 4 DENV serotypes, utilizing Bayesian skyline plots as an inference tool. Interestingly, with the exception of DENV-3, the relative genetic diversity of all 4 DENV were generally similar through time, with a number of distinct peaks and troughs. These population dynamic profiles also correlated well with the epidemiological pattern of DENV circulation in Cambodia in the last 10 years (Figure 6 A-D). Accordingly, the skyline plot is characterized by a downturn in genetic diversity at around 2006 for DENV-1 (Figure 6A) and during the 2007-2008 inter-epidemic seasons for DENV-2 (Figure 6B). The genetic diversity of DENV-4 (Figure 6D) seems to decrease progressively from 2002, while the skyline plot of DENV-3 (Figure 6C) exhibited a continued growth reaching its peak in 2007, before declining.

Where sample sizes were large enough we also analyzed the epidemiological dynamics of different lineages within serotypes. In particular, in the case of DENV-2, the genetic diversity of America/Asian genotype seemed to be stable except that a decline that was observed between 2002-2003 and which corresponds to the emergence of the Asian I genotype in Cambodia, and which appears to be spreading throughout Southeast Asia likely due to an

elevated fitness (Vu et al., 2010). In addition, the skyline plot Asian I virus showed 2 peaks in genetic diversity and which correlated well with the fluctuation of DENV-2 cases identified by the national surveillance system (Figure 6B). Finally, the overall genetic diversity of DENV-4 seemed to decrease over time, again with 2 discrete peaks. After the a first small peak in 2002, there was a continuous decrease in genetic diversity until a second peak was observed between 2008 and 2009 which mainly corresponded to an increase in the diversity of lineage 2.

Recombination and positive selection

We found no evidence for recombination in the 4 DENV serotypes sampled in our study. Similarly, an analysis of d_N/d_S revealed no evidence for positive selection in the Cambodian DENV strains, when mean values of d_N/d_S ranging from 0.001-0.844.

DISCUSSION

The burden of disease due to dengue in Cambodia is significant (Huy et al., 2009; Vong et al., 2011). The annual incidence over the sampling period ranged from 13.4 to 57.8/1000 person-season which was 4 to 29 fold higher than that reported by NDCP. Since 1980, two major dengue epidemics have been reported in Cambodia – in 1998 and 2007 – and all four dengue virus serotypes have been circulating each year in this country. Notably, the predominant circulating serotype changed from DENV-3 to DENV-2 in 2002 and then switched back to DENV-3 four years later (Huy et al., 2010). A similar pattern was seen with DENV-1 and DENV-4 in Thailand (Zhang et al., 2005), and where there was also an inverse correlation between the prevalence of DENV-1 and that of DENV-4, such that they are out-of-phase. Interestingly, there is a clear sequential replacement in dominance of one of the four co-circulating DENV serotypes in Vietnam in the period between 1994 and 2008 (Lourenco and Recker, 2010). They observed that the prevalence of DENV-3 was highest in relation to that of other 3 serotypes in 1998 followed by DENV-4 in 2001, DENV-2 in 2004 and 2005 and DENV-1 in 2008. A variety of epidemiological models have been proposed to explain these complex serotype dynamics, either invoking some degree of immunological cross-protection (Adams et al. 2006; Wearing and Rohani, 2006) or enhancement for transmission (Ferguson et al., 1999). It is currently unclear which of these models best explains the epidemiological dynamics of dengue in Cambodia: although some serotypes appear to be out-of-phase as implied in models invoking cross-protection (Recker et al., 2009), an increase in DENV-2 incidence was observed after introduction of genotype Asian 1, suggestive of a role for enhancement. Finally, our analysis of the epidemiological dynamic of dengue in Cambodia through changing patterns of genetic diversity through time revealed a strong concordance with the actual number of DENV serotypes identified by the NDCP. There is no significant difference in clinical dengue cases reported to NDCP annually, except during the large outbreak that occurred in 2007.

A number of notable phylogenetic patterns were observed within the DENV serotypes circulating in Cambodia. All Cambodian DENV-1 strains belong to genotype I that is the predominant genotype currently circulating in other Asian countries such as Singapore, Vietnam and Thailand (Raghwani et al., 2011; Schreiber et al., 2009; Zhang et al., 2005). In

366 the case of DENV-2, it is striking the Asian1 genotype was detected in 2002 and has
367 seemingly completely replaced the Asian/American genotype after 2004. This is indicative
368 of a major difference in fitness between these lineages (Vu et al. 2002). According to our
369 molecular dating study, this strain first entered Cambodia during 1980s or early 1990s.
370 Although this late date is of course dependent on the sample of Asia I viruses used in the
371 dating analysis, at face value it suggests that this genotype entered Cambodia later than
372 some other countries. For example, the Asia I genotype may entered Viet Nam in the late
373 1990's (Vu et al., 2010).

374 All 57 DENV-3 sequences generated in our study were assigned to genotype II, with at least
375 2 time points of introduction; lineage 1 at or before 2000 (lineage 1 viruses), and lineage 2
376 at around 2003 and which later replaced the lineage 1 viruses. This replacement event was
377 also documented in Thailand around 1991 or 1992 (Wittke et al., 2002; Zhang et al., 2005),
378 such that Thailand is likely the source of those viruses circulating in Cambodia. The
379 phylogenetic analysis of 389 DENV-4 strains confirms the existence of 4 genotypes in this
380 virus (Klungthong et al., 2004; Weaver and Vasilakis, 2009). The Cambodian genotype 1
381 viruses were probably introduced from Thailand in the late 1980s or early 1990s. Indeed,
382 although 3 genotypes (genotype 1, 2 and 3) of DENV-4 were circulating in Thailand,
383 genotype 1 viruses dominate more recent samples in Thailand (Klungthong et al., 2004).
384 Conversely, lineage 2 strains composed of mainly recent samples (2007-2010) grouped
385 closely to Thai viruses isolated in the 2000s. These data are indicative of a likely second
386 introduction of Thai strains in Cambodia during the early 2000s.

387 Our phylogenetic analysis also revealed a complex pattern of lineage turnover within each
388 serotype, with viral lineages increasing and decreasing in frequency through time, and which
389 has previously been documented in other populations in South-East Asia (Klungthong et al.,
390 2004; Myat Thu et al., 2005; Vu et al., 2010; Zhang et al., 2005). Although such a process of
391 lineage turnover is compatible with the action of natural selection favoring some lineages
392 over others, and fitness differences between dengue strains have been documented in
393 nature (Armstrong and Rico-Hesse, 2001; Rico-Hesse et al., 1997; Vu et al., 2010), we did not
394 observe any positive selection on any codon of the complete genome or of the E gene of
395 Cambodian DENVs, and a variety of neutral evolutionary processes can produce the same

phylogenetic pattern (Lourenco and Recker, 2010). It is therefore clear that more work is needed to document the precise selection pressures acting on dengue virus.

In support of the neutral hypothesis we noted a marked downfall of different lineages in DENV-1, DENV-2 and DENV-3 occurred between 2000-2004 (i.e. lineage 1 and 2 of DENV-1, Asian/American genotype of DENV-2, lineage 1 of DENV-3) and emergence of new lineages in the 4 DENVs around 2003 (i.e. lineage 4 of DENV-1, Asian 1 genotype, lineage 2 of DENV-3 and lineage 2 of DENV-4). That this event is synchronized across serotypes suggests the action of a stochastic event. For example, Cambodia experienced a major flood in 2000 which devastated provinces along the Mekong and Tonle Sap lake. This flood was then followed by consecutive droughts in 2003 and 2004 (MAO, 2005). These large scale natural disasters may have reduced the number of larvae habitats and mosquitoes population and consequently number of dengue cases observed in 2000 (Figure 1). Similarly, the period 2006-2007 was characterized by a large dengue outbreak mainly due to DENV-3. Interestingly, our phylogenetic analysis showed evidence of new clades in multiple serotypes from 2007 onwards; namely lineage 4 of DENV-1, lineage 4 of the DENV-2 Asian 1 genotype, and lineage 2 of DENV-4, while the dominant DENV-3 strains experienced a marked increase in genetic diversity as revealed by the BSP analysis. In addition, the prevalence and relative genetic diversity of DENV-3 s decreased sharply from 2008 onwards. These observations are suggestive of a second bottleneck that was probably generated by temporary cross-immunity protection. A similar hypothesis has been proposed to explain the replacement event of DENV-1 genotypes in Myanmar (Thu et al., 2004) and in Thailand (Zhang et al., 2005). However, further study demonstrating whether DENV-3 isolated during 2006-2007 outbreak were more virulence or had better fitness than other serotypes and serological survey should be done in order to confirm the hypothesis of the cross-immunity protection induced by this serotype.

Our phylogenetic analysis was also notable in that it revealed a clear evolutionary relationship between DENV in Cambodia and that in neighboring countries, particularly Vietnam and Thailand, and indicative of relatively frequent international viral gene flow, and which has been observed in previous molecular epidemiological studies of this region

(Holmes et al., 2009; Vu et al., 2010). Indeed, some Cambodian strains (2 DENV-1, 2 DENV-2 and 3 DENV-4) fall within the genetic diversity of Thai or Vietnamese viruses. In addition, that the Thai strains tend to fall basal to the Cambodian viruses in most of our phylogenetic trees, and perhaps most notably in the case of the Asian I genotype of DENV-2, suggests that many of the viral lineages circulating in Cambodia are of Thai origin. The virological link between these countries can be explained by the movement of both people and vectors. In particular, labor migration is reported as an important event in Cambodia due to the poverty and lack of employment, and both Thailand and Vietnam are the most common destinations of migration (CDRI, 2007). More generally, these trans-boundary movements of viruses are very common and DENVs have been already reported to be transported between Asian countries (Holmes et al., 2009; Kuan et al., 2010; Raghwani et al., 2011; Villabona-Arenas and Zanotto, 2011) or from Asia to as far as South America (Aquino et al., 2009; de Melo et al., 2009; Rico-Hesse et al., 1997; Uzcategui et al., 2001).

Competing interests

The authors declare that they have no competing interests.

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453 **Tables and figures**

454

455 **Table 1: Nucleotide substitution rates and times to the most recent common ancestor**
456 **(TMRCA) of dengue virus in Cambodia.**

457 **Figure 1: Number of dengue fever cases reported nationally (clinical surveillance) and**
458 **percentage of each DENV serotype detected in Cambodia.** Number of clinical dengue cases
459 declared to National Dengue Control Program per year in 5 sentinel sites (right-hand axis,
460 bar) and the percentage of dengue virus serotypes detected among samples tested by RT-
461 PCR (left-hand axis, line) : DENV-1 (line with ■marker), DENV-2 (line with▲ marker), DENV-
462 3 with ● marker) and DENV-4 (line with ◆ marker)**.**

463 **Figure 2: ML phylogenetic tree of 82 complete genome sequences of DENV-1.** Isolates
464 sampled in Cambodia are shown in bold and marked by "■". The tree is mid-point root for
465 clarity only and all horizontal branch lengths are drawn to a scale of nucleotide substitution
466 rates per site (0.005). Bootstrap support is shown for key nodes. Genotypes that do not
467 contain Cambodian DENV-1 were compressed to increase visibility.

468 **Figure 3: ML phylogenetic tree of 139 E gene sequences of DENV-2.** Isolates sampled in
469 Cambodia are shown in bold and marked by "▲". The tree is mid-point root for clarity only
470 and all horizontal branch lengths are drawn to a scale of nucleotide substitution rates per
471 site (0.01). Bootstrap support is shown for key nodes. Genotypes that do not contain
472 Cambodian DENV-2 were compressed to increase visibility.

473 **Figure 4: ML phylogenetic tree of 57 complete genome sequences of DENV-3.** Isolates
474 sampled in Cambodia are shown in bold and marked by "●". The tree is mid-point root for
475 clarity only and all horizontal branch lengths are drawn to a scale of nucleotide substitution
476 rates per site (0.01). Bootstrap support is shown for key nodes. Genotypes that do not
477 contain Cambodian DENV-3 were compressed to increase visibility.

478 **Figure 5: ML phylogenetic tree of 64 E gene sequences of DENV-4.** Isolates sampled in
479 Cambodia are shown in bold and marked by "◆". The tree is mid-point root for clarity only
480 and all horizontal branch lengths are drawn to a scale of nucleotide substitution rates per

site (0.01). Bootstrap support is shown for key nodes Genotypes that do not contain Cambodian DENV-4 were compressed to increase visibility.

Figure 6: Population dynamics of DENV in Cambodia. Changes in relative genetic diversity ($N_e\tau$) of (**A)** DENV-1 from 2000-2009y, (**B)** DENV-2 from 2000-2009, (**C)** DENV-3 from 2000-2008 , and (**D)** DENV-4 from 1998-2010. The solid line represents the mean estimate of $N_e\tau$ (right axis), while the 95% HPD intervals are shown in dot line. Time is shown as year of the first and last sampled isolates. The marked line is the percentage (%; left axis) of DENV serotype confirmed by RT-PCR and the bar is the number of clinical DENV cases declared to the National Dengue Control Program by year (x 1000; left axis).

Supplementary materials

Supplementary Table 1: List of complete genome sequences of DENV-1, DENV-2 and DENV-3 in Cambodia used in this study and GenBank accession number.

Supplementary Table 2: Mix preparation and RT-PCR condition for sequencing E gene of DENV-2 and DENV-4.

Supplementary Table 3: Primers list for sequencing E gene of DENV-2 and DENV-4.

Supplementary Table 4:List of E gene sequences of DENV-2 and DENV-4 in Cambodia generated in this study and GenBank accession number.

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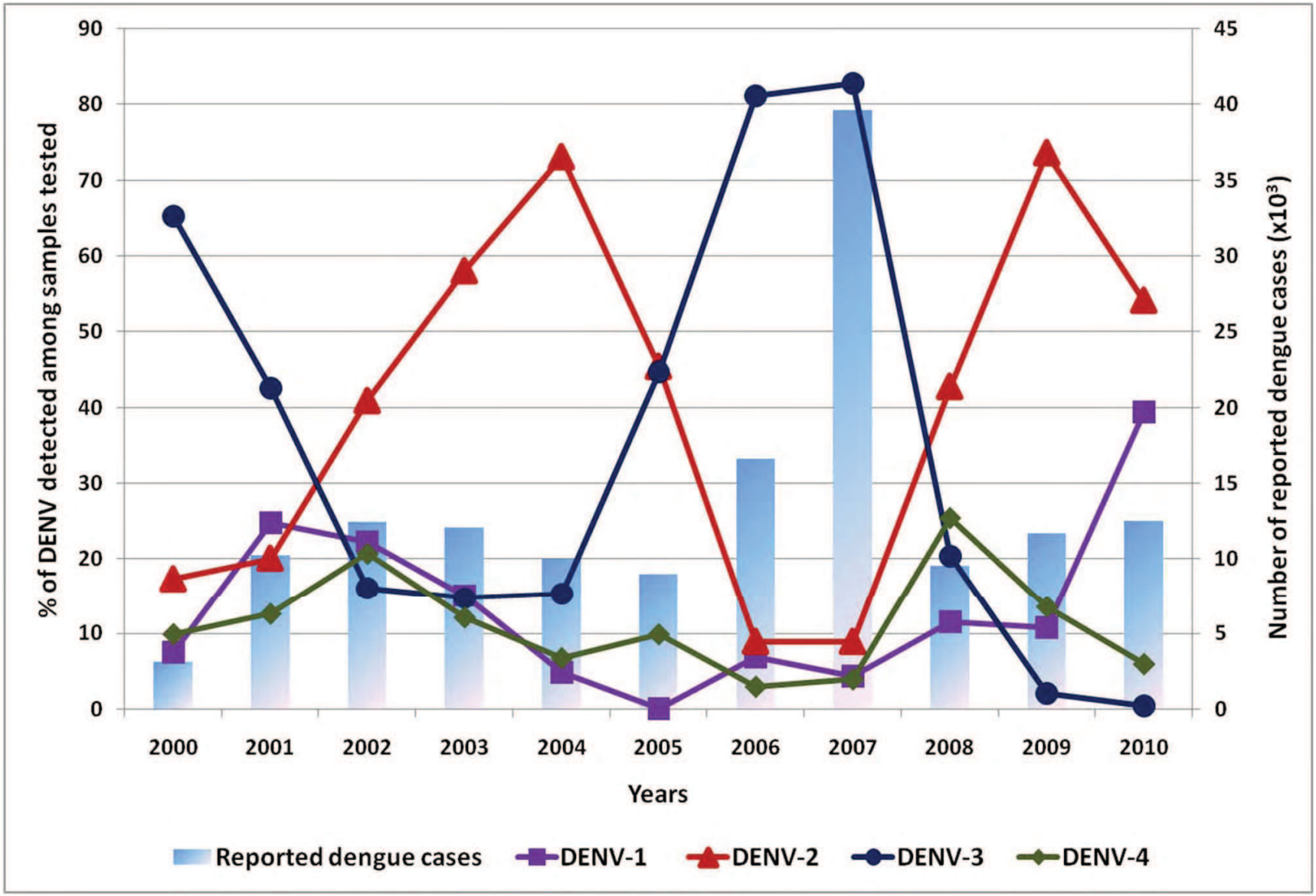
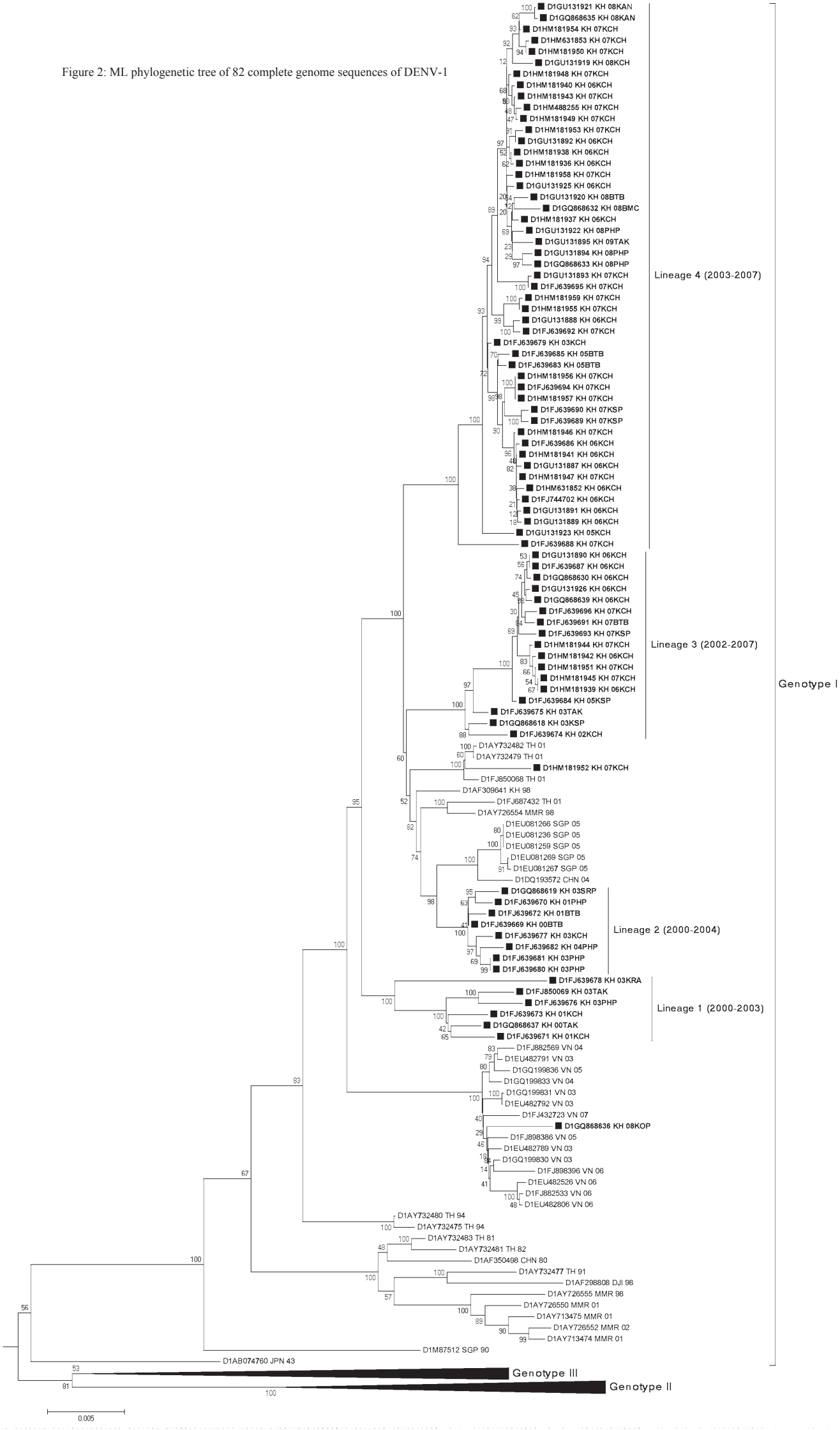


Figure 1: Number of dengue fever cases reported nationally (clinical surveillance) and percentage of each DENV serotype detected in Cambodia.

Figure 2: ML phylogenetic tree of 82 complete genome sequences of DENV-1



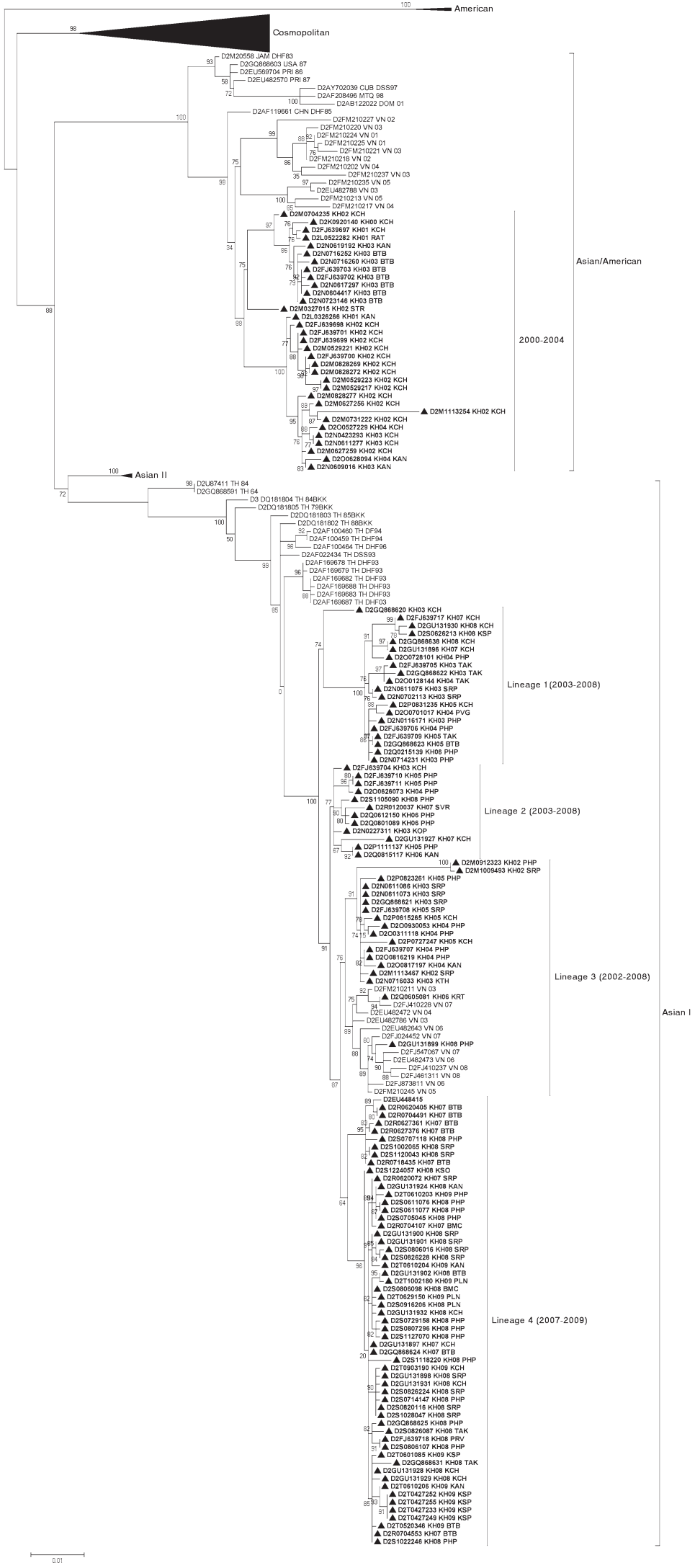


Figure 3: ML phylogenetic tree of 139 E gene sequences of DENV-2

Figure 4: ML phylogenetic tree of 57 complete genome sequences of DENV-3

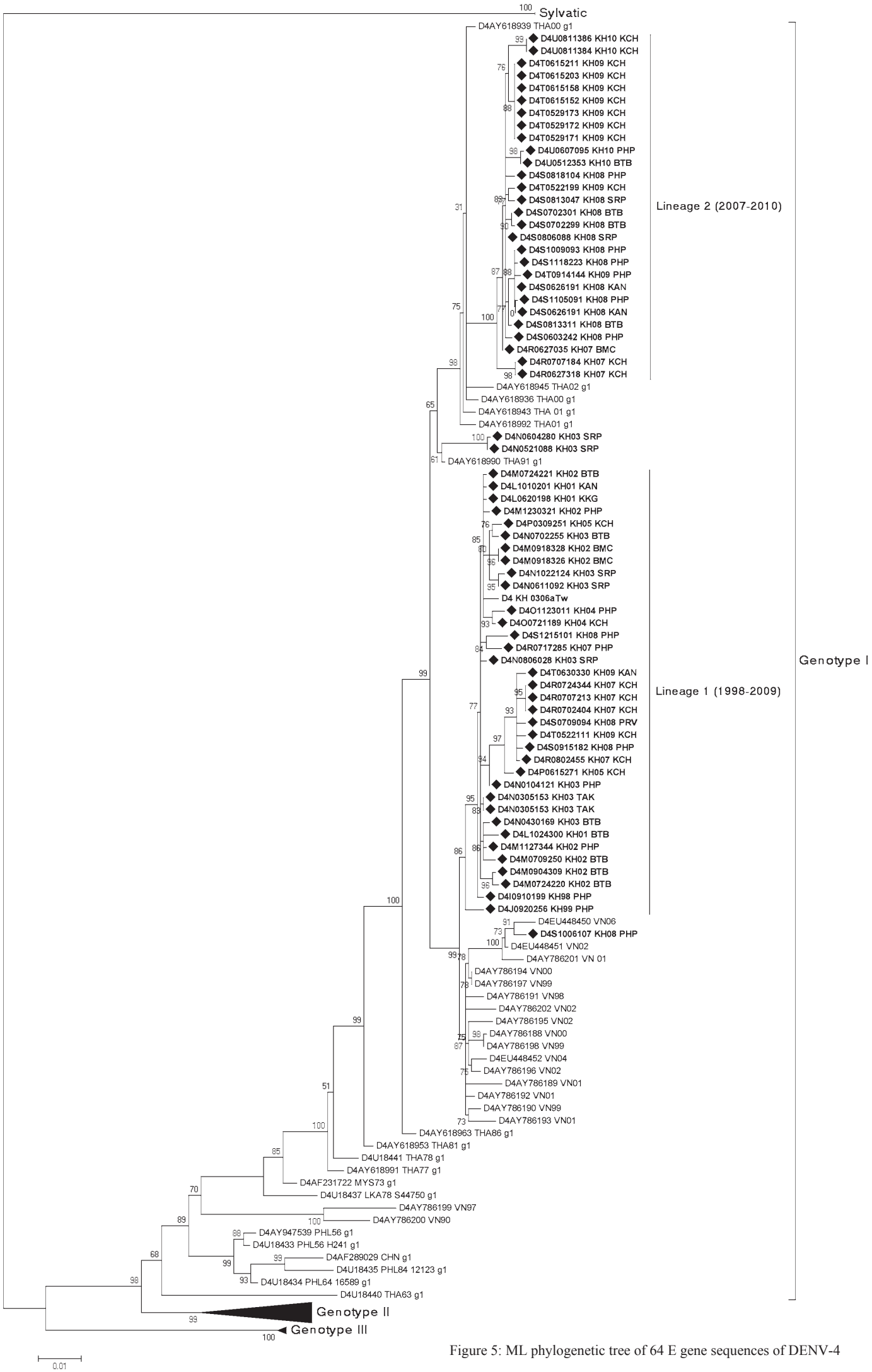


Figure 5: ML phylogenetic tree of 64 E gene sequences of DENV-4

Figure 6A: Population dynamics of DENV in Cambodia.
Changes in relative genetic diversity ($N_e\tau$) of DENV-1

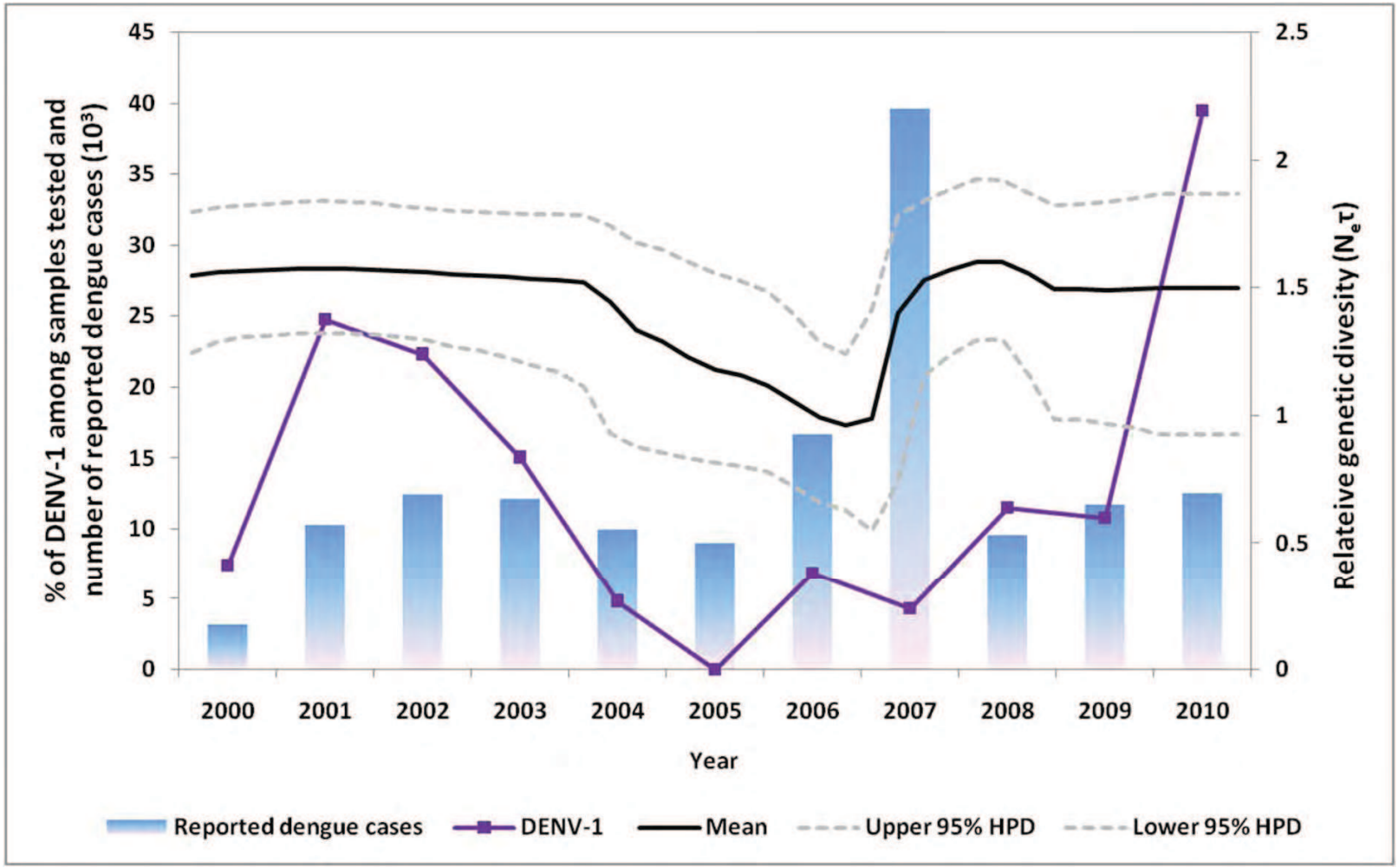


Figure 6B: Population dynamics of DENV in Cambodia.
Changes in relative genetic diversity of DENV-2

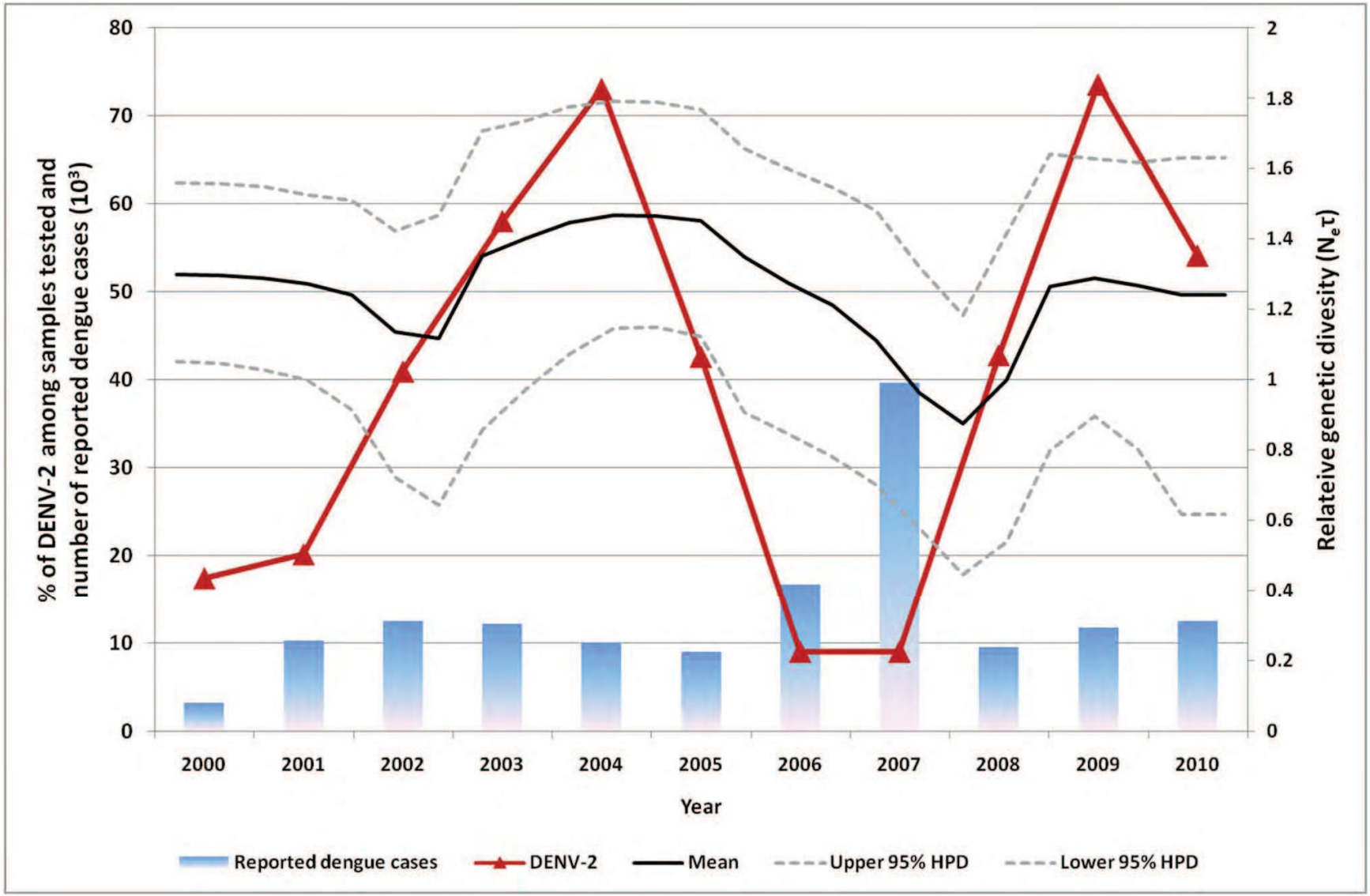


Figure 6C: Population dynamics of DENV in Cambodia.
Changes in relative genetic diversity of DENV-3

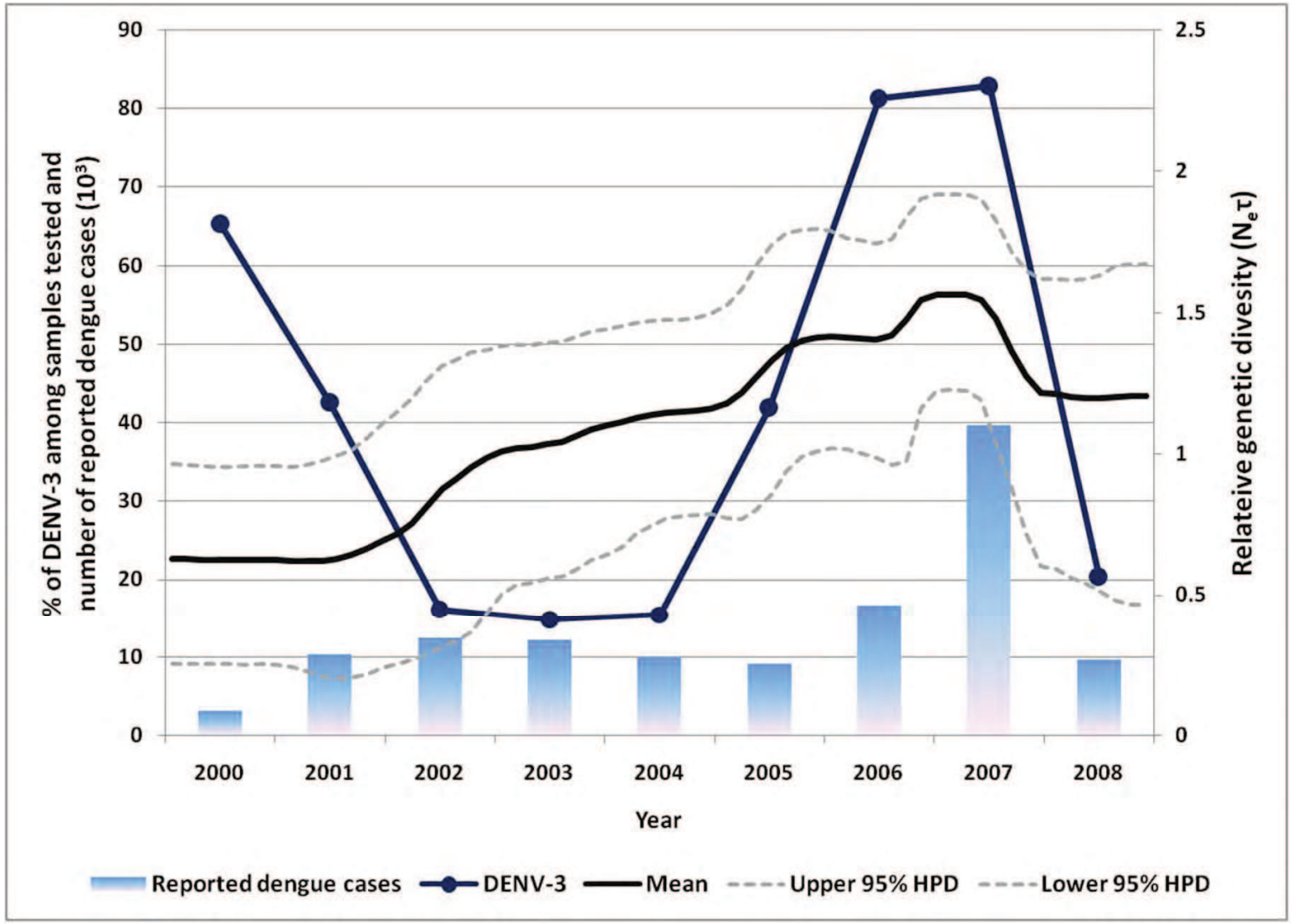


Figure 6D: Population dynamics of DENV in Cambodia.
Changes in relative genetic diversity of DENV-4

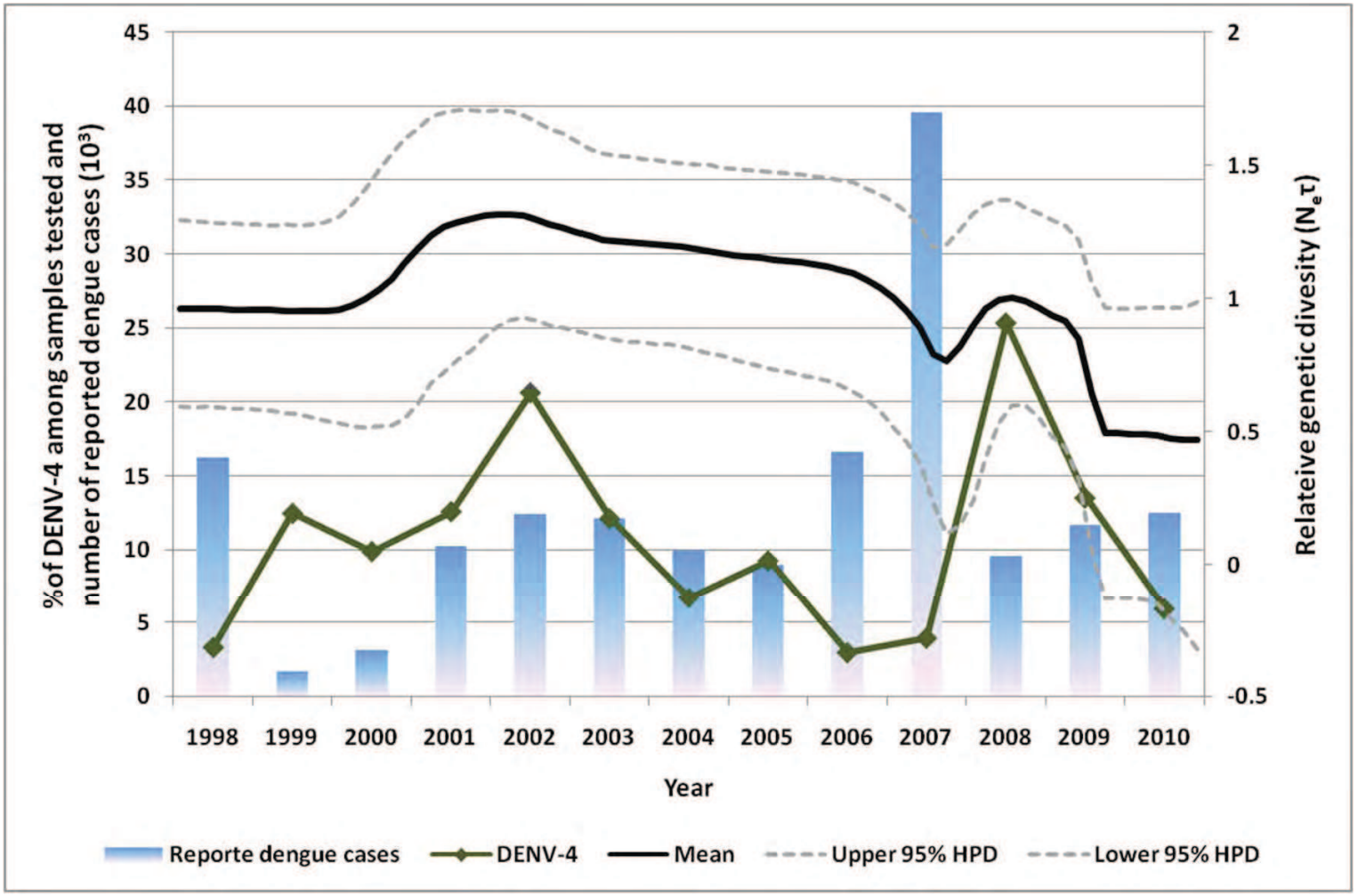


Table 1: Nucleotide substitution rate and time to the most recent common ancestor (TMRCA) of dengue virus in Cambodia

DENV	Number of strains	Year of isolation (oldest-youngest)	Posterior (95% HPD)	Likelihood (95% HPD)	Mean rate x 10^{-4} subs/site/year (95% HPD)	tMRCA in year (95% HPD)
DENV-1	83	2000-2009	-26410.8 (-26423, -26338)	-26052 (-26068, -26039)	6.9 (6-7.8)	25.8 (22.4-29.6)
DENV-1_L1-3	33	2000-2007	-21071.7 (-21087.5, -21056.5)	-20931.2 (-20941, -20921.5)	7.7 (6.5-9)	20.7 (17.9-24)
DENV-1_L4	49	2003-2007	-18406.3 (-18423.8, -18388.8)	-18222.9 (-18234.4, -18211.5)	6.7 (5.6-7.8)	10.7 (8.8-12.6)
DENV-2*	139	2000-2009	-5712.6 (-57467, -5678)	-5157.7 (-5194.6, -5158.5)	11.5 (9.2-14)	48 (37.0-59.0)
DENV-2_A/A*	33	2000-2004	-3009.9 (-3034.2, -2987.9)	-2883.4 (-2893, -2874.3)	13.1 (7.2-19.2)	16 (8.6-24.0)
DENV-2_A1*	106	2002-2009	-4438.6 (-4472.5, -4405.4)	-4026.5 (-4041.7, -4012.2)	9.7 (7.3-11.9)	21 (16.0-27.0)
DENV-2_A1_L1-3*	50	2002-2008	-3585.0 (-3615.1, 3556.9)	-3363.6 (-3374.9, -3353.0)	9.4 (6.5-12.9)	20.7 (14.4-27.7)
DENV-2_A1_L4*	56	2007-2009	-2857.4 (-2888.1, -2827.3)	-2681.8 (-2691.9, -2670.8)	10.5 (7-14.5)	6 (3.9-8.5)
DENV-3	57	2000-2008	-22041.3 (-22059.3, -22024.4)	-21803.1 (21815.5, -21791.1)	8.7 (7.4-10.5)	14.5 (12.9-16.5)
DENV-3_L1	20	2003-2008	-16779.5 (-16796.7, -16764.0)	-16704.0 (-16711.8, -16697.0)	9.3 (6.3-12.9)	6.3 (5.1-7.6)
DENV-3_L2	30	2003-2008	-18178.8 (-18198.3, -18159.9)	-18057.7 (-18067.0, 18049.0)	9.2 (6.2-12.1)	7.0 (5.6-8.8)
DENV-4*	64	1998-2010	-3829.3 (-3855.2, -3804.3)	-3608.5 (-3621.7, -3595.8)	11.3 (8.5-14.2)	19.8 (15.8-24.4)
DENV-4_L1*	34	1998-2009	-3003.9 (-3024.7, -2984.5)	-2883.3 (-2892.8, 2874.7)	10.7 (7.4-14.1)	14.2 (12.2-16.5)
DENV-4_L2*	29	2003-2010	-2632.5 (-2652.4, -2613.1)	-2578 (-2588.7, -2571.8)	19.7 (11.8-28.3)	11.5 (8.3-15.3)

DENV: dengue virus
HPD: highest posterior density
L: lineage
A/A: Asian/American genotype
A1: Asian 1 genotype
tMRCA: time to the most recent common ancestor
Effective sample size is higher than 200 in all cases and is not shown here.
* HKY+ Γ4 model was used.

Supplementary Table 1: List of complete genome sequences of DENV-1, DENV-2 and DENV-3 in Cambodia used in this study and GenBank accession number.

Order No.	Sequence ID used in this study	Accession No.	Serotype	Year of sampling	Country
1.	D1FJ639669_KH00_BTB	FJ639669	DENV-1	2000	Cambodia
2.	D1GQ868637_KH00_TAK	GQ868637	DENV-1	2000	Cambodia
3.	D1FJ639670_KH01_PHP	FJ639670	DENV-1	2001	Cambodia
4.	D1FJ639671_KH01_KCH	FJ639671	DENV-1	2001	Cambodia
5.	D1FJ639672_KH01_BTB	FJ639672	DENV-1	2001	Cambodia
6.	D1FJ639673_KH01_KCH	FJ639673	DENV-1	2001	Cambodia
7.	D1FJ639674_KH02_KCH	FJ639674	DENV-1	2002	Cambodia
8.	D1FJ639675_KH03_TAK	FJ639675	DENV-1	2003	Cambodia
9.	D1FJ639676_KH03_PHP	FJ639676	DENV-1	2003	Cambodia
10.	D1FJ639677_KH03_KCH	FJ639677	DENV-1	2003	Cambodia
11.	D1FJ639678_KH03_KRA	FJ639678	DENV-1	2003	Cambodia
12.	D1FJ639679_KH03_KCH	FJ639679	DENV-1	2003	Cambodia
13.	D1FJ639680_KH03_PHP	FJ639680	DENV-1	2003	Cambodia
14.	D1FJ639681_KH03_PHP	FJ639681	DENV-1	2003	Cambodia
15.	D1FJ850069_KH03_TAK	FJ850069	DENV-1	2003	Cambodia
16.	D1GQ868618_KH03_KSP	GQ868618	DENV-1	2003	Cambodia
17.	D1GQ868619_KH03_SRP	GQ868619	DENV-1	2003	Cambodia
18.	D1FJ639682_KH04_PHP	FJ639682	DENV-1	2004	Cambodia
19.	D1FJ639683_KH05_BTB	FJ639683	DENV-1	2005	Cambodia
20.	D1FJ639684_KH05_KSP	FJ639684	DENV-1	2005	Cambodia
21.	D1FJ639685_KH05_BTB	FJ639685	DENV-1	2005	Cambodia
22.	D1GU131923_KH05_KCH	GU131923	DENV-1	2005	Cambodia
23.	D1FJ639686_KH06_KCH	FJ639686	DENV-1	2006	Cambodia
24.	D1FJ639687_KH06_KCH	FJ639687	DENV-1	2006	Cambodia
25.	D1FJ744702_KH06_KCH	FJ744702	DENV-1	2006	Cambodia
26.	D1GQ868630_KH06_KCH	GQ868630	DENV-1	2006	Cambodia
27.	D1GQ868639_KH06_KCH	GQ868639	DENV-1	2006	Cambodia
28.	D1GU131887_KH06_KCH	GU131887	DENV-1	2006	Cambodia
29.	D1GU131888_KH06_KCH	GU131888	DENV-1	2006	Cambodia
30.	D1GU131889_KH06_KCH	GU131889	DENV-1	2006	Cambodia
31.	D1GU131890_KH06_KCH	GU131890	DENV-1	2006	Cambodia
32.	D1GU131891_KH06_KCH	GU131891	DENV-1	2006	Cambodia
33.	D1GU131892_KH06_KCH	GU131892	DENV-1	2006	Cambodia
34.	D1GU131925_KH06_KCH	GU131925	DENV-1	2006	Cambodia
35.	D1GU131926_KH06_KCH	GU131926	DENV-1	2006	Cambodia
36.	D1HM181936_KH06_KCH	HM181936	DENV-1	2006	Cambodia
37.	D1HM181937_KH06_KCH	HM181937	DENV-1	2006	Cambodia
38.	D1HM181938_KH06_KCH	HM181938	DENV-1	2006	Cambodia
39.	D1HM181939_KH06_KCH	HM181939	DENV-1	2006	Cambodia
40.	D1HM181940_KH06_KCH	HM181940	DENV-1	2006	Cambodia
41.	D1HM181941_KH06_KCH	HM181941	DENV-1	2006	Cambodia
42.	D1HM181942_KH06_KCH	HM181942	DENV-1	2006	Cambodia
43.	D1HM631852_KH06_KCH	HM631852	DENV-1	2006	Cambodia
44.	D1FJ639688_KH07_KCH	FJ639688	DENV-1	2007	Cambodia
45.	D1FJ639689_KH07_KSP	FJ639689	DENV-1	2007	Cambodia

46.	D1FJ639690_KH07_KSP	FJ639690	DENV-1	2007	Cambodia
47.	D1FJ639691_KH07_BTB	FJ639691	DENV-1	2007	Cambodia
48.	D1FJ639692_KH07_KCH	FJ639692	DENV-1	2007	Cambodia
49.	D1FJ639693_KH07_KSP	FJ639693	DENV-1	2007	Cambodia
50.	D1FJ639694_KH07_KCH	FJ639694	DENV-1	2007	Cambodia
51.	D1FJ639695_KH07_KCH	FJ639695	DENV-1	2007	Cambodia
52.	D1FJ639696_KH07_KCH	FJ639696	DENV-1	2007	Cambodia
53.	D1GU131893_KH07_KCH	GU131893	DENV-1	2007	Cambodia
54.	D1HM181943_KH07_KCH	HM181943	DENV-1	2007	Cambodia
55.	D1HM181944_KH07_KCH	HM181944	DENV-1	2007	Cambodia
56.	D1HM181945_KH07_KCH	HM181945	DENV-1	2007	Cambodia
57.	D1HM181946_KH07_KCH	HM181946	DENV-1	2007	Cambodia
58.	D1HM181947_KH07_KCH	HM181947	DENV-1	2007	Cambodia
59.	D1HM181948_KH07_KCH	HM181948	DENV-1	2007	Cambodia
60.	D1HM181949_KH07_KCH	HM181949	DENV-1	2007	Cambodia
61.	D1HM181950_KH07_KCH	HM181950	DENV-1	2007	Cambodia
62.	D1HM181951_KH07_KCH	HM181951	DENV-1	2007	Cambodia
63.	D1HM181952_KH07_KCH	HM181952	DENV-1	2007	Cambodia
64.	D1HM181953_KH07_KCH	HM181953	DENV-1	2007	Cambodia
65.	D1HM181954_KH07_KCH	HM181954	DENV-1	2007	Cambodia
66.	D1HM181955_KH07_KCH	HM181955	DENV-1	2007	Cambodia
67.	D1HM181956_KH07_KCH	HM181956	DENV-1	2007	Cambodia
68.	D1HM181957_KH07_KCH	HM181957	DENV-1	2007	Cambodia
69.	D1HM181958_KH07_KCH	HM181958	DENV-1	2007	Cambodia
70.	D1HM181959_KH07_KCH	HM181959	DENV-1	2007	Cambodia
71.	D1HM488255_KH07_KCH	HM488255	DENV-1	2007	Cambodia
72.	D1HM631853_KH07_KCH	HM631853	DENV-1	2007	Cambodia
73.	D1GQ868632_KH08_BMC	GQ868632	DENV-1	2008	Cambodia
74.	D1GQ868633_KH08_PHP	GQ868633	DENV-1	2008	Cambodia
75.	D1GQ868635_KH08_KAN	GQ868635	DENV-1	2008	Cambodia
76.	D1GQ868636_KH08_KOP	GQ868636	DENV-1	2008	Cambodia
77.	D1GU131894_KH08_PHP	GU131894	DENV-1	2008	Cambodia
78.	D1GU131919_KH08_KCH	GU131919	DENV-1	2008	Cambodia
79.	D1GU131920_KH08_BTB	GU131920	DENV-1	2008	Cambodia
80.	D1GU131921_KH08_KAN	GU131921	DENV-1	2008	Cambodia
81.	D1GU131922_KH08_PHP	GU131922	DENV-1	2008	Cambodia
82.	D1GU131895_KH09_TAK	GU131895	DENV-1	2009	Cambodia
83.	D2FJ639697_KH01_KCH	FJ639697	DENV-2	2001	Cambodia
84.	D2FJ639698_KH02_KCH	FJ639698	DENV-2	2002	Cambodia
85.	D2FJ639699_KH02_KCH	FJ639699	DENV-2	2002	Cambodia
86.	D2FJ639700_KH02_KCH	FJ639700	DENV-2	2002	Cambodia
87.	D2FJ639701_KH02_KCH	FJ639701	DENV-2	2002	Cambodia
88.	D2FJ639702_KH03_BTB	FJ639702	DENV-2	2003	Cambodia
89.	D2FJ639703_KH03_BTB	FJ639703	DENV-2	2003	Cambodia
90.	D2FJ639704_KH03_KCH	FJ639704	DENV-2	2003	Cambodia
91.	D2FJ639705_KH03_TAK	FJ639705	DENV-2	2003	Cambodia
92.	D2GQ868620_KH03_KCH	GQ868620	DENV-2	2003	Cambodia
93.	D2GQ868621_KH03_SRP	GQ868621	DENV-2	2003	Cambodia
94.	D2GQ868622_KH03_TAK	GQ868622	DENV-2	2003	Cambodia
95.	D2FJ639706_KH04_PHP	FJ639706	DENV-2	2004	Cambodia
96.	D2FJ639707_KH04_PHP	FJ639707	DENV-2	2004	Cambodia
97.	D2FJ639708_KH05_SRP	FJ639708	DENV-2	2005	Cambodia

98.	D2FJ639709_KH05_TAK	FJ639709	DENV-2	2005	Cambodia
99.	D2FJ639710_KH05_PHP	FJ639710	DENV-2	2005	Cambodia
100.	D2FJ639711_KH05_PHP	FJ639711	DENV-2	2005	Cambodia
101.	D2GQ868623_KH05_BTB	GQ868623	DENV-2	2005	Cambodia
102.	D2FJ639717_KH07_KCH	FJ639717	DENV-2	2007	Cambodia
103.	D2GQ868624_KH07_BTB	GQ868624	DENV-2	2007	Cambodia
104.	D2GU131896_KH07_KCH	GU131896	DENV-2	2007	Cambodia
105.	D2GU131897_KH07_KCH	GU131897	DENV-2	2007	Cambodia
106.	D2GU131927_KH07_KCH	GU131927	DENV-2	2007	Cambodia
107.	D2FJ639718_KH08_PRV	FJ639718	DENV-2	2008	Cambodia
108.	D2GQ868625_KH08_PHP	GQ868625	DENV-2	2008	Cambodia
109.	D2GQ868631_KH08_TAK	GQ868631	DENV-2	2008	Cambodia
110.	D2GQ868638_KH08_KCH	GQ868638	DENV-2	2008	Cambodia
111.	D2GU131898_KH08_SRP	GU131898	DENV-2	2008	Cambodia
112.	D2GU131899_KH08_PHP	GU131899	DENV-2	2008	Cambodia
113.	D2GU131900_KH08_SRP	GU131900	DENV-2	2008	Cambodia
114.	D2GU131901_KH08_SRP	GU131901	DENV-2	2008	Cambodia
115.	D2GU131902_KH08_BTB	GU131902	DENV-2	2008	Cambodia
116.	D2GU131924_KH08_KAN	GU131924	DENV-2	2008	Cambodia
117.	D2GU131928_KH08_KCH	GU131928	DENV-2	2008	Cambodia
118.	D2GU131929_KH08_KCH	GU131929	DENV-2	2008	Cambodia
119.	D2GU131930_KH08_KCH	GU131930	DENV-2	2008	Cambodia
120.	D2GU131931_KH08_KCH	GU131931	DENV-2	2008	Cambodia
121.	D2GU131932_KH08_KCH	GU131932	DENV-2	2008	Cambodia
122.	D3FJ639719_KH00_PHP	FJ639719	DENV-3	2000	Cambodia
123.	D3FJ639720_KH01_PHP	FJ639720	DENV-3	2001	Cambodia
124.	D3GQ868626_KH01_PHP	GQ868626	DENV-3	2001	Cambodia
125.	D3FJ639721_KH02_KCH	FJ639721	DENV-3	2002	Cambodia
126.	D3FJ639722_KH02_KCH	FJ639722	DENV-3	2002	Cambodia
127.	D3GQ868627_KH02_KCH	GQ868627	DENV-3	2002	Cambodia
128.	D3FJ639723_KH03_KCH	FJ639723	DENV-3	2003	Cambodia
129.	D3FJ639724_KH03_PHP	FJ639724	DENV-3	2003	Cambodia
130.	D3FJ639725_KH03_KSO	FJ639725	DENV-3	2003	Cambodia
131.	D3GU131906_KH03_KSO	GU131906	DENV-3	2003	Cambodia
132.	D3FJ639726_KH04_PHP	FJ639726	DENV-3	2004	Cambodia
133.	D3FJ639727_KH05_BTB	FJ639727	DENV-3	2005	Cambodia
134.	D3FJ639728_KH05_PHP	FJ639728	DENV-3	2005	Cambodia
135.	D3GQ868628_KH05_BTB	GQ868628	DENV-3	2005	Cambodia
136.	D3GQ868629_KH05_PHP	GQ868629	DENV-3	2005	Cambodia
137.	D3GU131904_KH05_KCH	GU131904	DENV-3	2005	Cambodia
138.	D3FJ639729_KH06_BTB	FJ639729	DENV-3	2006	Cambodia
139.	D3FJ639730_KH06_PHP	FJ639730	DENV-3	2006	Cambodia
140.	D3GQ868634_KH06_KCH	GQ868634	DENV-3	2006	Cambodia
141.	D3GU131907_KH06_KCH	GU131907	DENV-3	2006	Cambodia
142.	D3GU131908_KH06_KCH	GU131908	DENV-3	2006	Cambodia
143.	D3GU131909_KH06_KCH	GU131909	DENV-3	2006	Cambodia
144.	D3GU131910_KH06_KCH	GU131910	DENV-3	2006	Cambodia
145.	D3GU131911_KH06_KCH	GU131911	DENV-3	2006	Cambodia
146.	D3GU131933_KH06_KCH	GU131933	DENV-3	2006	Cambodia
147.	D3HM181933_KH06_KCH	HM181933	DENV-3	2006	Cambodia
148.	D3HM181934_KH06_KCH	HM181934	DENV-3	2006	Cambodia
149.	D3FJ639712_KH07_KCH	FJ639712	DENV-3	2007	Cambodia

150.	D3FJ639713_KH07_PHP	FJ639713	DENV-3	2007	Cambodia
151.	D3FJ639714_KH07_KCH	FJ639714	DENV-3	2007	Cambodia
152.	D3FJ639731_KH07_SRP	FJ639731	DENV-3	2007	Cambodia
153.	D3GU131912_KH07_KCH	GU131912	DENV-3	2007	Cambodia
154.	D3GU131913_KH07_KCH	GU131913	DENV-3	2007	Cambodia
155.	D3GU131914_KH07_KCH	GU131914	DENV-3	2007	Cambodia
156.	D3GU131915_KH07_KCH	GU131915	DENV-3	2007	Cambodia
157.	D3GU131916_KH07_KCH	GU131916	DENV-3	2007	Cambodia
158.	D3GU131917_KH07_KCH	GU131917	DENV-3	2007	Cambodia
159.	D3GU131918_KH07_KCH	GU131918	DENV-3	2007	Cambodia
160.	D3GU131934_KH07_KCH	GU131934	DENV-3	2007	Cambodia
161.	D3GU131935_KH07_KCH	GU131935	DENV-3	2007	Cambodia
162.	D3GU131936_KH07_KCH	GU131936	DENV-3	2007	Cambodia
163.	D3GU131937_KH07_KCH	GU131937	DENV-3	2007	Cambodia
164.	D3GU131938_KH07_KCH	GU131938	DENV-3	2007	Cambodia
165.	D3GU131939_KH07_KCH	GU131939	DENV-3	2007	Cambodia
166.	D3GU131940_KH07_KCH	GU131940	DENV-3	2007	Cambodia
167.	D3GU131941_KH07_KCH	GU131941	DENV-3	2007	Cambodia
168.	D3GU131942_KH07_KCH	GU131942	DENV-3	2007	Cambodia
169.	D3GU131943_KH07_KCH	GU131943	DENV-3	2007	Cambodia
170.	D3GU131944_KH07_KCH	GU131944	DENV-3	2007	Cambodia
171.	D3GU131945_KH07_KCH	GU131945	DENV-3	2007	Cambodia
172.	D3HM181935_KH07_KCH	HM181935	DENV-3	2007	Cambodia
173.	D3FJ639715_KH08_PRV	FJ639715	DENV-3	2008	Cambodia
174.	D3FJ639716_KH08_PRV	FJ639716	DENV-3	2008	Cambodia
175.	D3GU131903_KH08_BMC	GU131903	DENV-3	2008	Cambodia
176.	D3GU131905_KH08_KCH	GU131905	DENV-3	2008	Cambodia
177.	D3GU131946_KH08_KCH	GU131946	DENV-3	2008	Cambodia
178.	D3HM631854_KH08 KCH	HM631854	DENV-3	2008	Cambodia

Supplementary Table 2: Mix preparation and RT-PCR condition for sequencing E gene of DENV-2 and DENV-4.

Reagents	1 reaction (µl)
RNase-free water	12.72
5x QIAGEN OneStep RT-PCR Buffer	6
dNTP Mix (10 mM)	1.2
Forward primer (10 µM)	1.44
Reverse primer (10 µM)	1.44
QIAGEN OneStep RT-PCR Enzyme Mix	1.2

Step	Temperature	Time	Cycles
Reverse transcription	45°C	60 min	1
Initial PCR activation	92°C	2 min	
Denaturation	94°C	30 sec	45
Annealing*	##°C	30 sec	
Extension	72°C	90 sec	
Final extension	72°C	10 min	1
Hold	4°C	∞	

* Annealing temperature of the primers is referred to the primer table.

Supplementary Table 3: Primers list for sequencing E gene of DENV-2 and DENV-4.

Primer name	Sequence 5'-3'	Length	Annealing temperature (^{o}C)
D2_E-817	CACCATAATGGCAGCAATCCTGG	703 bp	60
D2_E-1520	CCATCTGCAGCAACACCATCTCA		
D2_E-1370	GGAAATGACACAGGAAAACACGG	772 bp	60
D2_E-2142	GCCCCTCTCATCGTTGTCTCA		
D2_E-2018	GAAGCAGAACCTCCATTCGGAG	502 pb	60
D2_E-2520	GAAGGGGATTCTGGTTGGAACTT		

bp: base pair

D2: dengue virus serotype 2

E: gene coding for envelop gene

Primer name	Sequence 5'-3'	Length	Annealing temperature (^{o}C)
D4_E-834F	GGATTCGCTCTCTTGGCAGGATTTATG	855 bp	60
D4_E-1689R	CACATCCTGTCTCTTGGCATGAGGAAC		
D4_E-1263F	GGAAAAGGAGGAGTTGTGAC	1194 bp	60
D4_E-2454R	TCCGCTTCCACACTTCAATTC		
D4_E-2001F	ACCCCTTTTGCTCACAATAC	1098 bp	50
D4_E-2536R	GGGGACTCTGGTTGAAATTTGTACTGTTCTGTCCA		

bp: base pair

D4: dengue virus serotype 4

E: gene coding for envelop gene

Supplementary Table 4: List of E gene sequences of DENV-2 and DENV-4 in Cambodia generated in this study and GenBank accession number.

Order No.	Sequence ID used in this study	Accession No.	Serotype	Year of sampling	Country
1.	D2K0920140_KH00_KCH		DENV-2	2000	Cambodia
2.	D2L0326266_KH01_KAN		DENV-2	2001	Cambodia
3.	D2L0522282_KH01_RAT		DENV-2	2001	Cambodia
4.	D2M0912323_KH02_PHP		DENV-2	2002	Cambodia
5.	D2M1009493_KH02_SRP		DENV-2	2002	Cambodia
6.	D2M1113467_KH02_SRP		DENV-2	2002	Cambodia
7.	D2M1113254_KH02_KCH		DENV-2	2002	Cambodia
8.	D2M0731222_KH02_KCH		DENV-2	2002	Cambodia
9.	D2M0627259_KH02_KCH		DENV-2	2002	Cambodia
10.	D2M0627256_KH02_KCH		DENV-2	2002	Cambodia
11.	D2M0529223_KH02_KCH		DENV-2	2002	Cambodia
12.	D2M0828269_KH02_KCH		DENV-2	2002	Cambodia
13.	D2M0828272_KH02_KCH		DENV-2	2002	Cambodia
14.	D2M0327015_KH02_STR		DENV-2	2002	Cambodia
15.	D2M0704235_KH02_KCH		DENV-2	2002	Cambodia
16.	D2M0828277_KH02_KCH		DENV-2	2002	Cambodia
17.	D2M0529221_KH02_KCH		DENV-2	2002	Cambodia
18.	D2M0529217_KH02_KCH		DENV-2	2002	Cambodia
19.	D2N0611075_KH03_SRP		DENV-2	2003	Cambodia
20.	D2N0702113_KH03_SRP		DENV-2	2003	Cambodia
21.	D2N0116171_KH03_PHP		DENV-2	2003	Cambodia
22.	D2N0714231_KH03_PHP		DENV-2	2003	Cambodia
23.	D2N0227311_KH03_KOP		DENV-2	2003	Cambodia
24.	D2N0611073_KH03_SRP		DENV-2	2003	Cambodia
25.	D2N0611086_KH03_SRP		DENV-2	2003	Cambodia
26.	D2N0716033_KH03_KTH		DENV-2	2003	Cambodia
27.	D2N0423293_KH03_KCH		DENV-2	2003	Cambodia
28.	D2N0611277_KH03_KCH		DENV-2	2003	Cambodia
29.	D2N0609016_KH03_KAN		DENV-2	2003	Cambodia
30.	D2N0619192_KH03_KAN		DENV-2	2003	Cambodia
31.	D2N0716260_KH03_BTB		DENV-2	2003	Cambodia
32.	D2N0716252_KH03_BTB		DENV-2	2003	Cambodia
33.	D2N0617297_KH03_BTB		DENV-2	2003	Cambodia
34.	D2N0604417_KH03_BTB		DENV-2	2003	Cambodia
35.	D2N0723146_KH03_BTB		DENV-2	2003	Cambodia
36.	D2O0728101_KH04_PHP		DENV-2	2004	Cambodia
37.	D2O0128144_KH04_TAK		DENV-2	2004	Cambodia
38.	D2O0701017_KH04_PVG		DENV-2	2004	Cambodia
39.	D2O0626073_KH04_PHP		DENV-2	2004	Cambodia
40.	D2O0930053_KH04_PHP		DENV-2	2004	Cambodia
41.	D2O0817197_KH04_KAN		DENV-2	2004	Cambodia
42.	D2O0311118_KH04_PHP		DENV-2	2004	Cambodia
43.	D2O0816219_KH04_PHP		DENV-2	2004	Cambodia
44.	D2O0628094_KH04_KAN		DENV-2	2004	Cambodia
45.	D2O0527229_KH04_KCH		DENV-2	2004	Cambodia

46.	D2P0831235_KH05_KCH		DENV-2	2005	Cambodia
47.	D2P1111137_KH05_PHP		DENV-2	2005	Cambodia
48.	D2P0727247_KH05_KCH		DENV-2	2005	Cambodia
49.	D2P0823261_KH05_PHP		DENV-2	2005	Cambodia
50.	D2P0615265_KH05_KCH		DENV-2	2005	Cambodia
51.	D2Q0215139_KH06_PHP		DENV-2	2006	Cambodia
52.	D2Q0612150_KH06_PHP		DENV-2	2006	Cambodia
53.	D2Q0801089_KH06_PHP		DENV-2	2006	Cambodia
54.	D2Q0815117_KH06_KAN		DENV-2	2006	Cambodia
55.	D2Q0605081_KH06_KRT		DENV-2	2006	Cambodia
56.	D2R0620405_KH07_BTB		DENV-2	2007	Cambodia
57.	D2R0704491_KH07_BTB		DENV-2	2007	Cambodia
58.	D2R0627361_KH07_BTB		DENV-2	2007	Cambodia
59.	D2R0627376_KH07_BTB		DENV-2	2007	Cambodia
60.	D2R0718435_KH07_BTB		DENV-2	2007	Cambodia
61.	D2R0620072_KH07_SRP		DENV-2	2007	Cambodia
62.	D2R0704107_KH07_BMC		DENV-2	2007	Cambodia
63.	D2R0704553_KH07_BTB		DENV-2	2007	Cambodia
64.	D2R0120037_KH07_SVR		DENV-2	2007	Cambodia
65.	D2S0626213_KH08_KSP		DENV-2	2008	Cambodia
66.	D2S0707118_KH08_PHP		DENV-2	2008	Cambodia
67.	D2S1002065_KH08_SRP		DENV-2	2008	Cambodia
68.	D2S1120043_KH08_SRP		DENV-2	2008	Cambodia
69.	D2S1118220_KH08_PHP		DENV-2	2008	Cambodia
70.	D2S0826087_KH08_TAK		DENV-2	2008	Cambodia
71.	D2S0806016_KH08_SRP		DENV-2	2008	Cambodia
72.	D2S0826228_KH08_SRP		DENV-2	2008	Cambodia
73.	D2S0806107_KH08_PHP		DENV-2	2008	Cambodia
74.	D2S0826224_KH08_SRP		DENV-2	2008	Cambodia
75.	D2S0714147_KH08_PHP		DENV-2	2008	Cambodia
76.	D2S0820116_KH08_SRP		DENV-2	2008	Cambodia
77.	D2S1028047_KH08_SRP		DENV-2	2008	Cambodia
78.	D2S0611076_KH08_PHP		DENV-2	2008	Cambodia
79.	D2S0611077_KH08_PHP		DENV-2	2008	Cambodia
80.	D2S0705045_KH08_PHP		DENV-2	2008	Cambodia
81.	D2S1224057_KH08_KSO		DENV-2	2008	Cambodia
82.	D2S1022246_KH08_PHP		DENV-2	2008	Cambodia
83.	D2S0916206_KH08_PLN		DENV-2	2008	Cambodia
84.	D2S0806098_KH08_BMC		DENV-2	2008	Cambodia
85.	D2S0807296_KH08_PHP		DENV-2	2008	Cambodia
86.	D2S0729158_KH08_PHP		DENV-2	2008	Cambodia
87.	D2S1127070_KH08_PHP		DENV-2	2008	Cambodia
88.	D2S1105090_KH08_PHP		DENV-2	2008	Cambodia
89.	D2T0610204_KH09_KAN		DENV-2	2009	Cambodia
90.	D2T0903190_KH09_KCH		DENV-2	2009	Cambodia
91.	D2T1002180_KH09_PLN		DENV-2	2009	Cambodia
92.	D2T0610203_KH09_PHP		DENV-2	2009	Cambodia
93.	D2T0610206_KH09_KAN		DENV-2	2009	Cambodia
94.	D2T0427252_KH09_KSP		DENV-2	2009	Cambodia
95.	D2T0427255_KH09_KSP		DENV-2	2009	Cambodia
96.	D2T0427233_KH09_KSP		DENV-2	2009	Cambodia
97.	D2T0427249_KH09_KSP		DENV-2	2009	Cambodia

98.	D2T0520346_KH09_BTB		DENV-2	2009	Cambodia
99.	D2T0601085_KH09_KSP		DENV-2	2009	Cambodia
100.	D2T0629150_KH09_PLN		DENV-2	2009	Cambodia
101.	D4I0910199_KH98_PHP		DENV-4	1998	Cambodia
102.	D4J0920256_KH99_PHP		DENV-4	1999	Cambodia
103.	D4L0620198_KH01_KKG		DENV-4	2001	Cambodia
104.	D4L1010201_KH01_KAN		DENV-4	2001	Cambodia
105.	D4L1024300_KH01_BTB		DENV-4	2001	Cambodia
106.	D4M0709250_KH02_BTB		DENV-4	2002	Cambodia
107.	D4M0724220_KH02_BTB		DENV-4	2002	Cambodia
108.	D4M0724221_KH02_BTB		DENV-4	2002	Cambodia
109.	D4M0904309_KH02_BTB		DENV-4	2002	Cambodia
110.	D4M0918326_KH02_BMC		DENV-4	2002	Cambodia
111.	D4M0918328_KH02_BMC		DENV-4	2002	Cambodia
112.	D4M1127344_KH02_PHP		DENV-4	2002	Cambodia
113.	D4M1230321_KH02_PHP		DENV-4	2002	Cambodia
114.	D4N0104121_KH03_PHP		DENV-4	2003	Cambodia
115.	D4N0305153_KH03_TAK		DENV-4	2003	Cambodia
116.	D4N0430169_KH03_BTB		DENV-4	2003	Cambodia
117.	D4N0521088_KH03_SRP		DENV-4	2003	Cambodia
118.	D4N0604280_KH03_SRP		DENV-4	2003	Cambodia
119.	D4N0611092_KH03_SRP		DENV-4	2003	Cambodia
120.	D4N0702255_KH03_BTB		DENV-4	2003	Cambodia
121.	D4N0806028_KH03_SRP		DENV-4	2003	Cambodia
122.	D4N1022124_KH03_SRP		DENV-4	2003	Cambodia
123.	D4O0721189_KH04_KCH		DENV-4	2004	Cambodia
124.	D4O1123011_KH04_PHP		DENV-4	2004	Cambodia
125.	D4P0309251_KH05_KCH		DENV-4	2005	Cambodia
126.	D4P0615271_KH05_KCH		DENV-4	2005	Cambodia
127.	D4R0627035_KH07_BMC		DENV-4	2007	Cambodia
128.	D4R0717285_KH07_PHP		DENV-4	2007	Cambodia
129.	D4R0627318_KH07_KCH		DENV-4	2007	Cambodia
130.	D4R0702404_KH07_KCH		DENV-4	2007	Cambodia
131.	D4R0707184_KH07_KCH		DENV-4	2007	Cambodia
132.	D4R0707213_KH07_KCH		DENV-4	2007	Cambodia
133.	D4R0724344_KH07_KCH		DENV-4	2007	Cambodia
134.	D4R0802455_KH07_KCH		DENV-4	2007	Cambodia
135.	D4S0603242_KH08_PHP		DENV-4	2008	Cambodia
136.	D4S0626191_KH08_KAN		DENV-4	2008	Cambodia
137.	D4S0702299_KH08_BTB		DENV-4	2008	Cambodia
138.	D4S0702301_KH08_BTB		DENV-4	2008	Cambodia
139.	D4S0709094_KH08_PRV		DENV-4	2008	Cambodia
140.	D4S0806088_KH08_SRP		DENV-4	2008	Cambodia
141.	D4S0813047_KH08_SRP		DENV-4	2008	Cambodia
142.	D4S0813311_KH08_BTB		DENV-4	2008	Cambodia
143.	D4S0818104_KH08_PHP		DENV-4	2008	Cambodia
144.	D4S0915182_KH08_PHP		DENV-4	2008	Cambodia
145.	D4S1006107_KH08_PHP		DENV-4	2008	Cambodia
146.	D4S1009093_KH08_PHP		DENV-4	2008	Cambodia
147.	D4S1105091_KH08_PHP		DENV-4	2008	Cambodia
148.	D4S1118223_KH08_PHP		DENV-4	2008	Cambodia
149.	D4S1215101_KH08_PHP		DENV-4	2008	Cambodia

150.	D4T0522111_KH09_KCH		DENV-4	2009	Cambodia
151.	D4T0522199_KH09_KCH		DENV-4	2009	Cambodia
152.	D4T0529171_KH09_KCH		DENV-4	2009	Cambodia
153.	D4T0529172_KH09_KCH		DENV-4	2009	Cambodia
154.	D4T0529173_KH09_KCH		DENV-4	2009	Cambodia
155.	D4T0615152_KH09_KCH		DENV-4	2009	Cambodia
156.	D4T0615158_KH09_KCH		DENV-4	2009	Cambodia
157.	D4T0615203_KH09_KCH		DENV-4	2009	Cambodia
158.	D4T0615211_KH09_KCH		DENV-4	2009	Cambodia
159.	D4T0630330_KH09_KAN		DENV-4	2009	Cambodia
160.	D4T0914144_KH09_PHP		DENV-4	2009	Cambodia
161.	D4U0512353_KH10_BTB		DENV-4	2010	Cambodia
162.	D4U0607095_KH10_PHP		DENV-4	2010	Cambodia
163.	D4U0811384_KH10_KCH		DENV-4	2010	Cambodia
164.	D4U0811386_KH10_KCH		DENV-4	2010	Cambodia

CHAPTER 3 : CLINICAL MANIFESTATION IN DENGUE INFECTIONS

II.1.7 Context of study

DENV infection induces life-long protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infections by any of the other three serotypes. Secondary infection by a different DENV type is the most significant risk factors for severe dengue. Viral strain (Rico-Hesse, 2007; Watts et al., 1999) and human host genetic polymorphisms (Sakuntabhai et al., 2005) also influence the clinical outcome of DENV infection, and much has yet to be learned about the complex interplay between host and pathogen in the pathogenesis of dengue. Unapparent dengue infection was detected mostly through large serological cohort studies (Burke et al., 1988; Porter et al., 2005; Yew et al., 2009) and is estimated to represent 50-90% of all DENV infections (Kyle and Harris, 2008). However, these kind of data are still very limited and very little is known regarding the biological characteristics of the infections in these asymptomatic individuals.

The burden and the severity of dengue disease in a population are always difficult to evaluate because laboratory confirmation is frequently unavailable and there is a great selection bias towards more severe cases. When laboratory diagnosis of dengue disease is accessible, it is mainly performed using serological tools with limited performances that only become positive in the early convalescent phase of infection; more specific viral diagnosis tools, based on viral isolation and genome detection methods that detect acute dengue infection, are generally only available in well-equipped reference centers for arboviruses (Guzman et al., 2004; Shu and Huang, 2004).

II.1.8 Objectives

In order to evaluate the real burden and severity of the dengue infection in general population and not only in hospitalized patients, a household investigation was performed to compare clinical data and biological markers from subjects with a broad range of dengue disease clinical presentations, including pauci-or a-symptomatic cases that are not captured in most of the clinical studies. For this purpose, a multinational, prospective study in South-East Asia (Cambodia and Viet Nam) and Latin America (Brazil and French Guiana) was conducted. This clinical study aimed (i) to estimate the proportion of pauci-or a-symptomatic dengue infections among household members of a laboratory-confirmed symptomatic dengue case, and (ii) to compare clinical and biological data from unapparent dengue-infected subjects and symptomatic dengue-infected patients. In addition, an ELISA test to detect DENV NS1 protein (Alcon et al., 2002; Young et al., 2000) that

could theoretically be implemented in any laboratory for diagnosis of acute dengue (Blacksell et al., 2008; Dussart et al., 2006; Dussart et al., 2008) was evaluated for its performance in early dengue diagnosis.

II.1.9 Results and conclusions

This study examined the clinical and virological spectrum of DENV infections in children and adults living in the same household of virologically-confirmed dengue cases in South-East Asia and in Latin America. During the study period, the etiologies of dengue-like illness were more diverse in Latin America than in South-East Asia, where acute dengue cases represented two-thirds of the symptomatic dengue-like illnesses.

Among the 443 participants recruited, 215 (48.5%) were identified as dengue index cases (DIC), 21 (4.7%) as dengue convalescent cases, 187 (42.2%) as non-dengue cases (NDC), and 20 (4.5%) were non classifiable. Among the 215 DIC, 28 (13%) were considered as severe dengue cases and all of them were from SEA and only 177 confirmed dengue cases had a familial investigation. Among the 497 household participants, 39 had a dengue infection and 29 (74.4%) of them were asymptomatic. Among households with at least two dengue-infected cases, 9 (31.0%) households were found to have 2 different DENV types circulating at the same period of time.

Logistic regression with familial cluster effect identified having low neutrophils and low monocytes as factors associated with clinical dengue symptoms compared to non-dengue participants. Symptomatic cases had significantly lower lymphocytes counts and more positive NS1 antigen detection than from unapparent dengue-infected individuals.

DENV-1, -2 and -3 were found with similar frequencies in South-East Asia while DENV-3 predominated in Latin America. Among severe dengue cases reported in South-East Asia, 15 (53.6%) were infected with DENV-2. In parallel with the virological techniques, NS1 antigen capture detection kit (BioRad) was used and had good sensitivity (83.6%) and specificity (98.9%) in Asia and Latin America, as reported in previous studies (Dussart et al., 2006; Kumarasamy et al., 2007). The use of viral detection antigen (NS1) was especially useful in the first 5 days of illness.

The next chapter (Chapter 4) will evaluate more explicitly the performance of NS1 antigen capture assay using well characterized data including the various factors that might interfere with this assay in the Cambodian context.

The result of this work is summarized in the article submitted to « PloS Neglected Tropical Diseases ».

PLoS Neglected Tropical Diseases

Clinical and virological study of dengue cases and the members of their households: the multinational DENFRAME project

--Manuscript Draft--

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Short Title:	Dengue Clinical Study in Asia and Latin America
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Abstract:	Background: Dengue has emerged as the most important vector-borne viral disease in tropical areas and its geographic range is continually expanding. Evaluations of the burden and severity of dengue disease in populations have been hindered by the frequent lack of laboratory confirmation and strong selection bias towards more severe cases. Methodology: A multinational, prospective clinical study was carried out in South-East Asia and Latin America, to ascertain the proportion of inapparent dengue infections in households of febrile dengue cases, and to compare clinical data and biological markers (including NS1 antigen) from subjects with various dengue disease patterns. Dengue infection was laboratory-confirmed during the acute phase, by virus isolation and detection of the genome. The four participating reference laboratories used standardized methods. Principal findings: Among 215 febrile dengue subjects - 114 in South-East Asia (SEA) and 101 in Latin America (LA) - 28 (13.0%) were diagnosed with severe dengue (from SEA only) using the WHO definition. Household investigations were carried out for 177 febrile subjects. Among household members at the time of the first home visit, 39 acute dengue infections were detected of which 29 were inapparent. A further 62 dengue cases were classified at early convalescent phase. The proportion of dengue infections was 47.5% (278/585; 95%CI: 43.5-51.6). Lymphocyte counts and detection of the NS1 antigen differed significantly between inapparent and symptomatic dengue subjects; among inapparent cases lymphocyte counts were normal and only 20% were positive for NS1 antigen. Primary dengue infection (defined as absence of IgG at acute phase) and dengue virus serotype were not associated with symptomatic dengue infection in this study. Conclusion: Household investigation demonstrated a high proportion of household members positive for dengue infection, including a number of inapparent cases, the frequency of which was higher in SEA than in LA.
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Clinical and virological study of dengue cases and the members of their households: the multinational DENFRAME project.

Dengue clinical study in Asia and Latin America

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Key words: Dengue disease, Inapparent dengue case, South-East Asia, Latin America, Household investigation.

Abstract

Background: Dengue has emerged as the most important vector-borne viral disease in tropical areas and its geographic range is continually expanding. Evaluations of the burden and severity of dengue disease in populations have been hindered by the frequent lack of laboratory confirmation and strong selection bias towards more severe cases.

Methodology: A multinational, prospective clinical study was carried out in South-East Asia and Latin America, to ascertain the proportion of inapparent dengue infections in households of febrile dengue cases, and to compare clinical data and biological markers (including NS1 antigen) from subjects with various dengue disease patterns. Dengue infection was laboratory-confirmed during the acute phase, by virus isolation and detection of the genome. The four participating reference laboratories used standardized methods.

Principal findings: Among 215 febrile dengue subjects – 114 in South-East Asia (SEA) and 101 in Latin America (LA) – 28 (13.0%) were diagnosed with severe dengue (from SEA only) using the WHO definition. Household investigations were carried out for 177 febrile subjects. Among household members at the time of the first home visit, 39 acute dengue infections were detected of which 29 were inapparent. A further 62 dengue cases were classified at early convalescent phase. The proportion of dengue infections was 47.5% (278/585; 95%CI: 43.5-51.6). Lymphocyte counts and detection of the NS1 antigen differed significantly between inapparent and symptomatic dengue subjects; among inapparent cases lymphocyte counts were normal and only 20% were positive for NS1 antigen. Primary dengue infection (defined as absence of IgG at acute phase) and dengue virus serotype were not associated with symptomatic dengue infection in this study.

Conclusion: Household investigation demonstrated a high proportion of household members positive for dengue infection, including a number of inapparent cases, the frequency of which was higher in SEA than in LA.

Author summary

Dengue is the most important mosquito-borne viral disease in humans. This disease is now endemic in more than 100 countries and threatens more than 2.5 billion people living in tropical countries. It currently affects about 50 to 100 million people each year. It causes a wide range of symptoms, from an inapparent to mild dengue fever, to severe forms, including dengue hemorrhagic fever. Currently no specific vaccine or antiviral drugs are available. We carried out a prospective clinical study in South-East Asia and Latin America, of virologically-confirmed dengue-infected patients attending the hospital, and members of their households. Among 215 febrile dengue subjects, 177 agreed to household investigation. Based on our data, we estimated the proportion of dengue-infected household members to be about 45%. At the time of the home visit, almost three quarters of (29/39) presented an inapparent dengue infection. The proportion of inapparent dengue infection was higher in South-East Asia than in Latin America. These findings confirm the complexity of dengue disease in humans and the need to strengthen multidisciplinary research efforts to improve our understanding of virus transmission and host responses to dengue virus in various human populations.

Introduction

Dengue is the most important mosquito-borne viral disease of humans. The disease is now endemic in more than 100 countries and threatens more than 2.5 billion people. It currently affects about 50 to 100 million people each year [1]. Dengue viruses (DENV) are enveloped, single-stranded positive-sense RNA viruses (family *Flaviviridae*, genus *Flavivirus*). There are four types of DENV: DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virus infection induces life-long protective immunity to the homologous serotype, but confers only partial and transient protection against subsequent infections with any of the other three serotypes [2]. The disease spectrum ranges from inapparent infection or mild dengue fever [3], probably the most common form, to a potentially severe form of dengue characterized by plasma leakage and hemorrhage, known as severe dengue. Uncommonly, severe dengue may manifest as hepatitis, encephalopathy or rhabdomyolysis [2,4,5,6,7]. About 500,000 people are estimated to have severe dengue and about 25,000, mostly children, die from it each year [8]. The underlying causes determining the outcome of DENV infection remain unknown. Although previous exposure, viral strain and human host genetic polymorphisms also influence the clinical outcome of DENV infection, we still know little about the complex interplay between host and pathogen in the pathogenesis of dengue [9,10,11,12].

Inapparent infections have largely been detected retrospectively through serology. The uses of genome detection or virus isolation have enabled detection of inapparent infections in cluster studies designed to detect natural infections in the community [13,14]. The present study was designed to identify symptomatic and inapparent dengue-infected subjects in genetically-related individuals living in the same household, in line with the main aim of the DENFRAME project which is to explore the influence of human genetic variants and their functional roles in the pathogenesis of dengue disease in humans. We based the identification of dengue-infected subjects upon virological techniques, namely virus isolation and detection of the genome. We also took this opportunity to evaluate prospectively a

commercial NS1 capture assay [15,16] that could potentially be implemented in laboratories for the diagnosis of acute dengue [17,18,19].

Methods

Objectives

A multinational, prospective study was conducted in South-East Asia (Cambodia and Vietnam) and Latin America (Brazil and French Guiana). We used virological techniques to identify dengue patients diagnosed at the acute phase of disease among the patients presenting with dengue-like illness. We then performed a household investigation, comparing clinical data and biological markers from subjects with a broad range of dengue disease patterns, including inapparent dengue cases that are rarely captured in clinical studies. This clinical study's aims were: (i) to estimate the proportion of inapparent dengue infections among members of the households of laboratory-confirmed symptomatic dengue cases, (ii) to calculate the proportion of dengue-infected subjects at the time of the household investigation, and (iii) to compare clinical and biological data from inapparent and symptomatic dengue-infected subjects.

Study sites

Five institutions were involved in this study during the recruitment period: Instituto Evandro Chagas (IEC) in Belém (Pará state, Brazil), *Institut Pasteur du Cambodge* (IPC) in Phnom Penh (Cambodia), *Institut Pasteur de la Guyane* (IPG) in Cayenne (French Guiana) and *Institut Pasteur de Ho Chi Minh Ville* (IPHCM) in Vietnam were responsible for the recruitment of patients and virological analyses; the *Institut Pasteur* (IP) in Paris (France) designed the study and was responsible for central monitoring and data analysis.

As shown in the two maps (Figure 1), volunteers were recruited at four clinical sites: Vinh Thuan District Hospital (Vietnam), Kampong Cham Referral Hospital (Cambodia), the IPG in Cayenne (French Guiana) and public outpatient and emergency rooms managed by the Belém Health Secretariat in the districts of Guamá, Marco, Marambaia and Sacramenta, and the outpatient unit of the IEC (Brazil). The virology laboratories of the four institutions responsible for recruitment are all National Reference Centers (NRC) for Arboviruses (IEC is

also a WHO collaborative center). These laboratories carried out virological, NS1 antigen (Platelia Dengue NS1 Antigen, Bio-Rad, Marnes La Coquette, France), and serological techniques.

Study design

We recruited subjects with acute dengue-like illness at the study sites. These subjects were identified by the treating physicians and were included if they satisfied the following criteria: (i) aged over 24 months; (ii) oral temperature > 38°C and onset of symptoms within the last 72 h; and (iii) presenting with at least one clinical manifestation suggestive of dengue-like illness: severe headache, retro-orbital pain, myalgia, joint pain, rash or any bleeding symptom. Furthermore, for inclusion in the second step of the study, the subject had to come from a familial household containing more than two people during the seven days preceding illness. We first identified the dengue-infected subjects (referred to in this study as Dengue Index Cases or DIC) and non-dengue-infected subjects (defined as Non-Dengue Cases - NDC) on the basis of virological results from an acute sample (see below). We then recruited individuals from the households of the DIC. We thus constituted three groups of participants: 1) DIC, 2) household members (HHM), and 3) NDC not related to the DIC. For all groups (DIC, HHM and NDC), we applied the same exclusion criteria: women who were pregnant or breastfeeding, individuals with a focal source of infection (e.g. otitis media, pneumonia, meningitis), patients presenting with a known chronic illness, and patients with malaria. Moreover, to ensure the feasibility of this study, each study site was asked to target a convenient sample of 50 households and to recruit subjects from July 2006 to June 2007 in line with the approval granted by the Institutional Review Board and the timing of the dengue season at each site.

Clinical data and blood sample collection

Participants were examined during sequential visits, as shown in the study design charts (Figure 2). At each visit, data were collected with a standardized questionnaire. Severe

dengue cases were classified, according to WHO recommendations on the basis of the clinical data. Biological data were also recorded at the sequential visits [2]. Blood samples were collected during the visits and were rapidly processed by the laboratories of each of the recruiting sites, for dengue diagnosis and biological testing. Blood sample volume was adapted for children weighing less than 20 kg.

Paired blood samples were collected for subjects presenting dengue-like illness to allow classification as DIC or NDC: during the acute phase (Visit 1) and during the convalescence phase (Visit 4: 15 to 21 days after the onset of fever). Blood samples were taken from hospitalized DIC within 24 hours of defervescence (Visit 3). HHM were visited at home for blood collection within 24 to 72 hours of DIC identification (Home Visit 1). For practical and logistical reasons this delay of up to 72 hours was unavoidable. HHM were supplied with a monitoring diary card and a thermometer, to enable them to follow their temperature over a 7-day period. For HHM with a positive diagnosis of dengue or with an onset of fever during the seven days of monitoring, a second visit with blood collection for dengue diagnosis was organized (Home Visit 2). Blood analyses included virological and serological dengue diagnosis, complete blood count, transaminases and bilirubin levels. Finally, the data were coded and entered into the computer via a secure website specifically developed with the PHP/MySQL system.

Classification of dengue cases on the basis of acute dengue diagnosis

All serum samples collected at Visit 1 or at Home Visit 1 or Home Visit 2 were tested: (i) for acute dengue diagnosis, defined as positive virus isolation on mosquito cells [20] and/or positive viral RNA detection by reverse transcriptase-polymerase chain reaction (RT-PCR) [21], and (ii) for the diagnosis of early convalescent dengue cases based on a standardized DENV IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) [22], and DENV IgG detection by indirect ELISA (in-house protocol developed by each NRC for Arboviruses). NS1 antigen detection was also performed.

Only subjects with febrile dengue infection diagnosis were classified as DIC. Subjects in the early stage of dengue convalescence at Visit 1 (*i.e.* positive NS1 antigen detection with concomitant DENV IgM detection, or isolated DENV IgM detection with no positive viral tests) were not classified as DIC; we did not perform a household investigation for them. For the classification of dengue-infected HHM at Home Visit 1, we included both HHM with an acute (febrile or inapparent) dengue infection diagnosis and HHM with isolated DENV IgM detection, presumably related to an infection preceding that of the DIC (i.e in the early convalescence phase). During the 7-day period of home monitoring, several new febrile cases of dengue-infected HHM were also confirmed through Home Visit 2.

We were unable to use the DENV IgM/IgG ratio to distinguish between primary and secondary dengue infections, due to a lack of standardization of DENV IgG tests among laboratories [23]. We therefore established two groups of dengue-infected participants, based on the presence or absence of DENV IgG during the acute phase of the disease. In this study, we considered the presence of DENV IgG in the acute phase of the study to be suggestive of previous dengue infection. All sera were also checked for DENV IgM and IgG at Visit 4. Finally, if all these dengue tests were negative, participants were classified as NDC.

Ethics

The study was approved by the Institutional Review Board of the *Institut Pasteur* and by the ethics committees of each of the countries concerned. It was conducted in accordance with the Declaration of Helsinki, and the participants or the parents of minors participating in the study gave written informed consent before inclusion. The clinical protocol, the questionnaires, the standard operating procedures and informed consent forms were adapted and translated for each clinical site. All the documentation was accessible through a dedicated website with a specific login access (www.denframe.org). The centralized

electronic database was based at the *Institut Pasteur* in Paris and registered with the *Commission Nationale de l'Informatique et des Libertés* (CNIL) in France.

Statistical methods

We present here the data from all four study sites in Latin America and South-East Asia. DIC are described according to region, disease severity, DENV type, age group and IgG status. We estimated the proportion of inapparent dengue infections among HHM, and we calculated the proportions of dengue-infected subjects among household subjects, in total and according to the IgG status at the time of household investigation. We compared clinical data and biological markers between inapparent dengue-infected subjects, symptomatic dengue-infected subjects, and non-dengue-infected participants at the time of the household investigation. We created binary variables to evaluate the effect of DENV infection on lymphocytes and neutrophils: we used a threshold of 2×10^9/l for adults, but variation with the age of the subject was taken into account. We used chi-squared or Fisher's exact tests to compare categorical variables between symptomatic cases, inapparent dengue-infected cases and non-dengue-infected subjects among HHM. Univariate and multivariable logistic regression models were used to assess the effect of covariates on the odds ratios (OR) of symptomatic dengue-infected cases, inapparent dengue-infected cases, and non-dengue-infected subjects among HHM. For the multivariable logistic regression models including data from household members (Tables 3 & 4 and supplementary Table S2), we used two-stage hierarchical regression models taking into account the family household structure [24]. Potential confounders with a P value of less than 0.20 in univariate analysis were retained for the final multivariable analyses. STATA version 10.0 (Stata Corp., College Station, TX, USA) and a significance level of 5% were used for all statistical analyses.

Results

Flowcharts for the recruitment of participants at each step are shown in Figure 3.

Step 1: identification of dengue index cases (DIC)

We screened 473 febrile subjects for dengue infection. Thirty (6.3%) had at least one criterion for non inclusion in the study at presentation; the remaining 443 (93.7%) were included in the study. We identified 215 (48.5%) of these 443 subjects as DIC, 21 (4.7%) as dengue convalescent cases, 187 (42.2%) as NDC, and 20 (4.5%) could not be classified because some biological markers were lacking. Recruitment levels during the study period were very low in French Guiana (9 DIC and 24 NDC), whereas there had been a large number of dengue cases during the rainy season of the previous year [25]. For the 215 subjects classified as DIC, 149 (69.3%) were positive by genome detection and viral isolation, 43 (20.0%) were positive by genome detection only, 15 (7.0%) were positive by viral isolation only, and a very few subjects (n=8, 3.7%) were ultimately classified as DIC by the virologists, based on positive NS1 detection, clinical data and serological results (negative IgM at Visit 1 followed by seroconversion IgM at convalescent phase).

The proportions of subjects classified as either NDC or DIC differed between Latin America and South-East Asia: 69.5% (130/187) of the total NDC in the study, and 47.0% (101/215) of the DIC, were recruited in Latin America whereas 30.5% (57/187) of the NDC and 53.0% (114/215) of the DIC were recruited in South-East Asia ($P< 10^{-4}$) (Figure 3A). In other words, in Latin America, in two thirds of subjects presenting with dengue-like illness, the cause was not related to dengue infection. Given the inclusion criteria, the dengue-like illness symptoms were not different between NDC and DIC (data not shown). However, all biological variables, including counts of platelets, lymphocytes and neutrophils, were significantly lower, whereas hematocrit and liver enzyme levels were higher in the DIC group than in the NDC group (data not shown).

Table 1 shows the distribution of DIC by region and according to IgG status at Visit 1 as a function of DENV type and age group. The proportions of severe dengue and dengue fever cases with DENV IgG (suggestive of previous DENV infection) and without DENV IgG in the acute phase were similar (Table 1): 15 (55.6%) severe dengue cases tested negative for DENV IgG and 12 (44.4%) tested positive for DENV IgG, versus 49 (31.8%) and 105 (68.2%) of the subjects with non severe disease, respectively (P = 0.017). DENV-1, -2 and -3 were found with similar frequencies in South-East Asia, whereas DENV-3 predominated in Latin America. Fifteen of the severe dengue cases reported in South-East Asia were infected with DENV-2 (53.6%; 15/28). Interestingly, seven severe dengue cases positive for DENV-2 virus and negative for DENV IgG in the acute phase but with subsequent DENV IgM and IgG seroconversion were identified. This serological pattern suggests that these patients had primary DENV infection. Two DIC in Vietnam were reported with co-detection of multiple DENV strains by RT-PCR: DENV-2/DENV-1 and DENV-4/DENV-2 respectively; the viral cultures were negative for both subjects. Only the first virus detected was considered for further statistical analysis (DENV-2 and DENV-4, respectively).

According to the WHO criteria, twenty-eight (13.0%) subjects were classified as severe dengue (based on severe plasma leakage and/or severe hemorrhages and/or severe organ impairment). All these cases were from clinical sites in South-East Asia (25 in Vietnam and 3 in Cambodia, as presented in supplementary Table S1). At visit 1, presentation with the following combination of features was significantly associated with the occurrence of severe dengue in this population: being male, over the age of seven years, with no retro-orbital pain but with bleeding, low monocyte count, normal liver enzyme levels and DENV-2 type infection.

For 163 (75.8%) DIC, data were available for all the biological markers at visits 1 and 4 (Figure 3A). All these markers had returned to normal levels by visit 4, and all participants,

including the 28 severe dengue cases displayed clinical recovery from dengue disease (data not shown).

Step 2: identification of household members (HHM)

Agreement for household investigations was obtained from 177 (82.3%) DIC, corresponding to a total of 651 household members. We compared the distribution of the covariates (as listed in supplementary Table S1) between the 38 DIC with no familial investigation and the 177 DIC who underwent familial investigation; no significant differences were found in the distribution of the covariates between these two groups (data not shown). All 28 patients with severe dengue infection underwent household investigation. In total, 141 (21.7%) of the 651 household members refused to participate in the study. We therefore screened 510 participants, 497 (97.5%) of whom were eligible for the study. All but one of these 497 household members were genetically related to the DIC. Eighty-four were not classifiable due to the lack of some biological results. Full assessment of DENV infection was carried out according to the study protocol for the remaining 413 of these subjects (Figure 3B) during Home Visit 1.

At the time of the household investigation (Home Visit 1), 39 subjects were identified as being in the acute phase of dengue infection: 29 (74.4%) cases were inapparent and 10 (25.6%) had symptomatic dengue infection. An additional 62 subjects were classified as being in the early phase of convalescence from dengue infection. The remaining 312 subjects were considered as non-dengue-infected at the time of Home Visit 1 (Figure 3B); however, five of them developed some clinical symptoms of dengue fever and were laboratory-confirmed as having acute dengue infection during the 7-day home monitoring. We excluded them (n=5) from the remaining analysis (n=312 subjects with 7-day home monitoring) that thus included 307 subjects (Figure 3B). It should be noted that a second home visit and blood sampling was not possible, for ethical and logistical reasons, for HHM without any clinical symptoms after the 7-day home monitoring. Hence, among the 307

remaining subjects, some may have had an inapparent dengue infection after Home Visit 1. Therefore, we considered that at least 101 (39 acute or 62 early convalescent) dengue infections were found amongst 408 HHM (24.8%; 95% CI: 20.6-28.9) at the time of Home Visit 1 (Figure 3B). Thus, adding together the 177 DIC and the 101 DENV-infected HHM, the overall proportion for dengue among the study participants was estimated at 47.5% (278/585; 95% CI: 43.5-51.6) (Figure 3B). We have also estimated these proportions according to the IgG status (Table 2) at the time of Home Visit 1 (excluding the 5 subjects with known symptomatic infection – 3 were IgG positive and 2 were IgG negative). Among the 585 subjects, 6 had missing IgG data. Among 425 subjects with positive IgG, the estimated proportion of dengue-infected subjects was 43.8% (186/425; 95% CI: 39.0-48.5) and, among the 154 with negative IgG, this estimated proportion was 57.1% (88/154; 95% CI: 49.3-65.0).

In 101 (57.1%) households, there was only one dengue-infected case. For the 76 (42.9%) households with at least two dengue-infected cases, DENV type had been determined for all subjects in 29 households. Nine (31.0%) households were found to have two different DENV types circulating during the same time period: DENV-1 & DENV-3 (n = 2 in Brazil, n = 4 in Cambodia), DENV-1 & DENV-2 (n = 1 in Vietnam), and DENV-2 & DENV-3 (n = 2 in Vietnam).

Tables 3 & 4 show comparisons between non-dengue-infected and inapparent dengue-infected cases, and symptomatic and inapparent dengue-infected subjects, respectively, among the household subjects. Supplementary Table S2 presents the main characteristics of subjects with acute dengue infection compared to non-dengue-infected subjects among the household subjects. In the comparisons between non-dengue-infected and inapparent dengue-infected subjects, taking into account potential confounders, only neutrophil and monocyte levels differed significantly whereas presence of IgG at Visit 1 was almost significant with the non-dengue-infected group. The comparison between symptomatic and

inapparent dengue-infected subjects (Table 4) showed significant difference between groups for lymphocyte counts and positive NS1 antigen detection. In this analysis, no significant difference was found for DENV types identified or IgG detection during the acute phase.

Discussion

Several previous epidemiological studies have focused on school-based surveillance aiming at improving dengue-vector control measures [3,14], studying the dynamics of patterns of dengue transmission [26,27,28] or describing a model that takes into account the role of human movement in the transmission dynamics of vector-borne pathogens [29]. Earlier cluster investigation methods were designed as an alternative approach to the commonly used prospective cohort study method for investigating the natural history of dengue virus infection in South-East Asia and Latin America [13,30]. Although different study designs have demonstrated the feasibility of identification of inapparent dengue cases, it remains difficult to recruit these subjects. We designed our study to include family household investigation in order to identify a group of inapparent dengue-infected subjects and to compare them with symptomatic dengue-infected and non-dengue-infected subjects living in the same family household. The study design was based on family household recruitment specifically in order to collect data and biological samples, and to study secondarily the host susceptibility to dengue infection and disease. Unlike studies based on cohorts from hospital referrals, this multi-country study captured dengue cases ranging from inapparent infections, through mild disease to severe dengue fever, using definitions of clinical cases and diagnostic methodology standardized across the four sites. The period of inclusion, from July 2006 to June 2007, spanned the dengue season at each site, although incidence of dengue was low that year in French Guiana.

The main objective of this study was to identify dengue infections and particularly inapparent infections among dengue patients' household family members in South-East Asia and Latin America. Based on our data, we estimated the proportion to be about 45% among those participating in the household study. Most of the dengue cases studied had symptomatic infections, covering the spectrum of disease from dengue fever to severe dengue cases. We also identified inapparent infections in the population. We observed dengue-infected subjects classified as DIC and some of their HHM without acute dengue infection but with a positive

IgM detection, suggesting an early convalescent phase after dengue infection with no clinical symptoms. In this study we identified 29 inapparent dengue infections but we believe this number underestimates the proportion of inapparent dengue cases because we were not able to take blood samples from non-symptomatic subjects at Home Visit 2.

We postulated that dengue is transmitted to members of the DIC's family household during the period of the index subject's infection, and thus designed our study to detect inapparent dengue infections with a home visit organized shortly after identification of DIC. Obviously, we cannot confirm whether the index subject's DIC was always the source of infection in other family members, but we can postulate that a non-hospitalized DIC who remains at home during acute illness represents a potential source of DENV transmission to *Aedes*. According to our study design, clustering of cases within a household could be the result of a single or very few infected mosquitoes biting different household members during a short period of time, perhaps within a single gonotrophic cycle as previously suggested [14,31]. This is also consistent with a previous observation that over periods from 1 to 3 days, dengue cases were clustered within short distances, i.e., within a household [32]. No mosquito captures were, however, conducted in our study to identify DENV-positive *Aedes* mosquitoes. DENV sequencing would help resolve the extent of localized transmission.

We characterized subjects with acute dengue infection using virus isolation and detection of the genome. We also used NS1 antigen detection, a more recently recognised diagnostic tool. As for many tropical infectious diseases, there is an urgent need for validated diagnostic tools for dengue. In parallel with the virological techniques, we evaluated detection of the NS1 antigen with the Platelia Dengue NS1 Ag test. In this study, this test was found to have good sensitivity (83.6%; 95% confidence interval (CI): 78.5-88.6) and specificity (98.9%; 95% CI: 96.6-99.9) in both Asia and Latin America, as reported in previous studies [17,33,34]. A recent multi-country study observed unequal sensitivity between geographical regions that remains unexplained, suggesting further assessments are needed [35]. The use of viral

detection antigen is particularly useful during the first five days of illness with NS1 assays that are significantly more sensitive for primary than secondary dengue [18,34,36]. However, NS1 antigen could be detected in only 20% of inapparent DENV-infection. This finding suggests that NS1 antigen may have a role in dengue disease pathogenesis and also indicates that this test cannot be relied upon for detection of inapparent dengue infection.

By comparing HHM not infected with dengue with those presenting with inapparent dengue infection, we showed that neutrophil and monocyte counts were early indirect biological markers of dengue infection, whereas platelet counts and the frequency of IgG detection at the first visit did not differ between the two groups (Table 3). A comparison of inapparent dengue-infected HHM with symptomatic dengue-infected subjects showed that lymphocyte counts and detection of the NS1 antigen differed significantly between these two groups (Table 4). Moreover, the NS1 antigen was detected during the acute phase in most of the dengue cases tested, and the sensitivity of this test was even higher in severe dengue cases (26/28, supplementary Table S1), possibly reflecting higher viral loads. These findings may indirectly reflect the progression of the immune response to DENV, leading in some cases to severe acute lymphopenia and a lack of virological control, with high rates of NS1 antigen circulation in the blood that may be correlated with high-level or prolonged viremia [7,36]. Severe dengue cases were also more likely to be male, to have lower monocyte counts or normal liver enzyme levels, and to be infected with DENV-2, although quantitative RT-PCR did no permit study of the magnitude of the viremia. We showed that half of the severe dengue cases had not previously been infected with DENV, as confirmed by the occurrence of DENV IgG seroconversion during convalescent phase [7]. In all dengue-infected subjects, including inapparent, we observed a decrease in neutrophil and monocyte counts. On one hand, it may suggest a direct effect of dengue illness on hematopoiesis, although such an effect is in conflict with data reported elsewhere in the literature [37]. On the other hand, DENV is detected in peripheral monocytes during acute disease, and the infection of monocytes leads to cytokine production, suggesting that virus-monocyte interactions are

relevant to pathogenesis [38,39,40]. Moreover, DENV can induce apoptosis in monocytes, and this may lead to decreases in the number of these cells in severe dengue cases [41].

In this study we only observed severe dengue cases in South-East Asia. Disease severity and pathogenesis remain largely unexplained and certainly related to complex interactions of several factors, including virus strain, immune response to previous dengue infection and host genetic background. The introduction of the Asian 1 DENV-2 genotype into the Americas in the 1980s led to the emergence of severe dengue cases on this continent. Following this introduction a new genotype emerged, named Asian/American DENV-2 genotype [42,43,44]. During the study period, this Asian/American genotype was circulating in French Guiana (Philippe Dussart, personal data) and probably in the north of Brazil, however DENV-2 did not cause an outbreak and we did not report any severe dengue case among Brazilian subjects.

Two constraints of the study design deserve mention. All methods (biological markers, virological testing, NS1 antigen detection and IgM serology) were standardized across the four reference laboratories, with the exception of the IgG ELISA. As a consequence, we were unable to calculate the IgM/IgG ratio [45,46]. However, as the intention was to include dengue cases during the acute phase of infection, this ratio was not a crucial endpoint for the study. Another constraint of this study was that we did not include infants and children below 24 months of age in the DENFRAME project. However, several previous reports already provide insight into the epidemiology of dengue in this specific population [47,48,49,50].

These findings confirm the complexity of dengue disease in humans and the need to strengthen multidisciplinary research efforts to improve our understanding not only of virus transmission but also host responses to DENV in various human populations. It will therefore be interesting, based on clinical data and biological samples collected in this study, to further evaluate the host susceptibility to dengue infection and disease using family-based

association analyses. Moreover, we think that technological transfer of standardized diagnostic methods in laboratories based in tropical countries is essential if we are to estimate disease burden and to optimize vector control interventions. Together with improvements in clinical care for dengue patients and better understanding of dengue pathogenesis, the development of a preventive vaccine and antiviral drugs would complete the arsenal of weapons for combating dengue worldwide.

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Figure legends

Figure 1. Localization of the four clinical sites. A: in South-East Asia (Cambodia and Vietnam). B in Latin America (Brazil and French Guiana).

Figure 2. Study design for the inclusion of patients. A: Step 1, identification of dengue index cases (DIC) and non-dengue-infected cases (NDC). B: Step 2, Identification of household members (HHM).

Figure 3. Identification of the dengue index cases (DIC) and of the household members (HHM). A: Identification of DIC in Step 1. B: Recruitment of HHM for 177 DIC during Step 2. * Full assessment of DENV infection was performed for a total of 413 HHM at Home Visit 1, and 312 subjects were considered as non-dengue-infected at that time. Five of them developed a dengue fever and were excluded from our analysis, defining a total of 408 HHM at Home Visit 1. Among them, 307 (312-5) subjects may have had an inapparent dengue infection after Home Visit 1 as we did not perform blood sample collection at Home Visit 2 for non-symptomatic subjects.

Table 1. Characteristics of dengue index cases (DIC, n = 215). Distribution of DIC is provided by region in relation to the presence of WHO criteria for severe dengue and IgG status during the acute phase.

	Acute serum samples (n = 215)											
	Latin America (n = 101)						South-East Asia (n = 114)*					
	Negative IgG (n = 14)			Positive IgG (n = 87)			Negative IgG (n = 60)			Positive IgG (n = 51)		
	Severe dengue n = 0 (%)	Dengue fever n = 6 (%)	Non classifiable n = 8 (%)	Severe dengue n = 0 (%)	Dengue fever n = 70 (%)	Non classifiable n = 17 (%)	Severe dengue n = 15 (%)	Dengue fever n = 43 (%)	Non classifiable N = 2 (%)	Severe dengue n = 12 (%)	Dengue fever n = 35 (%)	Non classifiable n = 4 (%)
Dengue type												
DENV-1	-	3 (50.0)	-	-	8 (11.4)	3 (17.6)	4 (26.7)	20 (46.5)	-	2 (16.7)	14 (40.0)	-
DENV-2	-	-	8 (100.0)	-	13 (18.7)	1 (5.9)	7 (46.7)	12 (27.9)	-	7 (58.3)	6 (17.2)	-
DENV-3	-	3 (50.0)	-	-	47 (67.1)	13 (76.5)	3 (20.0)	9 (21.0)	1 (50.0)	1 (8.3)	11 (31.4)	2 (50.0)
DENV-4	-	-	-	-	-	-	-	1 (2.3)	-	-	2 (5.7)	1 (25.0)
Missing data	-	-	-	-	2 (2.8)	-	1 (6.6)	1 (2.3)	1 (50.0)	2 (16.7)	2 (5.7)	1 (25.0)
Age-group (years)												
[2-7]	-	-	-	-	3 (4.3)	2 (11.8)	2 (13.3)	21 (48.9)	2 (100.0)	2 (16.7)	13 (37.1)	-
]7-10]	-	1 (16.7)	1 (12.5)	-	-	1 (5.9)	3 (20.0)	9 (20.9)	-	4 (33.3)	6 (17.1)	1 (25.0)
>10	-	5 (83.3)	7 (87.5)	-	67 (95.7)	13 (76.4)	10 (66.7)	13 (30.2)	-	6 (50.0)	16 (45.7)	3 (75.0)
Missing data	-	-	-	-	-	1 (5.9)	-	-	-	-	-	-

* For 3 subjects infected by DENV-2, data related to IgG status were missing: 2 dengue fever cases and 1 severe dengue case.

Table 2. Distribution of the participants to the clinical study (n = 590). All participants were identified at Visit 1 for Dengue Index Cases (DIC) and at Home Visit 1 for dengue-infected household members (HHM). Their distribution is presented by country, according to DENV-infected status and IgG status.

	Brazil	French Guiana	Cambodia	Vietnam	**Total**
	n = 134 (%)	n = 28 (%)	n = 180 (%)	n = 248 (%)	**n = 590 (%)**
	[IgG+ / IgG-]	[IgG+ / IgG-]	[IgG+ / IgG-]	[IgG+ / IgG-]	**[IgG+ / IgG-]**
Non DENV-infected subjects	47 (15.4)	9 (3.0)	98 (32.1)	151 (49.5)	**305 (51.7)**
	[44 / 3]	[3 / 6]	[95 / 3]	[97 / 54]	**[239 / 66]**
Missing IgG data	1	-	-	1	**2 (0.3)**
Early convalescent phase or convalescent phase (HHM only)	4 (6.5)	3 (4.9)	22 (36.1)	32 (52.5)	**61 (10.3)**
	[4 / 0]	[2 / 1]	[22 / 0]	[25 / 7]	**[53 / 8]**
Missing IgG data	-	-	-	1	**1 (0.2)**
DENV-infected at the acute phase (DIC + HHM)	82 (37.6)	16 (7.4)	60 (27.5)	60 (27.5)	**218 (37.0)**
Symptomatic	[69 / 6]	[3 / 10]	[30 / 19]	[16 / 36]	**[118 / 71]**
Missing IgG data	-	-	-	3	**3 (0.5)**
Inapparent dengue infection	[6 / 1]	[1 / 2]	[8 / 3]	[3 / 5]	**[18 / 11]**

Table 3. Main characteristics of subjects with inapparent dengue infections compared to non-dengue-infected subjects among Household members. Univariate and multivariable logistic regression were used for analyses.

	Non-dengue-infected n = 307 (%)	Inapparent dengue infection n = 29 (%)	Crude OR	95% CI	P	Adjusted OR	95% CI	P
Sex								
Male	135 (44.0)	16 (55.2)	1					
Female	172 (56.0)	13 (44.8)	0.64	[0.3-1.4]	0.25			
Age (years)								
[2 - 7]	16 (5.2)	5 (17.2)	1			1		
]7 - 10]	17 (5.5)	2 (6.9)	0.38	[0.1-2.2]	0.28	0.79	[0.1-6.5]	0.83
> 10	274 (89.3)	22 (75.9)	0.26	[0.1-0.7]	0.015	0.41	[0.1-1.8]	0.25
Weight-based Z-score								
[-1, 1]	89 (29.0)	6 (20.7)	1					
< -1	195 (63.5)	21 (72.4)	1.6	[0.6-4.1]	0.33			
> 1	23 (7.5)	2 (6.9)	1.3	[0.2-6.8]	0.76			
Hematocrit (%)								
≤ 36	93 (30.3)	7 (24.1)	1					
> 36	212 (69.1)	22 (75.9)	1.38	[0.6-3.3]	0.48			
Missing data	2 (0.6)	-						
Platelets (x 10^9/L)								
> 100	296 (96.4)	26 (89.7)	1			1		
≤ 100	10 (3.3)	3 (10.3)	3.42	[0.9-13.2]	0.075	1.71	[0.2-12.3]	0.6
Missing data	1 (0.3)	-						
Neutrophils (x 10^9/L)								
> 2	288 (93.8)	18 (62.1)	1			1		
≤ 2	18 (5.9)	11 (37.9)	9.8	[4-23.8]	<0.0001	7.75	[2.5-24]	**<0.0001**
Missing data	1 (0.3)	-						
Lymphocytes (x 10^9/L)								
> 2	243 (79.2)	15 (51.7)	1			1		
≤ 2	63 (20.5)	14 (48.3)	3.6	[1.6-7.8]	0.001	2.08	[0.7-5.6]	0.15
Missing data	1 (0.3)	-						
Monocytes (x 10^9/L)								
> 0.2	298 (97.1)	23 (79.3)	1			1		
≤ 0.2	8 (2.6)	6 (20.7)	9.72	[3.1-30]	<0.0001	9.1	[1.8-44]	**0.006**
Missing data	1 (0.3)	-						
ASAT (UI/L)								
≤ 30	225 (73.3)	17 (58.6)	1			1		
> 30	81 (26.4)	11 (37.9)	1.8	[0.8-4]	0.15	1.96	[0.7-5.2]	0.17
Missing data	1 (0.3)	1 (3.5)						
ALAT (UI/L)								
≤ 35	261 (85.0)	22 (75.9)	1					
> 35	45 (14.7)	6 (20.7)	1.58	[0.6-4.1]	0.35			
Missing data	1 (0.3)	1 (3.4)						
Bilirubin (µmol/L)								
≤ 17	262 (85.3)	24 (82.8)	1					
> 17	42 (13.7)	3 (10.3)	0.78	[0.2-2.7]	0.69			
Missing data	3 (1.0)	2 (6.9)						
IgG at visit 1								
Negative	66 (21.5)	11 (37.9)	1			1		
Positive	239 (77.8)	18 (62.1)	0.45	[0.2-1.0]	0.051	0.37	[0.1-1.04]	0.06
Missing data	2 (0.7)	-						

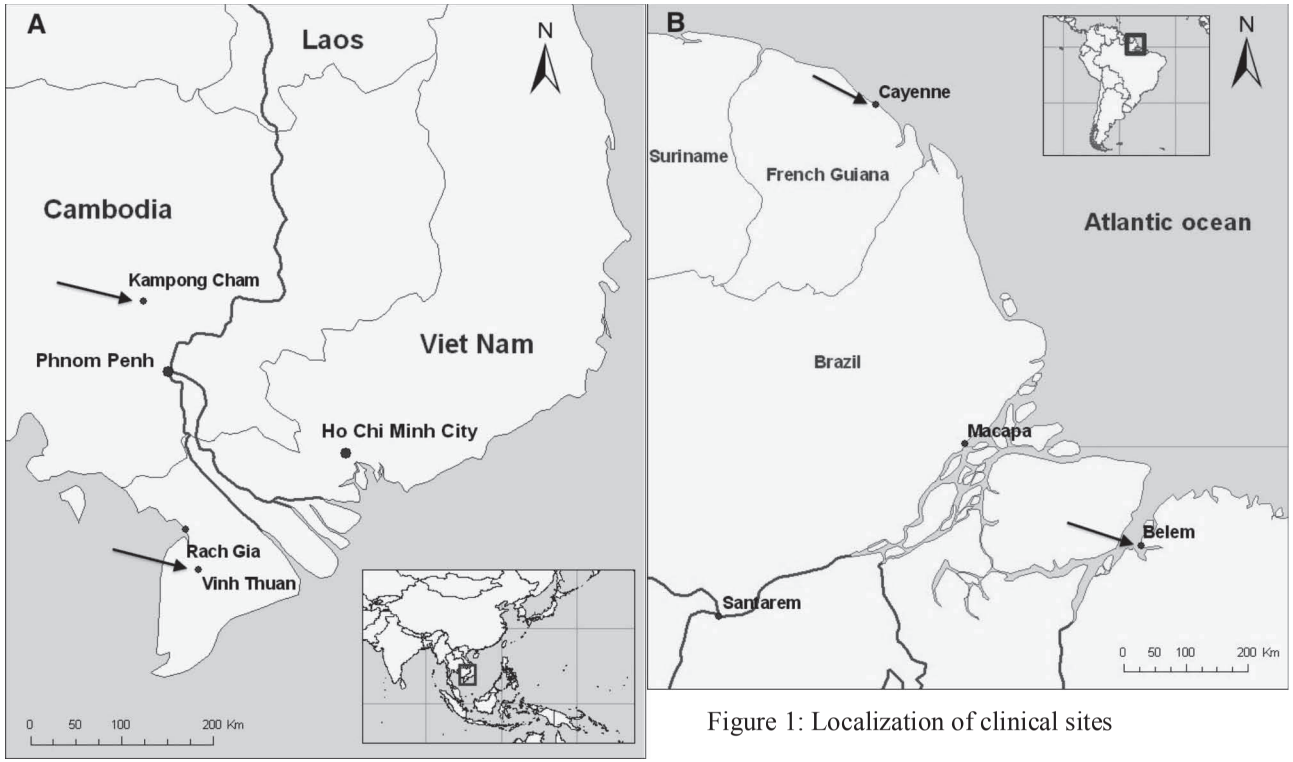
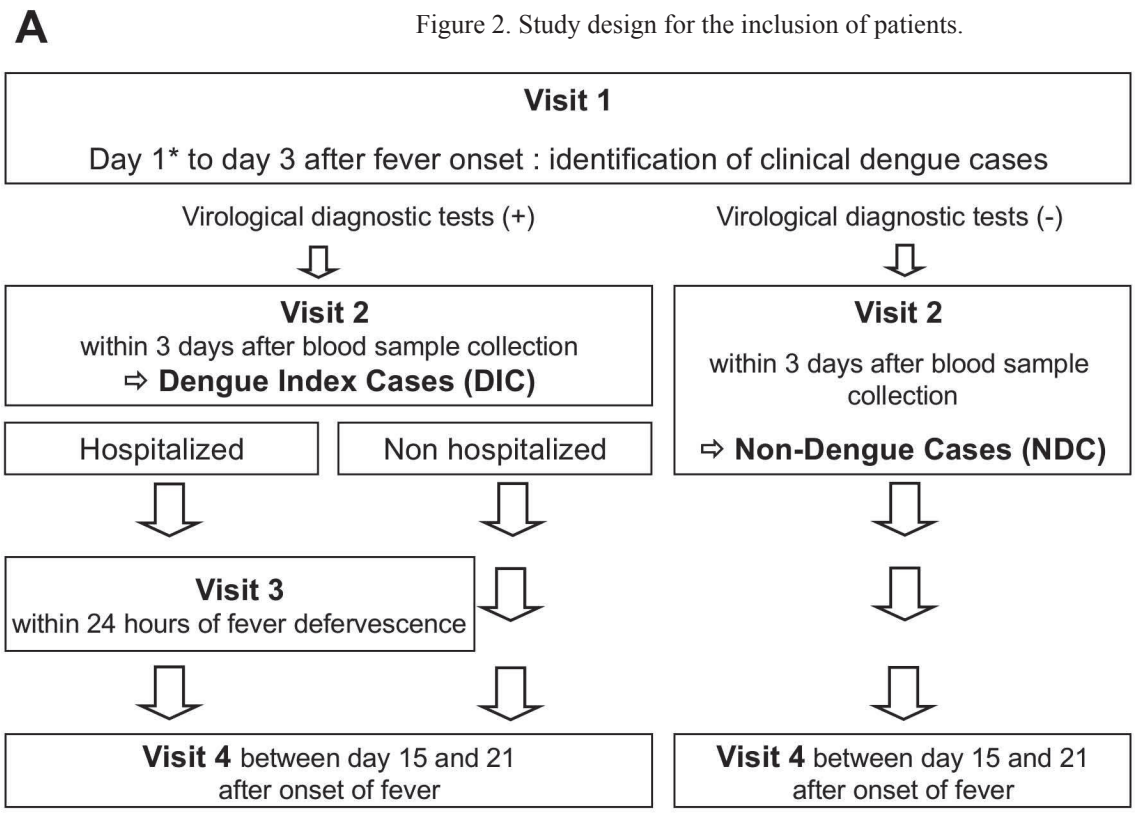
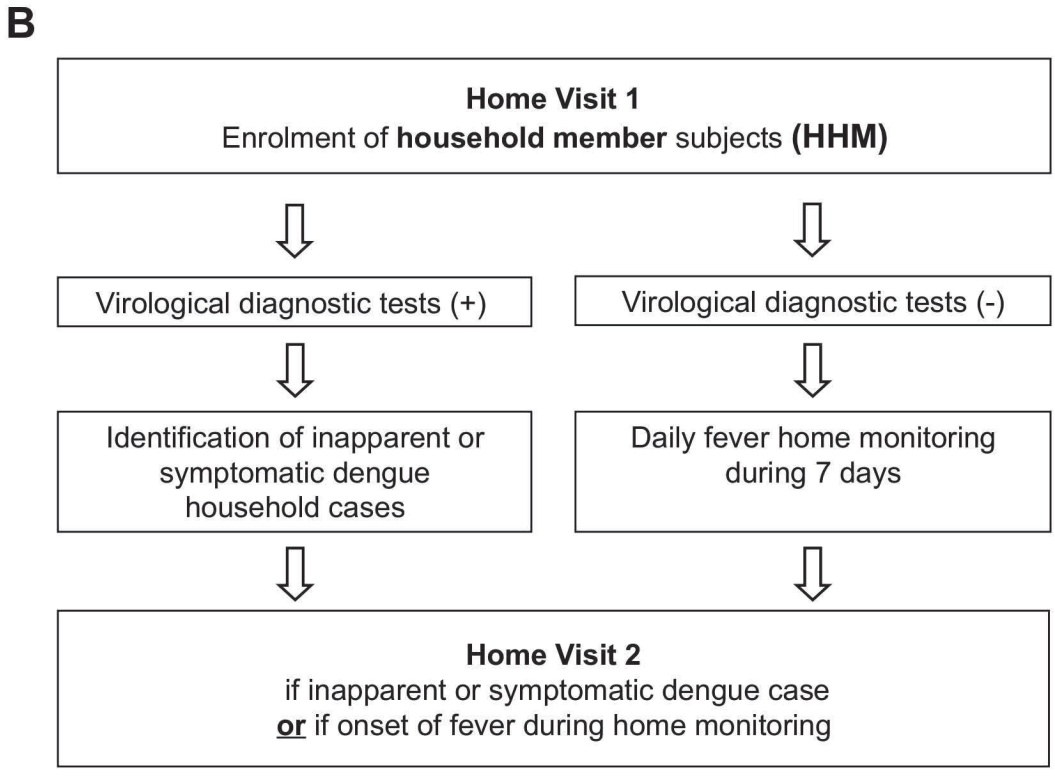


Figure 1: Localization of clinical sites

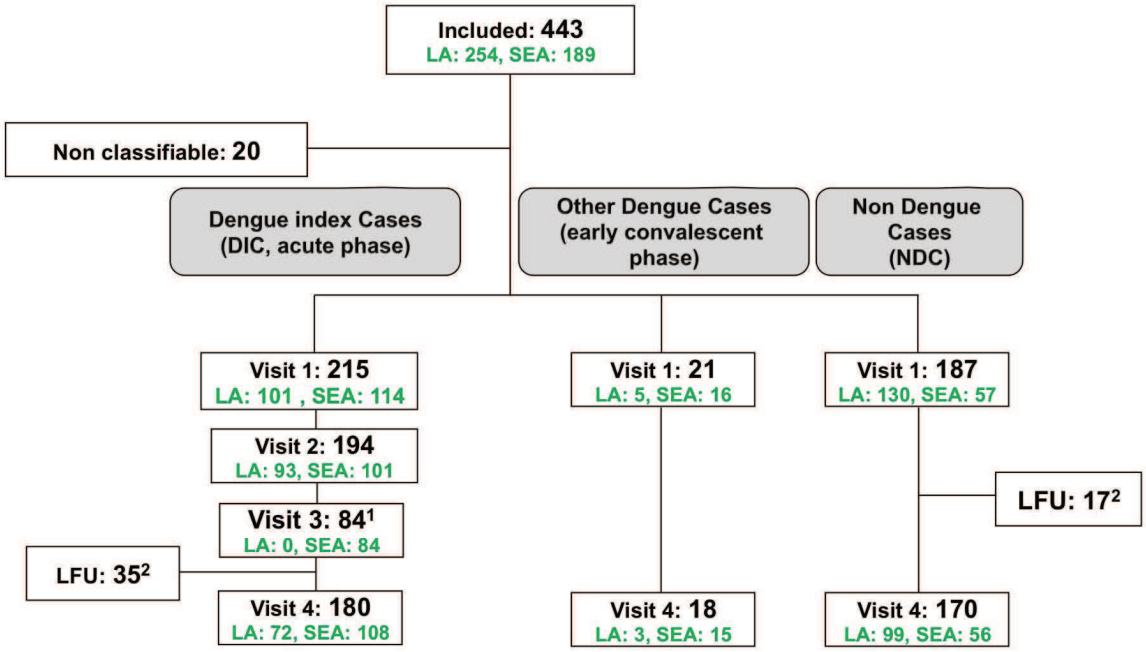
Figure 2. Study design for the inclusion of patients.





A

Figure 3. Identification of the dengue index cases (DIC) and of the household members (HHM)



[1] Only for hospitalized patients. [2] Lost from follow up

B

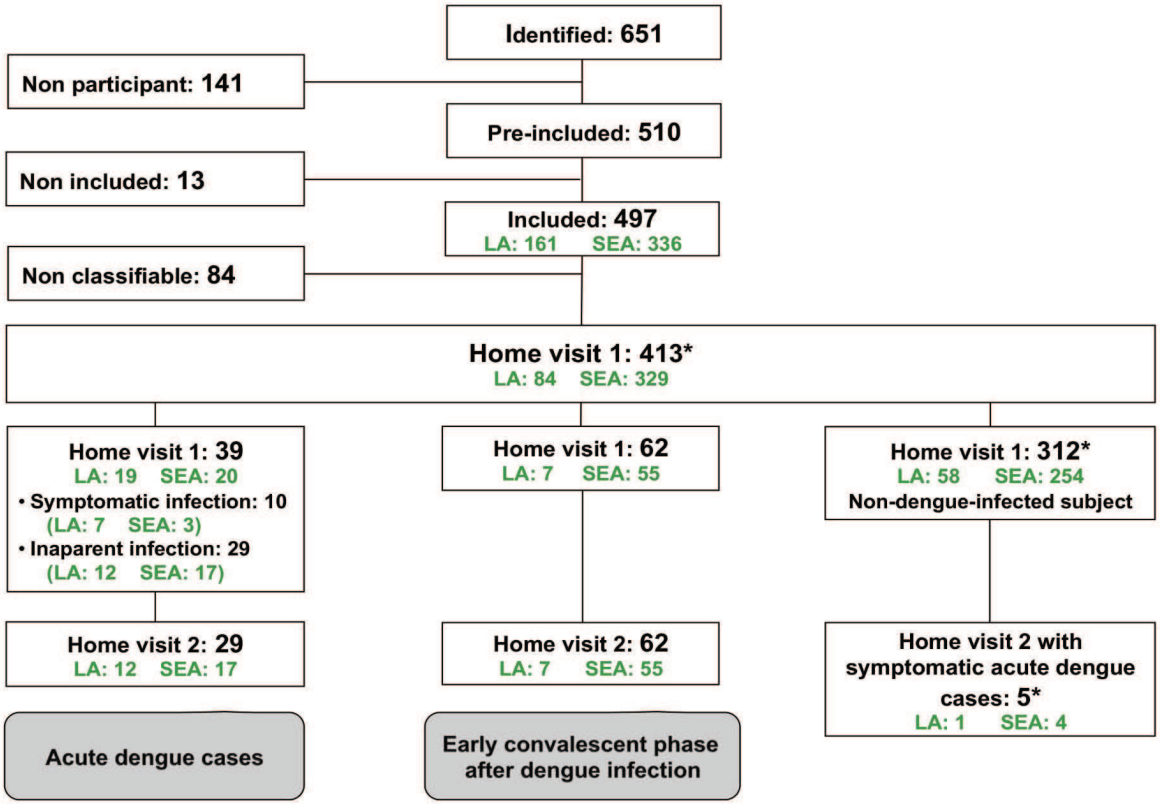


Table S1. Characteristics of dengue index cases from South-East Asia based on Visit 1 data (n =114). This table presents results of univariate analysis adjusted for sites.

	Dengue fever	Severe dengue			
	n = 86 (%)	n = 28 (%)	OR	95% CI	P
Sex					
Male	40 (46.5)	21 (75.0)	1		
Female	46 (53.5)	7 (25.0)	0.29	[0.1-0.7]	**0.01**
Age (years)					
[2 - 7]	37 (43.0)	4 (14.3)	1		
]7 - 10]	16 (18.6)	7 (25.0)	4.05	[1.04-16]	**0.04**
> 10	33 (38.4)	17 (60.7)	4.76	[1.4-15.6]	**0.01**
Weight-based Z-score					
[-1, 1]	8 (9.3)	6 (21.4)	1		
< -1	78 (90.7)	22 (78.7)	0.37	[0.1-1.2]	0.09
> 1	-	-	-	-	-
Fever (°C)					
≤ 39	69 (80.2)	26 (92.9)	1		
> 39	17 (19.8)	2 (7.1)	0.31	[0.07-1.4]	0.14
Headache					
No	23 (26.7)	10 (35.7)	1		
Yes	63 (73.3)	18 (64.3)	0.66	[0.3-1.6]	0.36
Retro-orbital pain					
No	51 (59.3)	24 (85.7)	1		
Yes	35 (40.7)	4 (14.3)	0.24	[0.07-0.7]	**0.015**
Myalgia					
No	66 (76.7)	22 (78.6)	1		
Yes	20 (23.3)	6 (21.4)	0.9	[0.3-2.5]	0.84
Joint pain					
No	79 (91.9)	24 (85.7)	1		
Yes	7 (8.1)	4 (14.3)	1.88	[0.5-6.9]	0.34
Bleeding symptom					
No	46 (53.5)	5 (17.9)	1		
Yes	40 (46.5)	23 (82.1)	5.29	[1.8-15.2]	**0.002**
Rash					
No	74 (86.1)	25 (89.3)	1		
Yes	12 (13.9)	3 (10.7)	0.74	[0.2-2.8]	0.66
Neutrophils (x 10^9/L)					
> 2	38 (44.2)	7 (25.0)	1		
≤ 2	48 (55.8)	21 (75.0)	2.37	[0.9-6.2]	0.076
Lymphocytes (x 10^9/L)					
> 2	13 (15.1)	2 (7.1)	1		
≤ 2	73 (84.9)	26 (92.9)	2.31	[0.5-11]	0.29
Monocytes (x 10^9/L)					
> 0.2	55 (64.0)	5 (17.9)	1		
≤ 0.2	31 (36.0)	23 (82.1)	8.16	[2.8-23]	**<0.0001**
ASAT[a] (UI/L)					
≤ 30	41 (47.7)	19 (68.0)	1		
> 30	45 (52.3)	9 (32.0)	0.43	[0.2-1.1]	0.067
ALAT[b] (UI/L)					
≤ 35	53 (61.6)	25 (89.3)	1		
> 35	33 (38.4)	3 (10.7)	0.19	[0.05-0.7]	**0.01**
Bilirubin (µmol/L)					
≤ 17	76 (88.4)	22 (78.6)	1		
> 17	10 (11.6)	6 (21.4)	2.07	[0.7-6.3]	0.2
DENV type					
DENV-2	20 (23.3)	15 (53.6)	1		
Other DENV	61 (70.9)	10 (35.7)	0.22	[0.1-0.6]	**0.002**
Missing data	5 (5.8)	3 (10.7)			
IgG at visit 1					
Negative	45 (52.3)	15 (53.6)	1		
Positive	39 (45.4)	12 (42.8)	0.92	[0.4-2.2]	0.86
Missing data	2 (2.3)	1 (3.6)			
NS1 antigen					
Negative	20 (23.3)	2 (7.1)	1		
Positive	66 (76.7)	26 (92.9)	3.94	[0.8-18]	0.08

[a]ASAT: Aspartate amino transferase. [b]ALAT: Alanine amino transferase.

Table S2. Main characteristics of subjects with acute dengue infection compared to non-dengue-infected subjects. Acute dengue-infected subjects belong to dengue index cases (DIC) and household members (HHM). Non-dengue-infected subjects belong to HHM. Univariate and multivariable logistic regression were used for analyses.

	Non-dengue-infected n = 307 (%)	Acute dengue-infected n = 221 (%)	Crude OR	95% CI	P	Adjusted OR	95% CI	P
Sex								
Male	135 (44.0)	119 (53.8)	1			1		
Female	172 (56.0)	102 (46.2)	0.67	[0.5-0.9]	0.025	0.79	[0.4-1.5]	0.48
Age (years)								
[2 - 7]	16 (5.2)	43 (19.5)	1			1		
]7 - 10]	17 (5.5)	29 (13.1)	0.63	[0.3-1.5]	0.28	0.34	[0.1-1.5]	0.16
> 10	274 (89.3)	149 (67.4)	0.2	[0.1-0.4]	<0.0001	0.12	[0.03-0.4]	**0.001**
Weight-based Z-score								
[-1, 1]	89 (29.0)	81 (36.6)	1			1		
< -1	195 (63.5)	123 (55.7)	0.69	[0.5-1.0]	0.056	0.8	[0.4-1.6]	0.55
> 1	23 (7.5)	17 (7.7)	0.81	[0.4-1.6]	0.56	0.35	[0.1-1.2]	0.11
Hematocrit (%)								
≤ 36	93 (30.3)	45 (20.4)	1			1		
> 36	212 (69.1)	176 (79.6)	1.72	[1.1-2.6]	0.01	2.34	[0.9-5.6]	**0.05**
Missing data	2 (0.6)	-						
Platelets (x 10^9/L)								
> 100	296 (96.4)	152 (68.8)	1			1		
≤ 100	10 (3.3)	69 (31.2)	13.4	[6.7-27]	<0.0001	3.35	[0.9-12]	0.06
Missing data	1 (0.3)	-						
Neutrophils (x 10^9/L)								
> 2	288 (93.8)	94 (42.5)	1			1		
≤ 2	18 (5.9)	127 (57.5)	21.6	[12.5-37]	<0.0001	8.08	[3.6-18]	**<0.0001**
Missing data	1 (0.3)	-						
Lymphocytes (x 10^9/L)								
> 2	243 (79.2)	31 (14.0)	1			1		
≤ 2	63 (20.5)	190 (86.0)	23.6	[14.8-38]	<0.0001	15.2	[6.7-34.6]	**<0.0001**
Missing data	1 (0.3)	-						
Monocytes (x 10^9/L)								
> 0.2	298 (97.1)	137 (62.0)	1			1		
≤ 0.2	8 (2.6)	84 (38.0)	22.8	[11-48.5]	<0.0001	9.23	[3.1-28]	**<0.0001**
Missing data	1 (0.3)	-						
ASAT (UI/L)								
≤ 30	225 (73.3)	92 (41.6)	1			1		
> 30	81 (26.4)	128 (57.9)	3.86	[2.7-5.6]	<0.0001	2.9	[1.4-6.1]	**0.004**
Missing data	1 (0.3)	1 (0.5)						
ALAT (UI/L)								
≤ 35	261 (85.0)	134 (60.6)	1			1		
> 35	45 (14.7)	86 (38.9)	3.72	[2.5-5.6]	<0.0001	1.68	[0.8-3.5]	0.20
Missing data	1 (0.3)	1 (0.5)						
Bilirubin (µmol/L)								
≤ 17	262 (85.3)	199 (90.0)	1			1		
> 17	42 (13.7)	17 (7.7)	0.53	[0.3-0.9]	0.037	0.42	[0.1-1.3]	0.14
Missing data	3 (1.0)	5 (2.3)						
IgG at visit 1								
Negative	66 (21.5)	82 (37.1)	1			1		
Positive	239 (77.8)	136 (61.5)	0.46	[0.3-0.7]	<0.0001	0.44	[0.2-1]	**0.04**
Missing data	2 (0.7)	3 (1.4)						

Table 4. Main characteristics of subjects with inapparent dengue infections compared to symptomatic dengue-infected subjects. Univariate and multivariable logistic regression were used for analyses.

	Symptomatic dengue-infected n = 192 (%)	Inapparent dengue infection n = 29 (%)	Crude OR	95% CI	P	Adjusted OR	95% CI	P
Sex								
Male	103 (53.6)	16 (55.2)	1					
Female	89 (46.4)	13 (44.8)	0.94	[0.4-2.1]	0.88			
Age (years)								
[2 - 7]	38 (19.8)	5 (17.2)	1					
]7 - 10]	27 (14.1)	2 (6.9)	0.56	[0.1-3.1]	0.51			
> 10	127 (66.1)	22 (75.9)	1.32	[0.5-3.7]	0.6			
Weight-based Z-score								
[-1, 1]	75 (39.1)	6 (20.7)	1			1		
< -1	102 (53.1)	21 (72.4)	2.57	[0.9-6.7]	0.052	2.54	[0.6-10.4]	0.20
> 1	15 (7.8)	2 (6.9)	1.66	[0.3-9.1]	0.55	4.11	[0.4-43]	0.24
Hematocrit (%)								
≤ 36	38 (19.8)	7 (24.1)	1					
> 36	154 (80.2)	22 (75.9)	0.77	[0.3-1.9]	0.59			
Platelets (x 10^9/L)								
> 100	126 (65.6)	26 (89.7)	1			1		
≤ 100	66 (34.4)	3 (10.3)	0.22	[0.1-0.7]	0.016	0.23	[0.4-1.4]	0.11
Neutrophils (x 10^9/L)								
> 2	76 (39.6)	18 (62.1)	1			1		
≤ 2	116 (60.4)	11 (37.9)	0.4	[0.2-0.9]	0.026	0.5	[0.15-1.6]	0.25
Lymphocytes (x 10^9/L)								
> 2	16 (8.3)	15 (51.7)	1			1		
≤ 2	176 (91.7)	14 (48.3)	0.08	[0.03-0.2]	<0.0001	0.09	[0.02-0.4]	**0.001**
Monocytes (x 10^9/L)								
> 0.2	114 (59.4)	23 (79.3)	1			1		
≤ 0.2	78 (40.6)	6 (20.7)	0.38	[0.1-0.9]	0.045	0.65	[0.16-2.7]	0.56
ASAT (UI/L)								
≤ 30	75 (39.1)	17 (58.6)	1			1		
> 30	117 (60.9)	11 (37.9)	0.4	[0.2-0.9]	0.034	0.4	[0.1-1.5]	0.17
Missing data	-	1 (3.5)						
ALAT (UI/L)								
≤ 35	112 (58.3)	22 (75.9)	1			1		
> 35	80 (41.7)	6 (20.7)	0.38	[0.15-0.9]	0.046	0.52	[0.14-1.9]	0.33
Missing data	-	1 (3.4)						
Bilirubin (µmol/L)								
≤ 17	175 (91.1)	24 (82.8)	1					
> 17	14 (7.3)	3 (10.3)	1.56	[0.4-5.8]	0.51			
Missing data	3 (1.6)	2 (6.9)						
DENV type								
DENV-1	50 (26.0)	5 (17.2)	1					
DENV-2	50 (26.0)	7 (24.2)	1.4	[0.4-4.7]	0.59			
DENV-3	79 (41.2)	13 (44.8)	1.64	[0.5-4.9]	0.37			
DENV-4	3 (1.6)	-						
Missing data	10 (5.2)	4 (13.8)						
IgG at visit 1								
Negative	71 (37.0)	11 (37.9)	1					
Positive	118 (61.4)	18 (62.1)	0.98	[0.4-2.2]	0.97			
Missing data	3 (1.6)	-						
NS1 antigen								
Negative	21 (10.9)	23 (79.3)	1			1		
Positive	171 (89.1)	6 (20.7)	0.03	[0.01-0.1]	<0.0001	0.05	[0.01-0.2]	**<0.0001**

CHAPTER 4 : DENGUE LABORATORY DIAGNOSIS (NS1 ANTIGEN CAPTURE)

II.1.10 Context of study

Commonly used diagnosis methods are often unable to confirm dengue infection during the acute febrile stage in a timely manner and at a reasonable cost (Gubler, 2002; WHO/TDR, 2009). Virus isolation is a time-consuming and fastidious process that requires specialized laboratory equipment and experienced personnel. The development of reverse transcriptase polymerase chain reaction (RT-PCR) and recently real time RT-PCR techniques have significantly reduced the processing time and permitted the detection of the virus in the early stage of the infection (Kao et al., 2005). However, these methods remain expensive and technically difficult, particularly in laboratory settings of the developing world. Serological diagnosis of dengue infection has many advantages including more flexibility, wide availability of reagents, lower cost, and less equipment required (WHO/TDR, 2009)., Cross-reactivity between flaviviruses, need of paired sera, and inability to detect IgM in acute phase of infection make the serological diagnosis more difficult to provide confirmation during the early phase of a dengue infection (WHO/TDR, 2009) .

Due to the drawbacks of serological methods to reliably diagnose acute infections, a number of alternative options have been explored (Kao et al., 2005) and one of the most promising methods is the detection of the NS1 antigen . NS1, produced in both membrane-associated and secreted forms, may play an essential role in viral replication. The amount of secreted NS1 (sNS1) in the serum of individuals infected with DENV has been shown to directly correlate with viraemia and with the pathogenesis of dengue infection (Hang et al., 2009; Libraty et al., 2002; Vaughn et al., 2000; Wang et al., 2006; Young et al., 2000).

The NS1 protein is detectable by ELISA as early as the first day of fever and can be found up to 9 days in serum even after RT-PCR detection has become negative (Alcon et al., 2002; Lapphra et al., 2008; Libraty et al., 2002; Schilling et al., 2004). Given all these advantages, NS1-based ELISA may be an important diagnostic tool for those acute samples in which IgM is not detectable and for which PCR is not available. Thus, NS1 antigen might be useful in early detection. Several NS1 antigen commercial kits are now available and most of them have been evaluated for their sensitivity and specificity in patients experiencing clinically apparent infections. The sensitivity observed for these assays varied from 63% to 94% (Dussart et al., 2008; Hang et al., 2009; Lapphra et al., 2008; Phuong et al., 2009).

II.1.11 Objectives

In this study, the performance of NS1 antigen-capture assay was evaluated in relation with various clinical and virological factors. Performances were for instance evaluated in confirmed dengue infections defined by detection of anti-DENV-specific IgM or a 4 fold increase of HI titer in the pair of sera collected with an interval of minimum 5 days and by the detection of NS1 antigen in serum and/or the isolation of DENV after inoculation into mosquito cell lines and/or the detection DENV RNA by RT-PCR or real time RT-PCR assay. Another objective was to assess the potential association of the level of NS1 antigenaemia (using simple semi-quantitative estimation) and that of viraemia with dengue disease severity using well characterized sera from hospitalized patients. NS1 antigen test could be an easy tool to confirm dengue infection in such individuals on a single blood sample during the acute phase of the disease rather than by indirect methods that require at least 2 samples. In addition, we also evaluated the test in asymptomatically dengue-infected individuals. Indeed, asymptomatic individuals and unreported patient with mild febrile disease who represent the vast majority of dengue infections are believed to constitute an important reservoir of virus in countries where dengue is highly endemic (Kyle and Harris, 2008; Teixeira Mda et al., 2002; Yew et al., 2009).

II.1.12 Results and conclusions

Sample collection was carried out during 2006 and 2007 dengue epidemics at Kampong Cham provincial hospital, Cambodia. Dengue infection was confirmed in 260 patients. Overall sensitivity and specificity of Platelia NS1 Ag kit were of 57.5% and 100% respectively.

The evaluation of the Platelia NS1 Ag detection kit demonstrated a quite low overall sensitivity. These data suggest that the NS1 antigen results should be interpreted with caution when used alone. However, NS1 Ag positivity rate was found significantly higher in DF than in DHF/DSS (72.3% versus 40.2%, $p<0.001$); higher in primary than in secondary infections (87.5% versus 53.5%, $p=0.001$); higher in patients with a high viraemia (> 5 log cDNA equivalents/mL) than in those with lower viraemia (91% versus 45%, $p<0.001$); and higher in patients infected with DENV-1 (80%) than with DENV-2 (40%), DENV-3 (63%) and DENV-4 (53%) ($p<0.05$). The NS1 antigen kit combined with MAC-ELISA detected a significantly higher number of acute dengue cases than NS1 antigen kit alone (overall sensitivity: 85.7% vs. 57.7%; $p<0.001$). In asymptomatic individuals, the NS1 Ag capture sensitivity tends to be lower than that in symptomatic patients. This result has shown the usefulness of qualitative result of NS1 antigen detection assay in early recognition of dengue

infection particularly in combination with IgM test. The bedside rapid diagnostic tests including NS1 antigen and IgM detection would be probably a helpful tool for early dengue infection diagnosis in clinical practice but these tests need to be further extensively evaluated.

The present study demonstrates a moderate correlation of the semi-quantitative result of NS1 antigen-capture assay with the level of viraemia quantified by real time RT-PCR. Multivariate analysis shown that milder disease severity was observed independently in patients with RNA copy number >5 log10 cDNA equivalents/mL or in high level of NS1 antigen ratio or in DENV-1 infection. As expected and already largely described, DHF/DSS cases are more frequently observed in secondary infection with an adjusted odd ratio of 6.6.

We have demonstrated in this chapter that low level of NS1 antigen can be likely an indication of progression to severe dengue. However, the mechanism and expression profiles that differ between DF and DHF/DSS were not covered in this study. Thus, a study on genomic-wide expression (Chapter 5) in blood collected from patients experiencing different degree of severity was carried out in order to characterize any potential molecular and cellular markers associated in plasma leakage in DHF/DSS.

The results of this work are summarized in the article below which was published in « PloS Neglected Tropical Diseases ».

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PLoS NEGLECTED TROPICAL DISEASES

Clinical and Virological Factors Influencing the Performance of a NS1 Antigen-Capture Assay and Potential Use as a Marker of Dengue Disease Severity

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Abstract

Background: Detection of dengue NS1 antigen in acute infection has been proposed for early diagnosis of dengue disease. The aim of this study was to evaluate the clinical and virological factors influencing the performance of the Platelia NS1 Ag kit (BioRad) and to assess the potential use of NS1 antigen and dengue viral loads as markers of dengue disease severity.

Methodology/Principal Findings: Blood specimens were collected from patients hospitalized at the Kampong Cham hospital during the 2006 and 2007 dengue epidemics in Cambodia. Dengue infection was confirmed in 243/339 symptomatic patients and in 17 asymptomatic individuals out of 214 household members tested. Overall sensitivity and specificity of Platelia NS1 Ag kit were 57.5% and 100% respectively. NS1 Ag assay combined with IgM antibody capture ELISA significantly increased the sensitivity for dengue diagnosis. NS1 Ag positivity rate was found significantly higher in DF than in DHF/DSS, in primary than in secondary infections, in patients with a high viremia (>5 log/mL) and in patients infected with DENV-1. In asymptomatic individuals, the NS1 Ag capture sensitivity tends to be lower than that in symptomatic patients. Milder disease severity was observed independently in patients with RNA copy number >5 log10 cDNA equivalents/mL or in high level of NS1 antigen ratio or in DENV-1 infection.

Conclusions: Overall sensitivity of NS1 Ag detection kit varied widely across the various forms of dengue infection or disease. Sensitivity was highest in patients sampled during the first 3 days after onset of fever, in patients with primary infection, DENV-1 infection, with high level of viremia and in DF rather than DHF/DSS. In asymptomatic patients, RT-PCR assay has proved to be more sensitive than NS1 antigen detection. The NS1 antigen level correlated significantly with viremia and a low NS1 antigen ratio was associated with more severe disease.

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Introduction

Dengue virus (DENV), a mosquito-borne virus (family *Flaviviridae*, genus *Flavivirus*) is an enveloped, single stranded positive-sense RNA virus. There are 4 serologically related but antigenically and genetically distinct dengue viruses (DENV-1, -2, -3, and -4) causing disease in human. While most infections result in asymptomatic response or mild febrile illness (dengue fever or DF), all 4 serotypes are capable of producing the more severe and potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) and non-specific complication of systemic diseases (e.g., encephalitis, hepatitis) [1,2,3].

With over 2.5 billion people living in area at high risk for infection and an estimated 50–100 million cases of dengue infection every year, DENV has become the most important arthropod-borne virus affecting human [2]. Several factors such as rapid urbanization, failure to control vector mosquitoes and rapid progress in air transportation have contributed to the emergence of endemic dengue in over 100 countries [2,4].

In Cambodia, there is a high incidence of reported diseases (DF, DHF) of 10,000–12,000 cases annually during 2002–2006 and the case-fatality rate was 1–2% over the past 5 years [5].

Commonly used diagnosis methods are often unable to confirm dengue infection during the acute febrile stage in a timely manner and at a reasonable cost [3,6]. Virus isolation is a time-consuming and fastidious process that requires specialized laboratory equipments and experienced personnel. The development of reverse transcriptase polymerase chain reaction (RT-PCR) and recently real time RT-PCR techniques have significantly reduced the processing time and permitted the detection of the virus in the early stage of the infection [7]. However, these methods remain expensive and technically difficult, particularly in laboratory

Author Summary

Dengue is the most prevalent arthropod-borne disease in tropical regions. The clinical manifestation may vary from asymptomatic to potentially fatal dengue shock syndrome. Early laboratory confirmation of dengue diagnosis is essential since many symptoms are not specific. Dengue non-structural protein 1 (NS1) may be used in simple antigen-capture ELISA for early detection of dengue virus infection. Our result demonstrated that the Platelia NS1 antigen detection kit had a quite low overall sensitivity. However, sensitivity rises significantly when used in combination with MAC-ELISA. When taking into account the various forms of dengue infection, the NS1 antigen detection was found relatively high in patients sampled during the first 3 days of fever onset, in patients with primary infection, DENV-1 infection, with high level of viremia and in mild form of dengue fever. In asymptomatically infected individuals, RT-PCR assay has proved to be more sensitive than NS1 antigen detection. Moreover, the NS1 antigen level correlated significantly with high viremia and low level of NS1 antigen was associated with more severe disease.

settings of the developing world. Serological diagnosis of dengue infection has many advantages including more flexibility, wide availability of reagents, lower cost, and less equipments required [3]. Unfortunately, cross-reactivity between flaviviruses, antibody half-life, need of paired sera, and inability to be detected in acute phase of infection make the serological diagnosis more complicated [3] .

Due to the drawbacks of serological methods to reliably diagnose acute infections, a number of alternative options have been explored [7] and one of the most promising methods was the detection of nonstructural protein 1 (NS1) . NS1, produced in both membrane-associated and secreted forms, may play an essential role in viral replication. The amount of secreted NS1 (sNS1) in the serum of individuals infected with DENV has been shown to directly correlate with viremia and the pathogenesis of dengue infection [8,9,10,11,12].

The NS1 protein is detectable by enzyme-linked immunosorbent assay (ELISA) as early as the first day of fever and can be found up to 9 days in serum even after RT-PCR detection has become negative [9,13,14,15]. Given all these advantages, NS1-based ELISAs may be an important diagnostic tool for those acute samples in which IgM is not detectable and for which PCR is not available. Thus, NS1 antigen might be useful in early detection and a potential prognosis parameter for severe dengue infections. Several NS1 antigen commercial kits are now available and most of them have been evaluated for their sensitivity and specificity in patients experiencing clinically apparent infections. The sensitivity observed for these assays varied from 63% to 94% [8,14,16,17].

In this study, we evaluated the clinical and virological factors influencing the performance of NS1 antigen-capture assay and assessed the potential association of the level of NS1 antigenemia (using simple semi-quantitative estimation) and the level of viremia with dengue disease severity using well characterized sera from patients presenting at hospitals. NS1 antigen test could be an easy tool to confirm dengue infection in such individuals on a single blood sample during the acute phase of the disease rather than by indirect methods that require at least 2 samples. We also evaluated the test in asymptomatically dengue-infected individuals. Indeed, asymptomatic individuals and unreported patient with mild febrile disease who represent the vast majority of dengue infection are believed to constitute an important reservoir of the virus in countries where dengue is highly endemic [18,19].

Materials and Methods

Patients' recruitment and samples processing

Patients were enrolled in the pediatric ward of the Kampong Cham provincial hospital during 2 consecutive dengue epidemics between May and October in 2006 and 2007. This study was approved by the National Cambodian Ethics Committee and patient's enrolment was subject to obtaining a written consent signed by the patients or the under 16 year old patient's legal representatives.

Patients clinically diagnosed with dengue infection and who fulfilled the following inclusion criteria were enrolled: age $\geq\geq$24 months, fever $>38^{\circ}$C and having at least one of the symptoms: rash or severe headache or retro-orbital pain or myalgia or joint pain or bleeding symptom. Patients' information and clinical data were collected by the physicians using a specific case report form and blood samples were taken on hospital admission and discharge. Patients diagnosed for other infections beside dengue and patients hospitalized with a non-infectious disease (e.g., cranial trauma, etc.) were recruited as control group.

Blood samples were tested for haematocrit and platelet count as well as for other biological parameters necessary for patients' follow-up. Sera were tested for dengue using serology and molecular methods at Institut Pasteur - Cambodia.

Patients diagnosed for dengue infections were classified as DF, DHF or DSS using the former WHO criteria [20] as recommended at the time of the study.

In order to identify non-symptomatic cases, family members of dengue-infected patients were visited the next day following laboratory confirmation of the dengue infection (which usually took approximately 24 hours). Their body temperature was followed for 7 days. Blood samples were taken at the first and 7^{th} day of follow-up and if a family member developed fever. A non-symptomatic dengue case was defined as a household member who tested positive for dengue infection but did not display any of the symptoms of the inclusion criteria.

Laboratory diagnosis

A confirmed dengue infection ("gold standard algorithm") was defined by the detection of anti-dengue virus (DENV)-specific IgM or a 4 fold increase of hemagglutination inhibition (HI) titer in the pair of sera collected with an interval of minimum 7 days and the detection of NS1 antigen in serum by the NS1 Platelia test (BioRad, Hercules, CA) and/or the isolation of DENV after inoculation into mosquito cell lines and/or the detection DENV RNA by RT-PCR or real time RT-PCR assay.

An "in-house" IgM capture Enzyme-Linked Immuno-Sorbent Assay (MAC-ELISA) was used to detect DENV and JEV IgM as the 2 viruses co-circulate in the country [21,22]. A result was considered positive when the optical density (OD) was $>$ mean OD of three negative control specimens +3 standard deviations. When the anti-JEV result was higher than the anti-DENV result, the subject was not considered to have DENV infection.

HI test was carried out according to the method described by Clarke and Casals [23] adapted to 96-well microtitre plate. Due to the serological cross-reactivity between arboviruses, paired specimens were tested for DENV and JEV hemagglutination-inhibiting antibodies. Primary or secondary acute dengue infection was determined by a fourfold increase in HI titer between the first and second sample according to criteria established by the WHO [20].

The NS1 Platelia antigen detection (BioRad, Marnes-la-Coquette, France) was performed on patient's sera according to the manufacturer's instructions. Samples with equivocal result were repeated and if they were still equivocal they were considered as negative. The optical density (OD) reading obtained with a spectrophotometer at 450/620 nm is proportional to the amount of NS1 antigen present in the sample [13]. The assay provides qualitative and semi-quantitative results in human serum or plasma. The semi-quantitative results were expressed as the ratio calculated by dividing the absorbance measured on the sample by the mean value of the optical densities of 2 cut-off controls. The cut-off value corresponds to the mean value of the OD of the cut-off control provided and tested in duplicate.

The isolation of DENV was performed using mosquito cell line (clone C6/36 of *Ae. Albopictus* cells). Briefly, each acute serum was diluted 1:20 with L15 Leibovitz Medium (Sigma Aldrich, Steinheim, Germany) in which 2% of fetal calf serum was added. Diluted sera were inoculated into 12-well plate containing 100% confluent C6/36 cells and then incubated for 7 days at 28°C. Cells were harvested, and DENV infection was confirmed by an immunofluorescence assay using dengue serotype-specific monoclonal antibodies as described previously [21,22].

Viral RNA was extracted from acute phase serum samples using the QIAmp Viral RNA Mini kit (Qiagen, Hilden, Germany). The DENV serotype was determined by RT-PCR based on the technique developed by Lanciotti *et al.* [24] and modified by Reynes *et al.* [25]. The positive samples by conventional RT-PCR were then tested for dengue viral loads by a serotype-specific real-time RT-PCR assay targeting NS5 gene using quantified internal controls [26]. The results were expressed as cDNA equivalents per milliliter of serum. The limit of detection for this assay was 500 cDNA equivalent/mL.

Statistical analysis

All statistical analyses were performed using Stata/SE version 9.0 (StataCorp, TX, USA). Significance was assigned at $P<0.05$ for all parameters and 95% of interval confidence was used. Categorical variables between groups were compared by Pearson's Chi-squared and Fisher's exact test. T-test and Kruskal-Wallis rank test were used for continuous variables. The correlation coefficients between 2 continuous variables were calculated by Spearman's rank correlation test. For multivariate analyses, we identified independent determinants using a logistic regression model. For clarity, adjustments by the day after onset of fever (DOF) was performed and presented using DOF as a categorical variable: ≤3 days and 4–8 days. In some analyses, DHF and DSS were grouped to increase statistical power.

Results

A total of 134 and 205 patients were enrolled in 2006 and 2007, respectively, of which 243 patients were diagnosed with acute dengue infection, 62 with non-dengue infection and 17 as having non infectious disease. The summary of patient's characteristics, clinical and virological data of this study is shown in Table 1.

Using the former WHO criteria [20], 101 dengue patients were classified as DF, 42 as DHF, 45 as dengue with DSS and 72 as indeterminate. The inability to classify these 72 patients was due to the lack of clinical and laboratory data necessary for the classification or they did not meet all the four WHO criteria (see materials and methods). After measuring HI titers on paired sera, dengue cases were classified in primary infections (n = 32, 14.5%), secondary infections (n = 189, 85.5%) but in 39 cases it was not possible to determine the immune status.

During the household investigation, 17 (8%) dengue-infected individuals who did not experience any symptoms and 2 (1%) symptomatic household members of 15 dengue index cases (DIC) were identified among 214 household members (Table S1).

Figure 1 shows the overall positive rate of NS1-antigen capture assay, RT-PCR and MAC-ELISA in relationship with the day after onset of fever. During the first 2 days in the course of the disease the sensitivity of NS1 assay and RT-PCR reached the highest values (81% and 90.5%, respectively) and then decreased to less than 20% and 45.5% respectively by day 7–8. On the contrary, the number of specimens positive by MAC-ELISA increased steadily from less than 20% at day 1–2 to 100% by day 7.

The sensitivity and specificity of the NS1-capture assay were 57.7% (95% CI: 51.4–63.8%) and 100% respectively (Table 2). When diagnosed solely by RT-PCR, the sensitivity was 77.3% (95% CI: 71.7–82.2) and the specificity 100%. When samples were collected during the first 3 days of illness, the sensitivity of the NS1 commercial test improved: 74% (95% CI: 62.8–83.4) in the dengue-confirmed case group and 82.3% (95% CI: 69.5–90.0) in the group of individuals who tested positive by RT-PCR only.

The NS1 antigen kit combined with MAC-ELISA detected a significantly higher number of acute dengue cases than NS1 antigen kit alone (overall sensitivity: 85.7% vs. 57.7%; $p<0.001$, Table S2). An increased sensitivity was also observed when combining RT-PCR and MAC-ELISA results (overall sensitivity: 95.4% vs. 77.3% for RT-PCR alone, $p<0.001$, Table S2). The comparison with DOF subgroups was detailed in Table S2.

When analyzing all dengue-infected individuals, the NS1 antigen ratio correlated with the RNA load of cDNA equivalents per milliliter ($r = 0.540$, $p<0.001$; Fig. 2).

The overall sensitivity of the NS1 detection kit was significantly higher in DF (72.3%; 95% CI: 63.5–81%) than in DHF/DSS (40.2; 95% CI: 29.85–51.3). But the difference in NS1-capture assay sensitivity was significant only for samples collected after day 3 of fever and not for specimen obtained during the very early phase of the disease (Table 3).

The sensitivity of the NS1-capture assay was significantly higher in primary dengue infection (87.5%; 95% CI: 70.0–96.5) than in secondary infection (53.5%; 95% CI: 46.1–60.7) ($p<0.001$). The difference was also significant in the DOF 4–8 group ($p = 0.002$) and at the limit of significance in the DOF 1–3 group ($p = 0.055$).

The sensitivity of the test also varied with the virus serotype. It was significantly higher in DENV-1-infected patients (80%; 95% CI: 67–89.6) than in DENV-2- (40%; 95% CI: 12.2–73.8, $p = 0.008$), DENV-3- (63.6%; 95% CI: 54.4–72.2, $p = 0.03$) and DENV-4- (53.3%; 95% CI: 26.6–78.7, $p = 0.03$) infected patients although the difference was only significant for the DOF 4–8 group (Table 4).

DF was significantly more frequent after infection with DENV-1, compared to other dengue virus infections (38/47, [80.85%, 95% CI: 66.7–90.8] vs. 4/8 [50%,95% CI: 15.7–84.3] for DENV-2, 42/89 [47.2%, 95% CI: 36.5–58] for DENV-3 and 5/9 [55.6%, 95% CI: 21.2–86.3] for DENV-4; $p = 0.002$). The proportion of primary infections in DENV-1-infected patients was 20% versus 12.3% for the overall studied population ($p = 0.13$).

Of 201 RT-PCR positive samples, 189 were tested for RNA quantification using real-time RT-PCR. Among these, 70 samples were containing RNA levels lower than the detection limit of the real time RT-PCR. During clinical classification, 44 cases were excluded from the analysis because the clinical information was not sufficiently precise to allow a classification according the WHO criteria. NS1 antigen-capture assay's sensitivity was

Table 1. Summary of demographic, clinical and virological information of studied population.

Variables	Years		
	2006	**2007**	**Total**
Acute dengueSymptomatic Asymptomatic	107[#]90/117 (77%)17/214 (8%)	153[#]153/205 (75.5%)0	260[#]243/322 (75.5%)17/214 (8%)
Non-dengue infection	27	35	62
Non infectious disease	0	17	17
Age (median, iqr*)	8 (5–11)	6 (4–8)	7 (4–9)
Sex (female, %)	50 (46.7%)	86 (56%)	136 (52.3%)
Median of day of illness (range)	4 (2–6)	5 (1–8)	4 (1–8)
Dengue diagnosis (n = 260)			
Virus isolation	48 (45%)	46 (30%)	94 (36%)
RT-PCR	91 (85%)	110 (72%)	201 (77%)
NS1 antigen assay	73 (68.2%)	77 (50.3%)	150 (57.7%)
MAC-ELISA Positive in acute serumSeroconversionNegative	97 (90.6%) 21 (19.5%) 76 (71%)10 (9.4%)	148 (96.7%)92 (60%) 56 (36.7%)5 (3.3%)	245 (94.2%)113 (43.5%) 132 (50.7%)15 (5.8%)
Hemagglutination-Inhibition assay (titer)Fourfold rise in antibodies on pair seraNo change or less than fourfold riseData not available[¥]	87 (81.3%) 7 (6.5%) 13 (12.2%)	134 (87.6%) 7 (4.6%) 12 (7.8%)	221 (85%) 14 (5.4%) 25 (9.6%)
DENV serotypes			
DENV-1	40 (37%)	15 (10%)	55 (21%)
DENV-2	2 (2%)	8 (5%)	10 (4%)
DENV-3	47 (44%)	74 (48.5%)	121 (46.5%)
DENV-4	2 (2 %)	13 (8.5 %)	15 (5.8%)
Unknown serotype	16 (15%)	43 (28%)	59 (22.7%)
Clinical manifestation			
DF	73 (68%)	28 (18%)	101 (39%)
DHF	17 (16%)	25 (16%)	42 (16%)
DSS	0	45 (29.5%)	45 (17%)
Indeterminate clinical status	17 (16%)	55 (36%)	72 (28%)
Serological status			
Primary	24/87 (28%)	8/134 (6%)	32/221 (14.5%)
Secondary	63/87 (72%)	126/134 (94%)	189/221 (85.5%)
Indeterminate or unknown	20	19	39

*interquartile range.
[#]Numbers used as denominator for each column, otherwise indicated.
[¥]Insufficient serum volume or no second serum.
doi:10.1371/journal.pntd.0001244.t001

significantly higher in patients with viremia >5 log cDNA equivalents/mL than <5 log cDNA equivalents/mL regardless the clinical severity and day of sample collection after onset of fever (Table 5). The overall NS1 antigen-capture sensitivity in patients with viremia >5 log cDNA equivalents/mL was 91% versus 45% in patients with viremia <5 log equivalents/mL ($p<0.001$).

In asymptomatic individuals the sensitivity of NS1 test was significantly lower than that in DIC (35.3% versus 86.7%, $p = 0.003$; Table S1) and at the limit of significance when compared to the sensitivity observed in all symptomatic cases (59.3%, $p = 0.053$). Seventy three percent (8/11) of the asymptomatic individuals experienced secondary infection which was lower than in DIC (100%, $p = 0.063$). The levels of viremia expressed in log10 cDNA equivalents/mL in asymptomatic individuals was significantly lower than in DIC (2.72, SD: 2.72, n = 13 vs. 4.96, SD: 2.37, n = 15; $p = 0.043$) but the difference was not significant if compared with the level of viremia in all dengue confirmed cases (3.79, SD: 3.06, n = 176; $p = 0.145$).

In these asymptomatic individuals, nested RT-PCR detection was significantly more sensitive than NS1 antigen-capture assay (76.5% vs. 35.3%, $p = 0.015$).

In multivariate analysis, DHF/DSS were independently associated with secondary infection (adjusted OR = 6.6, $p = 0.01$) when controlled with age, day of fever onset, DENV serotypes and immunity status (primary/secondary infection). Out of 77 DHF/DSS patients, 74 (96%) had secondary dengue infection. Milder disease severity was associated with high NS1 antigen level (adjusted OR: 0.21, $p = 0.002$) (Table S3A and Fig. 3A) or DENV-1 infection (adjusted OR: 0.083, $p = 0.006$). Similar results were found in multivariate analysis when using the number of cDNA copies instead of NS1 antigen OD ratio: association persisted between DHF/DSS and secondary infection (adjusted OR = 6.03, $p = 0.01$) and milder disease severity was observed in patients with

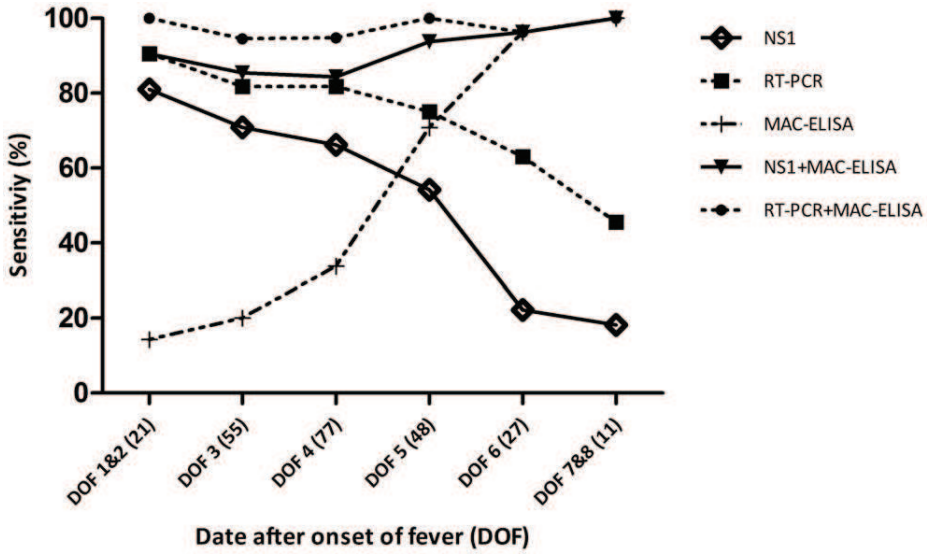

Figure 1. Sensitivity of Platelia NS1, MAC-ELISA and RT-PCR depending on DOF* (n = 239). *DOF: Day after onset of fever.
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cDNA copy number >5 log10 cDNA equivalents/mL (adjusted OR = 0.33, $p = 0.019$) (Table S3B and Fig. 3B).

Discussion

DENV NS1 antigen is detected in the blood circulation as early as viral RNA [8,9,12,13]. Thus its detection is useful for early dengue diagnosis and could be used as an easy, fast and feasible alternative to RT-PCR in developing countries. For this reason, the sensitivity of a commercial NS1 antigen detection kit was studied in context of several factors: severity of the infection including asymptomatic dengue-infected individuals, time of sampling, serological status (primary or secondary infection), DENV serotype and level of viremia in acute sample.

The overall sensitivity of Platelia Dengue NS1 Ag kit (58%) is slightly lower than that observed in previous studies (63–94%) [8,14,16,27,28], although, the excellent specificity reported here is in agreement with results provided by other authors (98.4–100%) [8,14,16,27,28]. The sensitivity of the test was better during the early stage of the illness (before day 4). The modest overall sensitivity reported here was comparable to that of a recent multi-country NS1 antigen assay evaluation [28] which showed a 66% (range: 34% to 76%) sensitivity of the NS1 antigen detection by Platelia kit. The relatively low sensitivity NS1 antigen detection in the current study is probably due to the high number of secondary infections (85.5%) which reflects of the true situation in Cambodia and other dengue hyper-endemic countries [14,29,30]. Indeed, we recorded a lower sensitivity of the NS1 antigen-capture assay in secondary infections in comparison to primary infections (87.5% vs. 53.5%). Anti-NS1 antibodies are more frequently detected in dengue secondary infection [31] and the antibody-antigen complex impedes the test's ability to detect free NS1 [9,12]. A dissociation of NS1 antibody-antigen immune complexes would increase the sensitivity of NS1 antigen detection [31] but such a method is unfortunately not offered in the commercial kits and was not performed in our study.

When the NS1 antigen assay was coupled with MAC-ELISA, the overall sensitivity increased by 28%. This combination of NS1-antigen capture assay and IgM antibody detection for dengue diagnosis showed higher sensitivity than RT-PCR alone and a slightly lower sensitivity than RT-PCR combined with IgM antibody detection. When performed together, NS1 antigen-capture and IgM assays appear to be highly sensitive and complementary, allowing a sufficiently good presumptive (IgM) or definitive (NS1) diagnosis during the acute and the convalescent phase of the disease. This advantage of the combination was

Table 2. Sensitivity, specificity, positive and negative predictive values of Platelia NS1 assay against dengue-confirmed cases.

	Studied population	Acute dengue infection	NS1 positive	Sensitivity % [CI95%]	Specificity % [CI95%]	PPV % [CI95%]	NPV % [CI95%]	*p* value[$]
Total	339*	260[#]	150[‡]	57.7 [51.4–63.8]	100	100	41.8 [34.7–49.2]	$p<0.001$
DOF 1–3	110	77	57	74 [62.8–83.4]	100	100	62.3 [47.9–75.2]	$p<0.001$
DOF 4–8	196	163	85	52.2 [44.2–60.0]	100	100	29.7 [21.4–39.1]	$p<0.001$

*33 cases with imprecise DOF were excluded.
[#]20 cases with imprecise DOF were excluded.
[‡]8 cases with imprecise DOF were excluded.
[$]*P* values refer to 2×2 contingency comparison between % of NS1 positive cases and % of dengue confirmed cases.
PPV: positive predictive value.
NPV: negative predictive value.
doi:10.1371/journal.pntd.0001244.t002

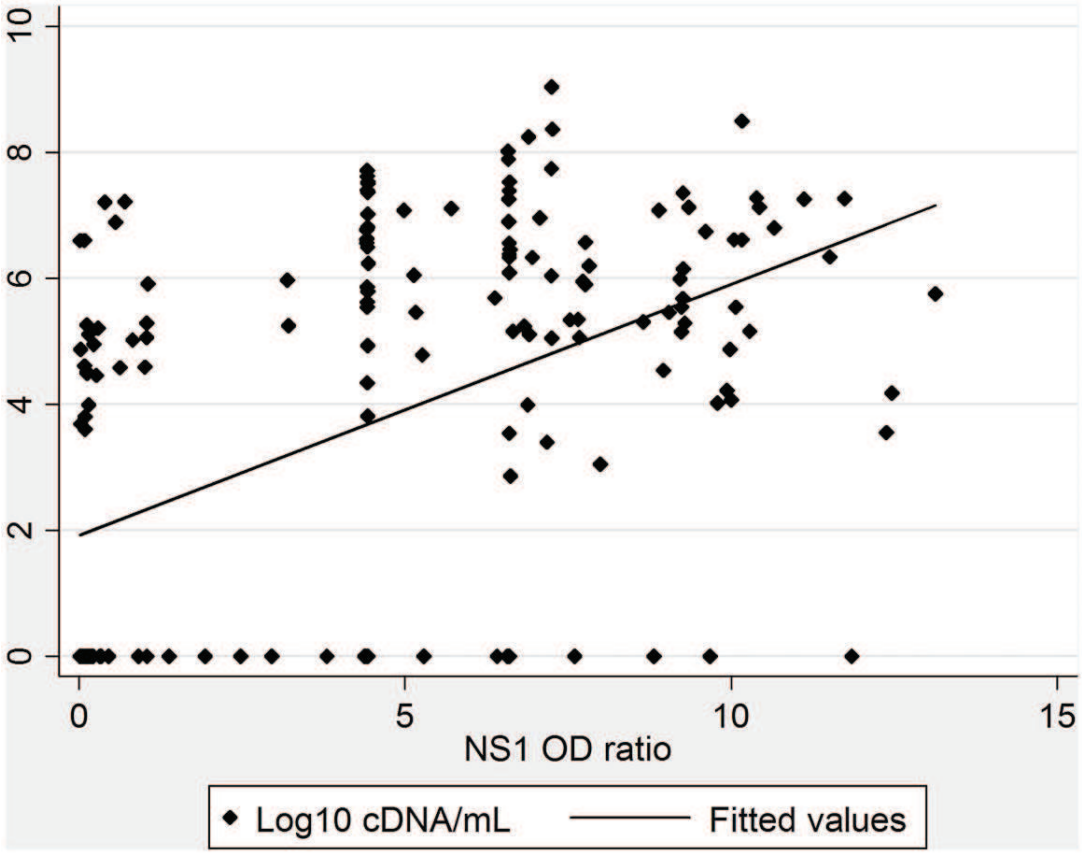

Figure 2. Comparison of NS1 antigen OD* ratio and viral load (log 10 cDNA equivalents/mL). *OD: optical density.
doi:10.1371/journal.pntd.0001244.g002

positively demonstrated in the multi-country study by Guzman et al. [28]. Moreover, both assays are easy to perform, fast, require limited equipments and expertise, and are affordable. The combination of NS1 antigen and dengue IgM/IgG used in rapid diagnostic test (RDT) format for dengue infection detection has shown in previous studies to be more sensitive than NS1 antigen detection alone and can be used as a "point of care" diagnosis [30,32].

The sensitivity of NS1 antigen-capture assay was significantly higher for DENV-1 than for the three other serotypes. This could be explained by the higher level of viremia measured during DENV-1 infection than that in patients infected with any of the three other DENV serotypes, although the difference was significant only when DENV-1 was compared with DENV-3 (data not shown). Other factors like a better affinity of the NS1 probe and monoclonal antibodies used in the assay for the DENV-1 strains circulating in Cambodia or the variations in the performances of the RT-PCR method used to establish the diagnosis [28] could also explain this observation. A multi-country evaluation of NS1 antigen capture assay has shown that the sensitivity was highest in DENV-1 infection and lowest in DENV-2 [28]. Additionally, two Vietnamese studies has also shown that

Table 3. Sensitivity of Platelia NS1 assay in DFand DHF/DSS patients according to timing of sample collection after DOF*.

	No. of sera tested	NS1 positive	DF (n = 101)		DHF/DSS (n = 87)		*p* value[$]
			No. of Positive/ total tested	Sensitivity % [95% CI]	No. of positive/ total tested	Sensitivity % [95% CI]	
Total	188[¤]	108[#]	73/101	72.3 [63.5–81]	35/87	40.23 [29.85–51.3]	$p<0.001$
DOF 1–3	55	43	31/41	75.6 [59.7–87.6]	12 /14	85.7 [57.2–98.2]	$p=0.407$
DOF 4–8	119	60	38/49	77.5 [63.4–88.2]	22/70	31.4 [20.8–43.6]	$p<0.001$

*DOF: Day after onset of fever.
[¤]14 cases with imprecise DOF were excluded.
[#]5 cases with imprecise DOF were excluded.
[$]*P* values refer to the comparison between NS1 positive rate of DF vs. DHF/DSS for total and for each DOF subgroup.
CI: confidence interval.
doi:10.1371/journal.pntd.0001244.t003

Table 4. Sensitivity of NS1 assay for each DENV serotype.

	DENV-1		DENV-2		DENV-3		DENV-4		*p* value[$]
	NS1 positive/ total tested[#]	Sensitivity% [95% CI]	NS1 positive/ total tested	Sensitivity% [95% CI]	NS1 positive/ total tested[‡]	Sensitivity% [95% CI]	NS1 positive /total tested	Sensitivity % [95% CI]	
Total	44/55	80.0 [67.0–89.6]	4/10	40.0 [12.2–73.8]	77/121	63.6 [54.4–72.2]	8/15	53.3 [26.6–78.7]	$p<0.05$
Day 1–3	21/25	84 [63.9–95.5]	2/3	66.6 [9.4–99.2]	27/32	84.3 [67.2–94.7]	3/5	60.0 [14.7–94.7]	$p>0.05$
Day 4–8	21/25	84.0 [63.9–95.5]	2/7	28.6 [3.7–70.9]	48/79	60.7 [49.1 –71.6]	5/10	50.0 [18.7–81.3]	$p<0.05$

[#]5 cases with imprecise DOF were excluded.
[‡]10 cases with imprecise DOF were excluded.
[$]*P* values refer to comparison between NS1 positive cases in DENV-1 and DENV-2, DENV-3 or DENV-4 groups.
CI: confidence interval.
doi:10.1371/journal.pntd.0001244.t004

the sensitivity in DENV-1 infection was significantly higher than in DENV-2 but not in DENV-3 infected patients and data on DENV-4 was not available [8,33]. The lowest sensitivity of NS1 antigen capture assay observed in DENV-2 infections (40%) - particularly at DOF 4–8 - might only be a bias due to the limited number of DENV-2 cases included in our study.

Unlike other self limited viral diseases, dengue infection may develop into the life threatening DSS form in a few days. A test allowing early diagnosis, which can predict a risk of subsequent evolution to the severe form is desirable in order to improve the clinical management of dengue infection. This could reduce unnecessary use of antibiotics, hospitalization of patients with milder disease in countries with limited resources and allow early hospitalization and supportive care of those developing potentially life threatening DHF. Quantification of viremia by real-time RT-PCR methods might be useful in this regard [9,10] but is expensive and not readily available in endemic regions, which hampers its use in clinical practice.

This present study demonstrates a moderate correlation of the semi-quantitative result of NS1 antigen-capture assay with the level of viremia quantified by real time RT-PCR. This finding is in agreement with previous studies in which NS1 antigen-capture assay was demonstrated *in vitro* to be applicable as an easy and fast method for semi-quantification of DENV in cell culture [12,13,34] and NS1 levels were found *in vivo* to correlate with viremia level [8,9]. However, as also stated by Ludert et al. [34], a limitation of the use of Platelia NS1 antigen capture kit as a semi-quantitative test was that we did not use quantified NS1 protein as internal control and our sera were not serially diluted.

As expected and already largely described, DHF/DSS cases are more frequently observed in secondary infection with an adjusted odd ratio of 6.6 [10,31,35,36]. The apparent lowest severity of DENV-1 infections observed in our study is partially in agreement with data published by Vaughn et al. [10] who reported that this serotype caused less severe pleural effusion than DENV-2 but not than DENV-3 and DENV-4 secondary infections. Due to the low number of DENV-2 and also DENV-4 cases recruited in our study but also at the country level (with DENV-2 representing 9.2% and 9.1% and DENV-4 accounting for 2.9% and 3.1% of the serotypes isolated out of the 16,635 and 39,618 dengue cases reported in 2006 and 2007, respectively) [5], we cannot discuss it further.

Interestingly, the mildest dengue infection was also associated with high NS1 antigen level semi-quantitatively measured by the Platelia Dengue NS1 Ag kit (OR = 0.21, $p = 0.002$) and in patients infected with DENV-1 (OR = 0.083, $p = 0.006$). These findings contrasted with those of studies which showed conversely that a higher viremia titer [10] and NS1 plasma levels were associated with more severe disease [9]. Of note, these studies were conducted on fewer cases and measured the viremia in patients recruited less than 72 hours after fever onset while our results were based on more patients, although only 14/84 DHF/DSS cases were included before DOF 4, and we included additional characteristics that were controlled in the multivariate analysis (i.e. patient's age, day of sample collection after fever onset, DENV serotypes and anti-DENV immune status with well characterized clinical and biological data from hospitalized patients). In addition, in our series, all ambiguous data in regard to severity or primary/secondary dengue infection classification were excluded. The multivariate analysis in principle would avoid the confounding factor introduced by the higher proportion of DENV-1 infections, associated with higher NS1 titers, observed among mild DF cases. Nonetheless, in a multi-country study, Guzman et al. did not find any association between the NS1 detection and disease severity

Table 5. Sensitivity of NS1 test compared with level of viral RNA in serum (log10 cDNA equivalents/mL).

	DF (n = 88*)NS1 positive/total tested [sensitivity; 95% CI]			DHF/DSS (n = 57[#])NS1 positive/total tested [sensitivity; 95% CI]		
	<5 log/ml	>5 log /ml	*P* value	<5 log/ml	>5 log /ml	*p* value
Total	19/33 [57.6%; 39.2–74.5]	51/55 [92.7%; 82.4–98]	$p<0.001$	14/41 [34% ; 20.1–50.6]	15/16 [93.8%; 69.8–99.8]	$p<0.001$
DOF 1–3	7/11 [63.6%; 30.8–89.1]	24/26 [92.3%; 74.8–99]	$p = 0.035$	1/2[50%; 1.2–98.7]	10/10 [100%; 69.2–100]	$p>0.05$
DOF 4–8	12/17 [70.6%; 44–89.7]	24/25 [96%; 79.6–99.9]	$p = 0.021$	13/38 [34.2%; 19.6–51.4]	5/5 [100%; 47.8–100]	$p = 0.009$

*9 cases with imprecise DOF were excluded.
[#]2 cases with imprecise DOF were excluded.
CI: confidence interval.
doi:10.1371/journal.pntd.0001244.t005

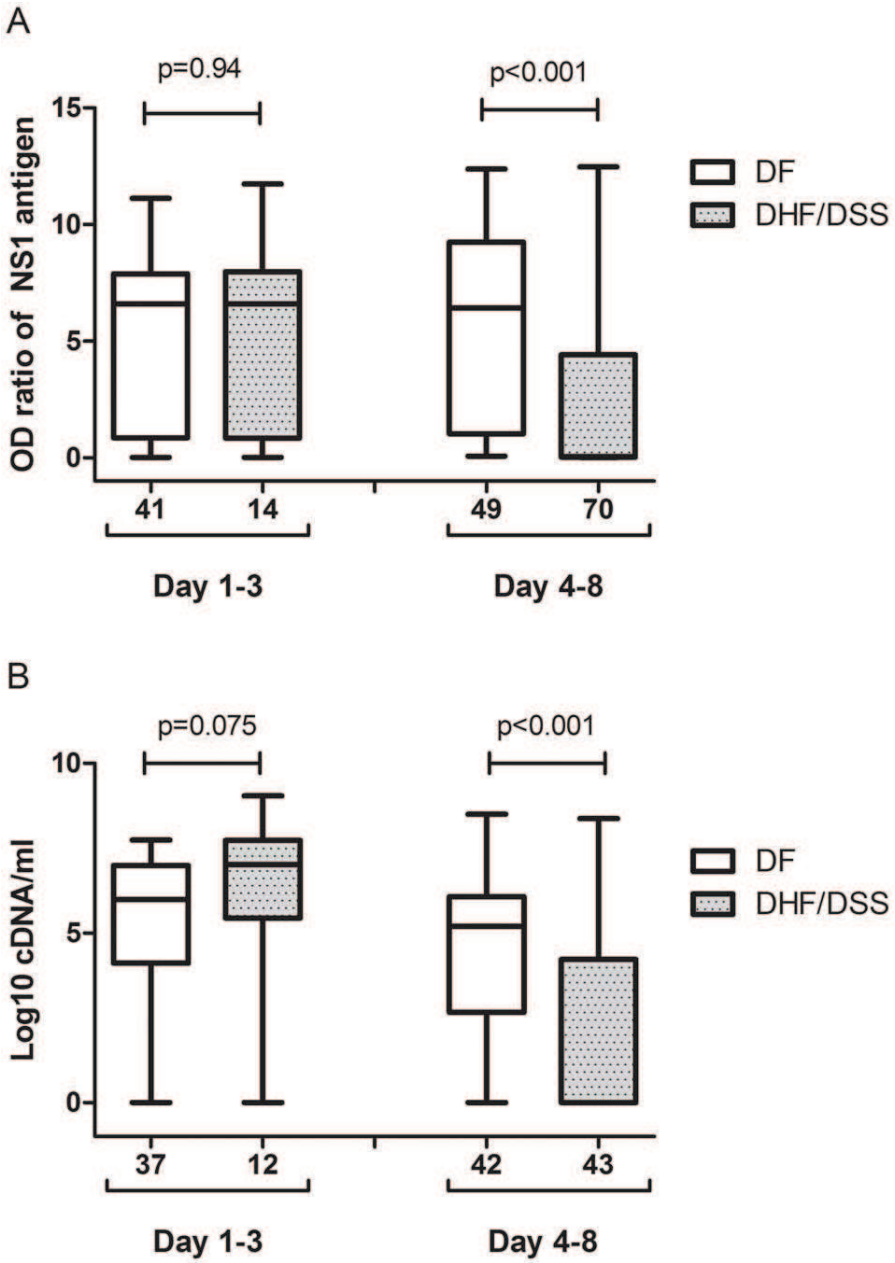

Figure 3. Level of NS1 antigen and viremia by disease severity. Shown are the median, interquartile and 95 percent range of OD ratio of NS1 antigen (A) and log10 cDNA equivalents/mL (B) distributed by disease severity (DF, DHF/DSS). The number of patients is shown under the X axis bar. NS1 antigen OD ratio was significantly higher in DF group than in DHF/DSS group ($p<0.001$) at DOF 4–8 (A). Log10 cDNA equivalents/ml was significantly higher in DF group than in DHF/DSS group at DOF 4–8 ($p<0.001$) (B).
doi:10.1371/journal.pntd.0001244.g003

[28]. Since our study was the first to find this association and considering the limitations in the use of Platelia kit for a semi-quantification of NS1 antigen, a more explicit study will be needed to confirm our results.

The same multivariate analysis but using viral load found that patients with viremia lower than 5 log10 cDNA equivalents/mL experienced more severe dengue infection (28% vs. 62.5%, adjusted OR = 0.33, $p = 0.019$). The result suggests that low level of viral load and NS1 antigen increases the likelihood of developing severe dengue infection at least in the context of Cambodian DENV strains in circulation and/or population enrolled during the period of this study. The enhanced anti-DENV immune response associated with the severity of the disease [37] and leading to an increased infected cell mass at the early stages of the disease may afterwards accelerate the virus clearance from the serum. Since the Platelia NS1 assay's sensitivity is enhanced after immune complexes dissociation [14,31], the lower antigenemia or viremia observed in the severe cases could be the result of a higher anti-NS1 immune response.

Indeed, it has already been suggested that the sNS1 can be trapped within immune complexes which impedes the detection by the antigen-capture assays by preventing the plate-bound or

probe monoclonal antibodies to access the NS1 target epitopes [31]. Koraka et al. have shown that the dissociation of NS1 antigen-antibody immune complexes improved the sensitivity of their in-house test, particularly in sera collected during secondary infections [31]. Lapphra et al. observed an increase of sensitivity of the Platelia kit by 10% using acid treatment [14]. Unfortunately, immune complex dissociation was not performed in our study to confirm these findings on Cambodian samples.

Immune complex formation with sNS1 [12,38] and sNS1 binding to endothelial cells [39] have been proposed as potential factors in DHF pathogenesis. In addition, antibodies directed against NS1 cross-react with human platelets and endothelial cells [40]. Anti-NS1 antibodies induce endothelial cells to undergo apoptosis and *in vitro* experiments demonstrated that these antibodies were responsible for an increased endothelial cell monolayer permeability [40]. NS1 may also activate complement by alternative pathway and this might explain the complement activation observed in infants with DHF during primary infections [41].

Our two former hypotheses in their attempts to explain the low antigenemia and viremia observed in the severe cases do not take into consideration the role of the virus. Some strains might be more virulent than others and we cannot rule out the possibility that a lower antigenemia and viremia could be at least partially also the consequence of a lower virus replication. Indeed, our observation is supported by *in vitro* experiments conducted by Tuiskunen et al (personal communication) using the DENV strains collected in this study during the same epidemic year. The *in vitro* study demonstrated that DENV-1 strain isolated from severe dengue infection (DSS) had lower level of replication in mammalian Vero cells than strains isolated from DF and DHF patients. Nonetheless, a non significant relationship between disease severity and level of NS1 antigen or DENV serotype detected in patients was reported elsewhere [8,28], Hence, our findings might be relevant only for the DENV-1 strains circulating in Cambodia.

Another interesting aspect of this study was the recruitment of individuals asymptomatically infected. In this group, NS1 antigen-capture assay was significantly less sensitive than in the DIC ($p = 0.003$). The lower sensitivity of NS1 antigen-capture in asymptomatic patients might be explained by the lower level of viremia in these individuals (Table S1). Along the same line, RT-PCR detection was more sensitive than NS1 antigen-capture assay in detecting infection in apparently healthy individuals (76.5% vs. 35.3% respectively, $p = 0.01$) but the difference was not significant in DIC. The level of viremia in asymptomatic cases was not significantly lower than in all dengue confirmed cases ($p = 0.145$). Since asymptomatic individuals did not experience more primary infections than DIC, this observation is probably not related to the presence of more anti-NS1 antibodies in one group rather in the other. In addition, the positivity rate of RT-PCR or real time RT-PCR was not significantly different between the two groups. Additional evaluations using greater numbers of asymptomatic cases would probably be helpful to address more explicitly the question of the mechanism of the lower sensitivity of NS1 antigen capture test in this particular group.

In conclusion, we have shown the usefulness of qualitative result of NS1 antigen detection assay in early recognition of dengue infection particularly in combination with IgM test. The point of care rapid diagnostic tests including NS1 antigen and IgM/IgG detection would be probably a helpful tool for early dengue infection diagnosis in clinical practice but these tests need to be further extensively evaluated.

The evaluation of the Platelia NS1 Ag detection kit exhibited a quite low overall sensitivity. These data suggest that the NS1 antigen results should be interpreted with caution when used alone. However, its sensitivity was relatively high in patients who were sampled during the first 3 days after the onset of fever, in patients with primary infection, in patients with DENV-1 infection, in patients experiencing a high level of viremia and in patients with dengue fever forms. In asymptomatic patients, RT-PCR assay has proved to be more sensitive than NS1 antigen detection.

Moreover, using the semi-quantitative approach of the test, we have demonstrated that the NS1 antigen level was significantly correlated to the level of viremia and that the low level of NS1 antigen was associated with more severe disease.

Supporting Information

Table S1 Virological results and clinical features of dengue index cases and household members (in 74 households).
(DOC)

Table S2 Comparison of NS1 kit or RT-PCR sensitivity against the combination of each assay with MAC-ELISA.
(DOC)

Table S3 A. Multivariate analysis of factors* associated with DHF/DSS. B. Multivariate analysis of factors* associated with DHF/DSS.
(DOC)

Checklist S1 STARD checklist.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: PB V. Duong V. Deubel. Performed the experiments: V. Duong SO. Analyzed the data: V. Duong SL SV PB. Contributed reagents/materials/analysis tools: PLT AT NC AL ILG. Wrote the paper: V. Duong AL ILG V. Deubel SV PB.

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Table S1. Virological results and clinical features of dengue index cases and household members (in 74 households).

	Household members (n=214)		Dengue index cases	*p* value*
	Presence of symptoms	Absence of symptom		
Total of positive cases	2 (1%)	17[#] (8%)	15	
NS1 antigen capture assay	2 (100%)	6/17 (35.3%)	13/15 (86.7%)	*p*=0.003
RT-PCR positive	2 (100%)	13/17 (76.5%)	14/15 (93.3%)	*p*=0.338
DENV serotype				
DENV-1	2	4 (30.8%)	7 (50%)	
DENV-3	-	9 (69.2%)	7 (50%)	
qRT-PCR				
Positive	2/2	7/13 (54%)	12/14 (85.7%)	*p*=0.103
Mean viremia (log cDNA equivalent/mL)	5.6 (SD: 0.43)	2.7 (SD: 2.7)	4.96 (SD: 2.37)	*p*=0.043
IgM				
Positive (acute serum)	2	1/17 (6%)	1/15 (6.7%)	
Seroconversion	-	9/17 (53%)	14/15 (93.3%)	*p*=0.011
Negative (on pair sera)[$]	-	3/17 (41%)	-	
Clinical manifestation				
DF	2	-	12/15 (80%)	
DHF/DSS	-	-	2/15 (13.3%)	
Indeterminate clinical status	-	-	1/15 (6.7%)	
Serological status				
Primary	-	3/11 (27%)	-	
Secondary	-	8/11 (73%)	15/15 (100%)	*p*=0.063
Unknown	2	6	-	

SD: standard deviation; DENV: dengue virus; qRT-PCR: quantitative RT-PCR; N/A: not available
[#] 2 households had 2 asymptomatic individuals
* *P* value for comparison between asymptomatic individual and dengue index case group
[$] Second serum was not available in 4 cases

Table S2. Comparison of NS1 kit or RT-PCR sensitivity against the combination of each assay with MAC-ELISA.

NS1 antigen capture assay				RT-PCR			
	NS1 positive [sensitivity , 95%CI]	**NS1 + IgM positive [sensitivity , 95%CI]**	***p* value**		**RT-PCR positive [sensitivity , 95%CI]**	**RT-PCR + IgM positive [sensitivity , 95%CI]**	***p* value**
Total (n=260)[#]	150 [57.7%, 51.4-63.8]	223* [85.7%, 80.9-89.8]	$p<0.001$	Total (n=260)[#]	201 [77%, 71.7-81.2]	248* [95.4%, 92.1-97.6]	$p<0.001$
DOF 1-3 (n=77)	57 [74%, 62.8-83.4]	67[¥] [87%, 77..4-93.6]	$p=0.042$	DOF 1-3 (n=77)	65 [84.4%, 74.5-91.7]	74[¥] [96%, 89-99.2]	$p=0.014$
DOF 4-8 (n=163)	85 [52%, 44.2-60]	147* [90.2%,84.5-94.3]	$p<0.001$	DOF 4-8 (n=163)	121 [74%, 66.8-80.7]	158* [97%, 93-99]	$p<0.001$

[#] 20 cases with imprecise DOF were excluded.
* Significant difference ($p<0.001$) for comparison between NS1+IgM positive and RT-PCR+IgM positive groups
[¥] No significant different ($p=0.35$) for comparison between NS1+IgM positive and RT-PCR+IgM positive groups

Table S3: Multivariate analysis of factors* associated with DHF/DSS using A) NS1 OD ration and B) Log10 cDNA.

A)

	Disease severity Number of positive/number tested (%)		**Logistic regression**		
	DF	**DHF/DSS**	**Adjusted Odds Ratio**	**95% CI**	***P* value**
Age (number, mean, SD)	111, 8.72, 9.74	87, 7.42, 3.75	1.087	0.947-0.237	0.237
DOF 1-3	41/90 (45.5)	14/84 (16.7)	1	-	-
DOF 4-8	49/90 (54.5)	70/84 (83.3)	2.408	0.959-0.061	0.061
NS1 group 1 (OD ratio <1)	28/101 (27.7)	55/87 (63.2)	1	-	-
NS1 group 2 (OD ratio 1-6)	20/101 (19.8)	14/87 (16.1)	0.363	0.121-0.07	0.070
NS1 group 3 (OD ratio > 6)	**53/101 (52.5)**	**18/87 (20.7)**	**0.214**	**0.08-0.002**	**0.002**
RT-PCR negative	12/101 (12)	23/87 (26.5)	1	-	-
DENV-1	**38/101 (37.6)**	**9/87 (10.3)**	**0.083**	**0.014-0.006**	**0.006**
DENV-2	4/101 (4)	4/87 (4.6)	0.124	0.014-0.064	0.064
DENV-3	42/101 (41.6)	47/87 (54)	0.368	0.077-0.212	0.212
DENV-4	5/101 (5)	4/87 (4.6)	0.203	0.026-0.126	0.126
Primary infection	26/86 (30.2%)	3/77 (3.9%)	1	-	-
Secondary infection	**60/86 (69.8%)**	**74/77 (96.1%)**	**6.606**	**1.58-0.01**	**0.010**

B)

	Disease severity Number of positive/number tested (%)		**Logistic regression**		
	DF	**DHF/DSS**	**Adjusted Odds Ratio**	**95% CI**	***P* value**
Age (number, mean, SD)	111, 8.72, 9.74	87, 7.42, 3.75	1.101	0.952 - 1.273	0.195
DOF 1-3	41/90 (45.5)	14/84 (16.7)	1	-	-
DOF 4-8	49/90 (54.5)	70/84 (83.3)	2.102	0.829 - 5.329	0.118
Log10 cDNA <5 /mL	33/88 (37.5)	41/57 (72)	1	-	-
Log10 cDNA >5 /mL	**55/88 (62.5)**	**16/57 (28)**	**0.329**	**0.13 - 0.834**	**0.019**
RT-PCR negative	12/101 (12)	23/87 (26.5)	1	-	-
DENV-1	38/101 (37.6)	9/87 (10.3)	0.579	0.102 - 3.276	0.536
DENV-2	4/101 (4)	4/87 (4.6)	1.433	0.158 - 12.974	0.749
DENV-3	42/101 (41.5)	47/87 (54)	2.136	0.465 - 9.799	0.329
DENV-4	No data				
Primary infection	26/86 (30.2%)	3/77 (3.9%)	1	-	-
Secondary infection	**60/86 (69.8)**	**74/77 (96.1)**	**6.032**	**1.511 - 24.085**	**0.010**

* Age, duration after fever onset, NS1 antigen level, DENV serotypes and immune status

SD: standard deviation

CI: confidence interval

CHAPTER 5: GENOME-WIDE EXPRESSION IN DSS

II.1.13 Context of study

The increasing incidence of severe cases in the last decades has strengthened the interest of both clinicians and research scientists for improving the knowledge on severe dengue pathophysiology. Few is known about the factors that determine the occurrence of severe forms and particularly of DSS in some dengue-infected patients, while it is now admitted that dengue is a multi-factorial disease which outcome is likely the result of complex interactions between vector, virus, host determinants and several environmental factors.

Clinically, dengue presentation encompasses a large spectrum of diseases going from asymptomatic infections of unknown incidence, to "classical DF", the most common, and finally to severe clinical forms out of which the life-threatening DSS. According to clinicians, the WHO criteria used until recently to classify dengue patients in different grades of severity, should be re-assessed (Deen et al., 2006; Phuong et al., 2004; Setiati et al., 2007). Indeed, those criteria likely over-estimate the percentage of severe cases while taking into account as signs of severity, clinical or biological disturbances also encountered in classical DF. Most importantly, about 20% of patients developing a DSS do not fill the criteria of severity defined by the WHO and are mistakenly classified as uncomplicated cases (Deen et al., 2006; Phuong et al., 2004; Setiati et al., 2007). Altogether this emphasizes the requirement for a better understanding of DSS pathophysiology and the identification of new biological markers predictive of evolution towards DSS.

DSS is regarded as a vascular disease involving a complex interplay between virus, whole blood cells and microvascular territories (Basu and Chaturvedi, 2008), and thought to result largely from an aberrant host response to infection. As for other major systemic diseases, a detrimental cytokine storm is thought to be central to the systemic microcirculatory failure and massive plasma leakage leading to cardiovascular decompensation characterizing DSS (Pang et al., 2007). However, controversies exist regarding the nature of pathogenic host immune responses supporting this life-threatening syndrome (Green and Rothman, 2006; Lin et al., 2006; Murgue, 2010). Indeed, reactivation of cross-reactive memory T lymphocytes and increased infection of monocytes mediated by cross-reactive antibodies acquired during previous infections by distinct DENV serotypes, are the main hypothetic mechanisms proposed to explain the putative cytokine storm leading to plasma leakage (Fink et al., 2006; Pang et al., 2007). However, these hypotheses fail to explain the occurrence of DHF/DSS in patients having primary dengue infection and their

relevance to the pathophysiology (Libraty et al., 2009; Murgue, 2010). Understanding the molecular basis of DSS and identifying relevant DSS biomarkers thus remains a major challenge (Green and Rothman, 2006; Pang et al., 2007). Attempting to decipher molecular mechanisms underlying DSS by analyzing circulating whole blood cell genome-wide expression profiles is a relevant approach regarding the study of other systemic inflammatory syndromes, where a cognate cross-talk between endothelial vascular cells and blood cells occurs (Cobb et al., 2005; Feezor et al., 2005). Whole blood represents a highly informative while complex cellular sample that may reflect host pathophysiological responses ongoing at the time of blood sampling (Cobb et al., 2005). Furthermore, whole blood cells are easy to collect and store during field studies on large cohorts, reducing samples volumes required and limiting technical bias due to cell purification. Such a bed-to-bench side medical research has gained more and more interest in the recent years. Indeed, it allowed improving the understanding of pathophysiological processes underlying systemic critical illnesses such as sterile and non-sterile systemic inflammatory responses syndromes (SIRS), allowing the identification of relevant disease biomarkers and of new putative therapeutic targets (Cobb et al., 2005; Feezor et al., 2005; Laudanski et al., 2006).

II.1.14 Objectives

Few are known about the pathological process leading to endothelial dysfunction in severe dengue infection. More recent evidences showed the contribution of immune-mediated mechanisms in the pathophysiological cascade leading to DSS. In this study, the role of immune inflammatory cells in the dysfunction of endothelium during dengue infection was assessed using a prospective study comparing the whole blood genome-wide expression profiles of 48 matched Cambodian children recruited during the large 2007 dengue outbreak. The objectives were to identify the critical mechanisms supporting the vascular leakage in the course of dengue infections. Such mechanisms may represent targets for future therapeutic strategies aimed at interfering with the occurrence of massive vascular leakage leading to DSS and to identify cellular markers that could be used as predictive factors of evolution towards DSS in dengue-infected patients.

II.1.15 Results and conclusion

To identify gene patterns specifically altered in DSS patients, genome-wide expression profiles of whole blood from 3 groups of carefully matched paediatric patients representing the main clinical

forms of symptomatic dengue infections DF (n= 16), DHF (n= 13) and DSS (n =19), according to the 1997 WHO classification criteria of dengue severity, were compared. Using multi-way analysis of variance (ANOVA) and adjustment of p-values to control the False Discovery Rate (FDR,10%), a signature of 2959 genes differentiating DSS patients from both DF and DHF were identified, and these DSS-gene signatures showed a strong association with the dengue disease phenotype. The 48 patients expression profiles were organized in two major subsets: subset 1 includes both DF and DHF patients without distinction; subset 2 encompasses a sub-group of DF and DHF patients, and a distinct sub-group including 17 out of the 19 DSS patients. These present results highlight the inadequacy of the 1997 WHO classification of dengue clinical forms that considers DF and DHF grades I/II as two separate disease phenotypes, and more likely support the recently proposed WHO classification.

Using a combined approach to analyze the molecular patterns associated with the DSS-gene signature, an integrative overview of the transcriptional responses altered in DSS children was revealed. In particular, the transcriptome of DSS children blood cells was characterized by a decreased abundance of transcripts related to T and NK lymphocyte responses. Those genes are critical to a number of T and NK-cell functions, including T and NK cell differentiation, receptor signaling, activation and proliferation, cytotoxic functions or recruitment of lymphocytes to peripheral tissues. An increased abundance of anti-inflammatory and repair/remodeling transcripts was also observed in DSS patients. Over-expressed anti-inflammatory genes identified encode molecules with diverse functions: the anti-inflammatory cytokine IL-10, a putative marker of severe dengue (Green et al., 1999), serine proteases and metalloprotease inhibitors, IL-1b cytokine decoy receptor, free haeme scavenger molecules, or complement-regulating receptors. Repair and remodeling genes over-expressed in the DSS-gene signature also encode a diversity of molecules: the MMP-9, a matrix metalloprotease with key role in tissue remodeling and a candidate to dengue plasma leakage (Luplertlop et al., 2006).

Unexpected pro-inflammatory gene patterns at the interface between innate immunity, inflammation and host lipid metabolism were identified and known to play pathogenic roles in acute and chronic inflammatory diseases associated with systemic vascular dysfunction. Three main pro-inflammatory genes were transcriptionnally active in the blood cells of DSS children: the first one was defined by a set of overexpressed genes strongly associated with the disease phenotype subtype DSS that encode highly pro-inflammatory microbicidal peptides and enzymes;

second pro-inflammatory gene pattern identified is typical of altered homeostasis of cholesterol in monocytes/macrophages that characterizes inflammatory lipid-laden monocytes/macrophages

(lipid-laden Mo/Mac), a subtype of foam cells initiating vascular lesions in metabolic inflammatory diseases; and the third pro-inflammatory gene pattern associated with the DSS-gene signature is characteristic of the metabolic proinflammatory arachidonic-acid pathway which is activated in the whole blood cells of DSS children at the time of cardiovascular decompensation.

The present study has also demonstrated that the shift from a ‘‘severe’’ to an ‘‘uncomplicated’’ transcriptional profile may occur within a very short time and this finding could explain the ‘‘uncomplicated’’ (non-destructive) characteristic of immune responses in dengue infection.

In conclusion, the results in this study provide a global while non-exhaustive overview of the molecular mechanisms altered in of DSS children and suggest how they may interact to lead to final vascular homeostasis breakdown. Some mechanisms identified should be considered putative therapeutic targets or biomarkers of progression to DSS.

The results of this work are summarized in the article below which was published in « PloS One » en 2010.

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PLoS one

Genome-Wide Expression Profiling Deciphers Host Responses Altered during Dengue Shock Syndrome and Reveals the Role of Innate Immunity in Severe Dengue

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Abstract

Background: Deciphering host responses contributing to dengue shock syndrome (DSS), the life-threatening form of acute viral dengue infections, is required to improve both the differential prognosis and the treatments provided to DSS patients, a challenge for clinicians.

Methodology/Principal Findings: Based on a prospective study, we analyzed the genome-wide expression profiles of whole blood cells from 48 matched Cambodian children: 19 progressed to DSS while 16 and 13 presented respectively classical dengue fever (DF) or dengue hemorrhagic fever grades I/II (DHF). Using multi-way analysis of variance (ANOVA) and adjustment of p-values to control the False Discovery Rate (FDR<10%), we identified a signature of 2959 genes differentiating DSS patients from both DF and DHF, and showed a strong association of this DSS-gene signature with the dengue disease phenotype. Using a combined approach to analyse the molecular patterns associated with the DSS-gene signature, we provide an integrative overview of the transcriptional responses altered in DSS children. In particular, we show that the transcriptome of DSS children blood cells is characterized by a decreased abundance of transcripts related to T and NK lymphocyte responses and by an increased abundance of anti-inflammatory and repair/remodeling transcripts. We also show that unexpected pro-inflammatory gene patterns at the interface between innate immunity, inflammation and host lipid metabolism, known to play pathogenic roles in acute and chronic inflammatory diseases associated with systemic vascular dysfunction, are transcriptionnally active in the blood cells of DSS children.

Conclusions/Significance: We provide a global while non exhaustive overview of the molecular mechanisms altered in of DSS children and suggest how they may interact to lead to final vascular homeostasis breakdown. We suggest that some mechanisms identified should be considered putative therapeutic targets or biomarkers of progression to DSS.

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Introduction

Acute dengue virus infections are a major public health problem for many tropical and sub-tropical countries and an increasing risk for the worldwide population [1]. Symptomatic infections occur under a spectrum of diseases ranging from classical dengue fever (DF) to the most severe life-threatening dengue shock syndrome (DSS), a leading cause of childhood hospitalisation and death in endemic countries with limited health resources [1,2].

DSS is regarded as a vascular disease involving a complex interplay between virus, whole blood cells and microvascular territories [3,4], and thought to result largely from an aberrant host response to infection. As for other major systemic diseases, a detrimental cytokine storm is thought to be central to the systemic microcirculatory failure and massive plasma leakage leading to cardiovascular decompensation characterizing DSS [5]. However, controversies exist regarding the nature of pathogenic host immune responses supporting this life-threatening syndrome [6–8]. Indeed, reactivation of cross-reactive memory T lymphocytes and increased infection of monocytes mediated by cross-reactive antibodies acquired during previous infections by distinct dengue virus serotypes, are the main hypothetic mechanisms proposed to explain the putative cytokine storm leading to plasma leakage [5,9]. However, those hypothesis fail to explain the occurrence of DSS in patients having primary dengue infection and their relevance to the pathophysiology of DSS disease is discussed [8,10].

Efforts to identify soluble biomarkers of severe dengue differentiating uncomplicated dengue infections from severe ones has led to the identification of a diversity of cytokines, chemokines, endothelial agonists or soluble endothelial molecules [11–18]. However, discrepancies in definition of dengue severity, variability in patients cohorts characteristics, as well as in techniques and markers investigated, have impaired the identification of reliable sets of DSS biomarkers and the possibility to get a global overview of biological markers altered during DSS.

Understanding the molecular basis of DSS and identifying relevant DSS biomarkers thus remains a major challenge [5,6]. Indeed, DSS occurs by the end of the acute infection in only a fraction of dengue-infected patients and current severity criteria, based on the 1997 World Health Organization (WHO) classification of dengue severity, fail to predict a significant proportion of patients who progress to life-threatening DSS [19–21]. Attempting to decipher molecular mechanisms underlying DSS by analyzing circulating whole blood cell genome-wide expression profiles is a relevant approach regarding the study of other systemic inflammatory syndromes, where a cognate cross-talk between endothelial vascular cells and blood cells occurs [22–24]. Whole blood represent a highly informative while complex cellular sample, that may reflect host pathophysiological responses ongoing at the time of blood sampling [22]. Furthermore, whole blood cells are easy to collect and store during field studies on large cohorts, reducing samples volumes required and limiting technical bias due to cell purification. However, due to the high cellular complexity of whole blood cells samples, whole gene expression patterns should be carefully analyzed and deciphered to allow returning to an integrative view of the molecular mechanisms altered during the pathophysiological process studied [25].

Such a bench-to-bedside medical research has gained more and more interest in the recent years. Indeed, it allowed improving the understanding of pathophysiological processes underlying systemic critical illnesses such as sterile and non sterile systemic inflammatory responses syndromes (SIRS), allowing the identification of relevant disease biomarkers and of new putative therapeutic targets [22–24,26].

Genome-wide expression studies aimed at deciphering molecular responses altered in the whole blood cells of adults [27] and children DSS patients [28] have been implemented recently by colleagues. They reported a decreased IFN type I-induced response and a benign transcriptional response at the time of cardiovascular decompensation [27,28], but failed in identifying biological pathways relevant to DSS pathophysiology and particularly inflammatory ones that could sustain microvascular dysfunction [28].

We report here the results of a prospective study comparing the whole blood genome-wide expression profiles of 48 matched Cambodian children recruited during the huge 2007 dengue outbreak who presented with classical dengue fever (DF), dengue hemorrhagic fever grades I/II (DHF) or dengue shock syndrome (DSS), according to the 1997 WHO classification of dengue severity [29]. Based on careful study design and statistic treatment of microarrays data, we identified a large and highly relevant gene signature of DSS never reported before, that discriminates DSS children from paediatric patients with DF or DHF grades I/II, who did not present severe clinical complications. Using an integrative analysis of the gene patterns altered in DSS children, we deciphered part of the complex interactive molecular processes occurring during DSS, highlighting similarities between DSS and other major inflammatory processes. Finally, we identified unexpected pro-inflammatory innate immune responses activated in the whole blood cells of DSS children that may play a major role in DSS pathophysiology. The implications of present findings to the improvement of DSS prognosis and treatment are discussed.

Materials and Methods

Ethics statement

The global study and all protocols presented here were approved by the national Cambodian ethical committee. Written informed consent was obtained from the legal guardians of each child. To ensure strict anonymity regarding the patients, samples were encoded as PLxxx (Plasma Leakage).

Patients and clinical data

Inclusion criteria retained were: age (1 to 15 years old); positive diagnosis of acute dengue infection assessed by different methods; absence of known chronic inflammatory disease or ongoing acute co-infection at the time of inclusion.

An eligible cohort of 83 dengue-infected children hospitalised at the Kampong Cham provincial hospital, Cambodia, was prospectively enrolled from July to September 2007 during the huge 2007 dengue outbreak in Cambodia, characterized by a high number of DSS cases.

Children diagnosed with acute dengue infections were classified at admission as classical dengue fever (DF), dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) based on the 1997 WHO criteria [29]. Clinical and biological follow-up was done daily for each hospitalised patient. DSS patients were admitted to hospital intensive care unit where they received appropriate fluid resuscitation and were monitored for vital parameters. Children who required blood transfusion were not included in the study.

To increase the probability to identify gene signatures specific of DSS, we chose to include only symptomatic dengue-infected classified DF, DHF and DSS, but no healthy or non-dengue children in the present study. This is based on the rationale that comparing DF, DHF and DSS patients together should improve the probability to identify a DSS-specific gene signature, while including an external non dengue control group should increase the probability to identify a general dengue-related signature but should be less powerful at identifying a signature of severe dengue disease.

DF, DHF and DSS patients whole blood samples selected for the present study corresponded to comparable duration of illness after onset of fever: all were collected within a window of time comprised between 3 days and 7 days after onset of fever (being considered day 0). For most DSS patients, this generally corresponded to the day of cardiovascular decompensation (shock) or the day after, except for 3 (PL017, PL033, PL047) and 2 DSS (PL005, PL101) for whom blood was collected respectively 2 and 3 days after onset of shock.

Patients' samples selected for the present study were also carefully matched for age, gender, viral serotype (when identified) and immunological status (primary or secondary, according to reference assays described in diagnosis methods) towards dengue infection.

Diagnosis assays carried out as described thereafter, indicated that about 90% of all dengue-infected children had secondary infection.

Dengue diagnosis and immunological status

All diagnosis assays were carried out at the Institut Pasteur in Cambodia, the National Reference Center for arboviral diseases in Cambodia. IgM capture ELISA and Hemagglutination-inhibition were performed on paired sera collected at admission and at discharge, and systematically tested for both dengue and Japanese

Encephalitis virus, another flavivirus endemic in Cambodia, as described previously [30]. Virus isolation was carried out on earliest serum samples by inoculating permissive C6/36 and VERO E6 cells, followed by serotype-specific immunofluorescence [30]. Viral RNA was detected in specimens collected at early stage of the disease using a nested RT-PCR [31]. Primary or anamnestic "secondary" antibody response indicating previous infections by dengue viruses was determined from paired serum samples by hemagglutination-inhibition assay. Interpretation followed WHO recommendations [29].

Blood sample preparation

Whole blood samples (2.5 ml) were collected on PAXgene^{TM} Tubes (PreAnalytiX^{TM}) further stored at −80°C, before being sent to France in dry ice. Extraction of series of 24 matched samples (DF, DHF and DSS) was done using PAXgene^{TM} Blood RNA kits (PreAnalytiX^{TM}) rapidly after collection. Purified total RNAs kept at −80°C were processed for hybridization on genome-wide DNA microarrays within one month.

cRNAs preparation and microarrays hybridization

All RNAs were checked for integrity using the 2100 BioAnalyzer (Agilent Technologies) and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies). Cyanine-3-labeled cRNA was generated from 0.3 µg of RNA using the One-Color Low RNA Input Linear Amplification kit (Agilent) according to the manufacturer's instructions, followed by purification on RNAeasy column (QIAGEN). All amplified cRNAs were checked for dye incorporation, cRNA yield and amplification profile. Only those fitting all quality criteria were fragmented for further hybridization on microarrays. Samples from DF, DHF and DSS patients were then carefully matched and hybridized onto Agilent Whole Human Genome (4×44K) Oligo Microarrays (G4112F). Microarrays were scanned using an Agilent DNA microarray scanner G2505B.

Microarray data analysis

All microarray data is MIAME compliant and the raw and normalized data have been deposited in the MIAME compliant database Gene Expression Omnibus [32] (GEO Series accession number GSE17924 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE17924).

Individual microarray quality was evaluated based on QC report, pair-wise MA-plots, and box plots. Intra-array normalization of raw signals from the 48 microarrays was done using Feature Extraction software 9.1.3.1 (Agilent). Microarrays normalized data were further exported into the Limma package [33], for inter-array normalization using the quantile method [34].

Statistical analysis were was performed using the TIGR MeV (MultiExperiment Viewer) v 4.4 software (http://www.tm4.org/mev.html), and the GeneANOVA program [35]. Multi-way ANOVA model was implemented: first, to identify differentially regulated genes when accounting for the multiple sources of variation in the microrray experiment; second, to evaluate the effect of the main variable, disease phenotype, relatively to that of other putative confounding variables such age, gender, duration of illness or microarray technical variability (independent extractions or hybridizations). Local ANOVA further determined the contribution of each covariate on the expression level of each gene. Multiple test correction was further carried out using the false discovery rate (FDR) method [36]. Cluster [37] and Tree View softwares [38] were used for unsupervised hierarchical clustering. Iterative SVM (Support Vector Machine) method associated with leave-one-out cross-validation [39] was used to assess the robustness of DSS-gene signature.

Real-time PCR validation of genes over and under-expressed in DSS patients

Briefly, total RNA extracted from whole blood samples was reverse-transcripted using the High Capacity cDNA RT kit (Applied Biosystems Inc) and random primers. Real-time PCR were carried out using the FastStart Universal Probe Master (ROX) (Roche) and real-time PCR primers designed using the Universal Probe Library (UPL) Assay Design Center (Roche). Amplification products were run on an ABI-PRISM 7900HT (Applied Biosystems). Cycle threshold Ct values were automatically calculated and value obtained for each gene amplified was normalized by subtracting the Ct corresponding to amplification of the HPRT1 gene (ΔCt) for the same sample. Correlation between ΔCt values obtained by real-time PCR and corresponding expression values from microarrays was estimated using Spearman correlation coefficient.

Comprehensive overview of functional patterns altered during DSS

Bio-informatics-based analysis using the demonstration version 7.1 of Ingenuity Pathway Analysis software (IPA; Ingenuity® Systems, www.ingenuity.com) associated with manual and litterature-based analysis was carried out to identify the most relevant functional processes associated with the identified DSS gene signature. This was done by combining most informative canonical pathways identified using IPA, genes having the strongest association with the disease phenotype based on ANOVA analysis, and similarities to molecular patterns altered in other systemic inflammatory processes associated with endothelial dysfunction.

Results

Patient characteristics

To identify gene patterns specifically altered in DSS patients, we compared three groups of carefully matched paediatric patients representing the main clinical forms of symptomatic dengue infections DF (n = 16), DHF (n = 13) and DSS (n = 19), according to the 1997 WHO classification criteria of dengue severity [29]. Altogether, DF, DHF and DSS represent different subtypes of the disease phenotype variable, further considered in this study. The clinical characteristics and values of haematological parameters are presented in table 1 (median values from each patient group) and table S1 (individual values from each of the 48 patients included). Supportive treatment provided to DSS patients are mentioned. As indicated, DSS children had significant lower relative neutrophil counts (median values / DF: 3900; DHF: 3950; DSS: 2500; p-value: 0.03; Kruskal Wallis test).

Unsupervised hierarchical clustering discriminates DSS children from DF/DHF ones, revealing a DSS-gene signature

Since microarray data analysis can be affected by a number of bias [40], we put a particular care on study design and analysed data from the 48 normalized microarrays using multi-way analysis of variance (ANOVA) [41]. Indeed ANOVA evaluates the statistical probability (p-value), for each individual gene, that a difference in expression between the three patient groups could have been observed by chance. This allows revealing genes that show even small but highly significant changes in expression, regarding the studied phenotype.

Table 1. Clinical and biological characteristics of DF, DHF and DSS patient groups at the time of hospital admission.

	DF ($n=16$)	DHF ($n=13$)	DSS ($n=19$)
Patients characteristics			
gender, male *n* (%)	7 (43%)	4 (31%)	7 (37%)
age, median (IQR), years	8 (4–9)	7 (5–8)	8 (7–9)
weight, median (IQR), kg	18 (13–20)	15 (14–18)	19 (15–23)
hospital admission, median (IQR), day after onset of fever (D0)	2 (1–3)	2 (2–3)	4 (3–4)
Dengue status			
viral serotype, *n* (DENV-1/DENV-2/DENV-3/DENV-4/unknown)	4/2/8/1/1	1/1/10/1/0	1/1/10/0/7
immunological status, secondary infections, *n* (%)	14 (88%)	12 (92%)	18 (95%)
Clinical manifestations			
tourniquet test (pos/neg/not done) (%)	56%/44%/0%	54%/38%/8%	37%/32%/31%
hepatomegaly, *n* (%)	3 (19%)	6 (46%)	17 (89%)
gastro-intestinal bleeding, gingivorragy, hematemesis, melena, *n* (%)	0	1 (8%)	6 (32%)
Blood pressure			
heart frequency, median (IQR), pulse per minute	113 (100–124) ($n=14^a$)	120 (112–120)	Not perceptible ($n=15^a$)
pulse pressure, median (IQR), mm Hg	40 (30–45)	30 (30–40)	15 (10–20) ($n=15^a$)
Haematological parameters			
thrombocytopenia (platelet count $<100000/mm^3$), %	15% ($n=14^a$)	55% ($n=11^a$)	94% ($n=17^a$)
hematocrit, median (IQR), %	36.5 (35–39) ($n=14^a$)	39.75 (38–42) ($n=12^a$)	42.5 (38–45) ($n=18^a$)
hemoconcentration (hematocrit >20%), *n* (%)	1 (6%)	3 (23%)	17 (89%)
white blood cells, median (IQR), number/mm^3	6600 (5500–9900) ($n=13^a$)	6450 (6200–7400) ($n=10^a$)	6900 (4800–6900) ($n=17^a$)
neutrophils, median (IQR), number/mm^3	3900 (2900–7600) ($n=13^a$)	3950 (3500–4200) ($n=10^a$)	2500 (2200–3800)($n=17^a$)
lymphocytes, median (IQR), number/mm^3	1600 (1400–2100) ($n=13^a$)	1850 (1500–1900) ($n=10^a$)	2200 (1500–3200) ($n=17^a$)
Supportive medical care			
oxygen supplementation, *n* (%)	0	0	15 (79%)
perfusion of colloid (dextran 40), *n* (%)	0	0	14 (74%)
perfusion of human plasma, *n* (%)	0	0	8 (42%)

DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; IQR, interquartile range; n, number.
[a]n = x : with x : number of patients for which the data is available.
doi:10.1371/journal.pone.0011671.t001

Based on ANOVA analysis, lists of genes differentially expressed between DF, DHF and DSS groups were generated using different false discovery rate (FDR) ranging from 0.05 up to 10%. Indeed, low FDR provide more stringent statistical filter while they reduce the number and thus the enrichment of genes differentially expressed. At the opposite, higher FDR, while statistically accepting a higher number of false positive genes, also provide larger and enriched gene lists that should be more informative when searching to identify molecular pathways. Based on this rationale, we chose to work using the gene list generated at FDR 10 after we verified by a different statistical method currently used for the analysis of microarrays data, SAM (Significant Analysis of Microarray) [33], that most significant genes were commonly found by the two types of analyses (data not shown). The gene list generated at FDR10 included 2959 genes differentially expressed between DF, DHF and DSS patients groups (Table S2).

The biological relevance of those differentially expressed genes was assessed using local ANOVA that allows evaluating the contribution of the main variable, disease phenotype, and that of other putative confounding variables related to patients (age, gender, day of blood sampling, viral serotype) and to technical steps (effect of independent RNA extractions, amplifications and hybridization) on variations of expression levels of those 2959 genes. This confirmed that the disease phenotype strongly influenced the variations of expression of the 2959 genes differentially expressed between the three patient groups, reinforcing the biological significance of this set of genes (Table S2).

Unsupervised hierarchical clustering based on the 2959 gene signature identified was then applied to the 48 children gene expression profiles. This allows clustering the patients whose gene expression profiles are the more similar independently of their disease phenotype subtype. As a result, the 48 patients expression profiles were organized in two major subsets (Figure 1): subset 1 (first dendrogram branch) includes both DF and DHF patients without distinction; subset 2 (second dendrogram branch) encompasses a sub-group (2a) of DF and DHF patients, and a distinct sub-group (2b) including 17 out of the 19 DSS patients, whatever they received or not plasma infusion, revealing a ''DSS-gene signature'' common to most DSS patients. Some few patients clustered however in unexpected subsets: two DSS patients (PL005, PL101) had gene expression profiles closer to those of the DF/DHF 2a subset, while one DF patient (PL064) and three DHF patients (PL037, PL058, PL070) gene expression profiles clustered within the DSS 2b subset.

We confirmed the robustness of the DSS-gene signature using the iterative Support Vector Machine (SVM) classifier learning method [39], which reclassified all the 19 DSS patients together.

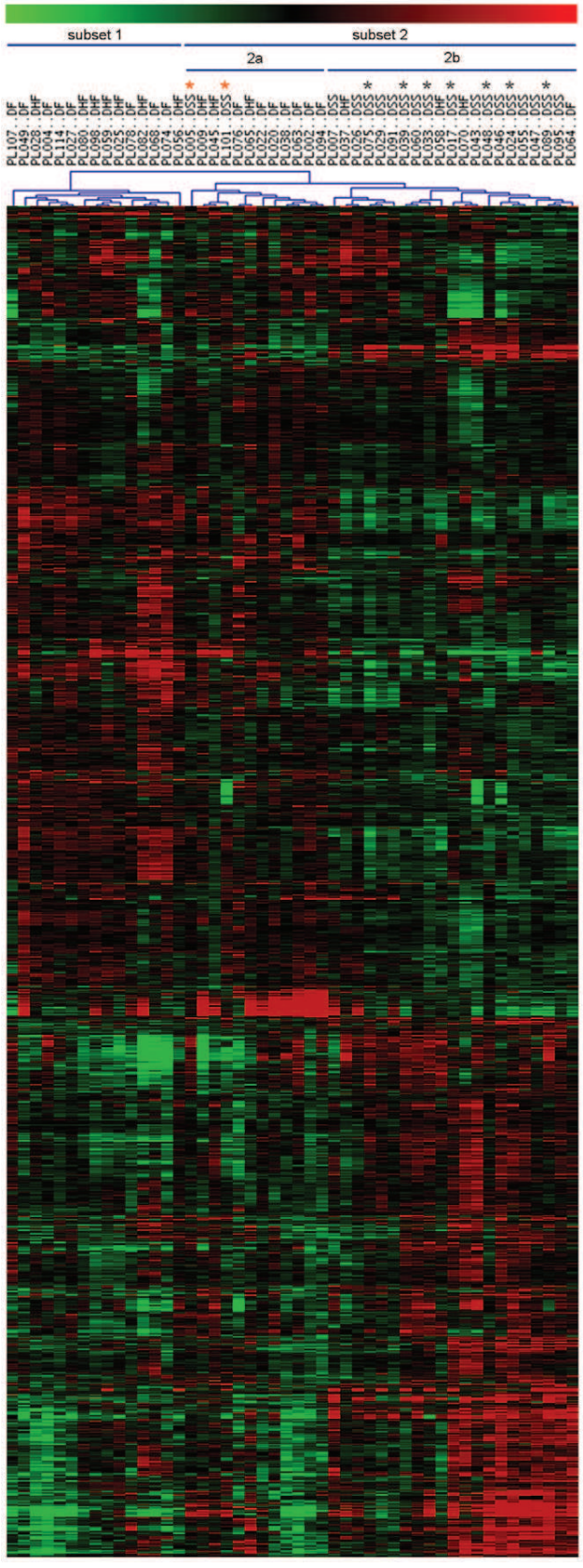

Figure 1. Unsupervised hierarchical clustering of whole blood cells expression profiles from the 48 dengue-infected children.

The clustering is based on the 2959 gene list (3515 clones, detailed in Table S2) discriminating dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) patients. Each row represents a single transcript and each column represents a patient's sample. Color scale indicates the range of gene expression: black indicates median expression level, red greater expression, green lower expression. The 2 patient subsets identified are indicated. PLxxx, code relative to one patient. Black star: DSS patient sampled 3 days after shock. Orange star: Patients who received perfusion of human plasma before collection of blood samples.
doi:10.1371/journal.pone.0011671.g001

To validate microarray data, we carried out real-time RT-PCR focusing on nine genes strongly associated with the DSS-gene signature, using 15 patients samples (five from each disease phenotype subtype: DF, DHF and DSS). Results obtained strongly correlate microarray data (Figure S1).

DSS-gene signature analysis identifies a diversity of genes and canonical molecular pathways related to immunity, inflammation and host metabolism

Filtering genes from those having the highest to the lowest statistical association with the disease phenotype variable (Table S2), relying on results from multi-way and local ANOVA revealed that the individual genes having the strongest association with the DSS phenotype subtype are, for a large part, related to innate immunity, inflammation and host lipid metabolism, a finding confirmed when the whole 2959 genes of the DSS-gene signature were processed through the knowledge-based IPA software.

Indeed, IPA analysis identified that 163 canonical pathways were significantly associated with those genes (data not shown) with a large proportion of immune-related pathways in the first top 30 (Figure 2). In particular, several under-expressed but partially redundant signaling canonical pathways related to T lymphocyte activation were identified, of which the T cell receptor (TCR) signaling pathway (Figure 3), which has the strongest association with the DSS-gene signature. Interestingly, a number of metabolic pathways, and particularly of lipid-signaling pathways, were significantly represented among the 163 DSS-related canonical pathways.

When comparing our results to those of colleagues who reported gene or protein signatures associated with DSS, we identified some transcripts encoding proteins considered putative markers of severe dengue. This includes non exhaustively the acute phase pentraxin-related protein PTX3 [15], the anti-inflammatory IL-10 [11] or the pro-inflammatory IL-18 [12] cytokine transcripts that have increased abundance in the DSS-gene signature, while having intermediate to low statistical association with the disease phenotype variable according to the multi-way ANOVA (Table S2).

IFN type I-related transcripts, of which abundance was shown to be decreased in DSS patients by others [27,28,42], represented only a limited number of genes associated with the DSS-gene signature. This was confirmed by the IPA analysis that did not identify IFN type I-related pathways among those strongly associated with the DSS-gene signature (Figure 2).

DSS is associated with impaired expression of T and NK cell-related genes but increased expression of anti-inflammatory and repair/remodeling transcriptional responses

Integrative analysis of the most significant individual genes and canonical pathways extended the finding that a large and diverse set of genes related to T but also to NK lymphocyte activity is

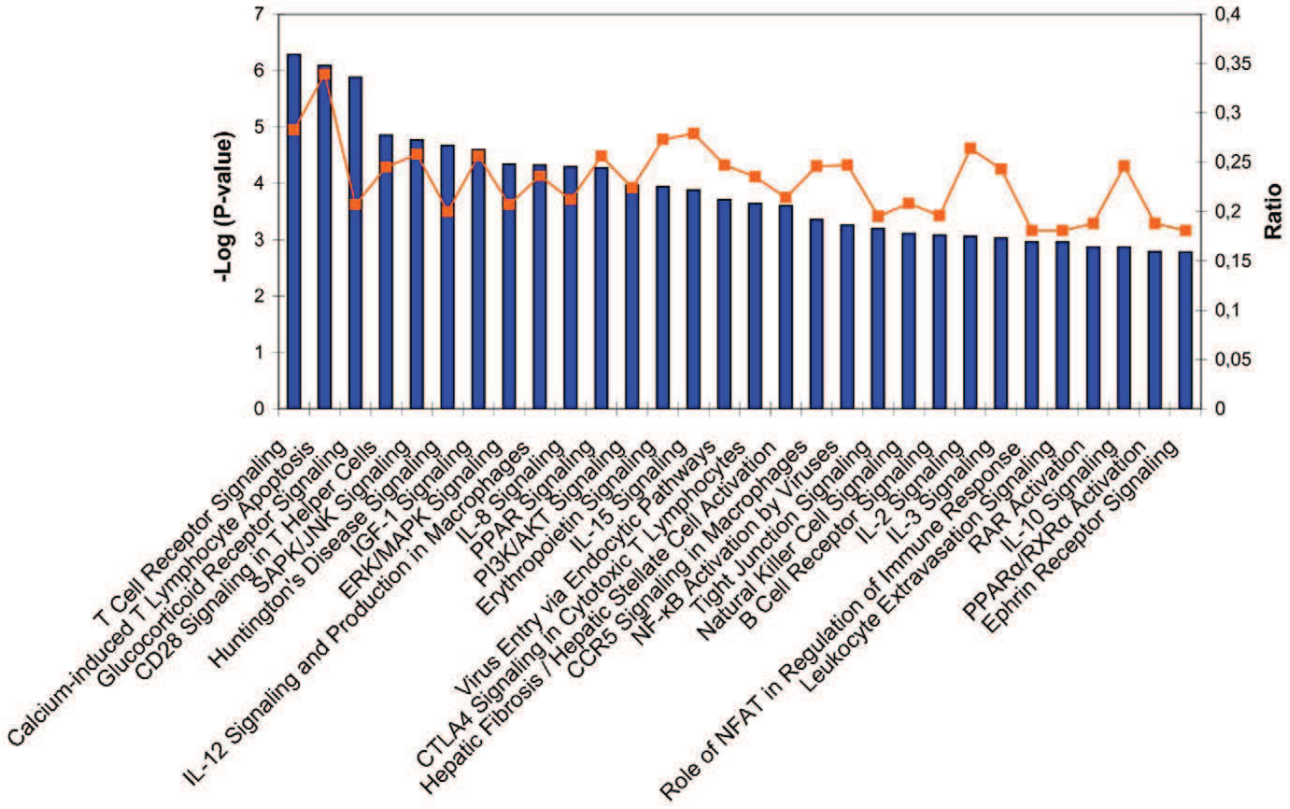

Figure 2. Top 30 canonical pathways identified from the DSS-gene signature using Ingenuity Pathway Analysis software. The significance of the association between data set and canonical pathway was estimated by the p-value (Fischer's exact test; left axis) and the ratio (right axis) of genes that maps to each canonical pathway.
doi:10.1371/journal.pone.0011671.g002

under-expressed in DSS patients compared to DF and DHF counterparts (Table 2; non exhaustive list; individual p-values available in Table S2; and Figure 3). Those genes are critical to a number of T and NK-cell functions, including T and NK cell differentiation, receptor signaling, activation and proliferation, cytotoxic functions or recruitment of lymphocytes to peripheral tissues. Since lymphocyte counts did not differ between the DF, DHF and DSS children ($p = 0.428$; Kruskal Wallis test), we searched whether genes encoding factors regulating negatively T and NK functions were over-expressed in the DSS-gene signature. We identified that the two genes having the strongest association with the disease phenotype variable, encode two major immunomodulatory factors, the microsomal prostaglandin E synthase (*PTGES*/Agilent clone number A-24-P478940) and the complement regulatory protein CRIg/VSIG4, considered potent negative regulators of T and NK cell responses [43–45]. The decreased abundance of *NFkB* signal transduction-related transcripts (Table 2), already reported in DSS patients by others [46], might be related to impaired expression of T and NK cell-related genes.

Our analysis also revealed that DSS whole blood cells from children over-expressed an enriched pattern of anti-inflammatory and repair/tissue remodeling genes (Table 3; non exhaustive list; individual p-values available in Table S2). Over-expressed anti-inflammatory genes identified encode molecules with diverse functions: the anti-inflammatory cytokine IL-10, a putative marker of severe dengue [11], serine proteases and metalloprotease inhibitors, IL-1β cytokine decoy receptor, free heme scavenger molecules, or complement-regulating receptors. Repair and remodeling genes over-expressed in the DSS-gene signature also encode a diversity of molecules: the MMP-9, a matrix metalloprotease with key role in tissue remodeling and a candidate to dengue plasma leakage [47], the extracellular matrix molecules fibronectin, versican and collagens, the angiogenin and VEGF [17] endothelial agonists as well as the arginase 1 repair enzyme, which competes with the endothelial NOS (*NOS3*) for L-arginine biodisponibility [48].

Thus, DSS children whole blood cells have a global decreased abundance of T and NK cell-related transcripts but an increased abundance of anti-inflammatory and repair/remodeling transcripts at the time of cardiovascular decompensation.

Pro-inflammatory innate defense and host-lipid metabolism-related transcriptional responses are activated in DSS children

When searching for pro-inflammatory gene patterns that may be relevant to DSS pathophysiology and particularly to systemic inflammation and vascular dysfunction, we identified three major pro-inflammatory gene patterns. Interestingly, all are related to innate defense and host lipid metabolism, and considered major pathogenic mechanisms in other systemic inflammatory diseases.

As shown in table 4 (non exhaustive list; individual p-values available in Table S2), the first one is defined by a set of over-expressed genes strongly associated with the disease phenotype subtype DSS that encode highly pro-inflammatory microbicidal peptides and enzymes. This pattern includes non exhaustively the alpha defensins DEFA1, DEFA3 and DEFA4, the cathelicidin (CAMP) and lactoferrin (LTF) peptides, the neutrophil enzymes

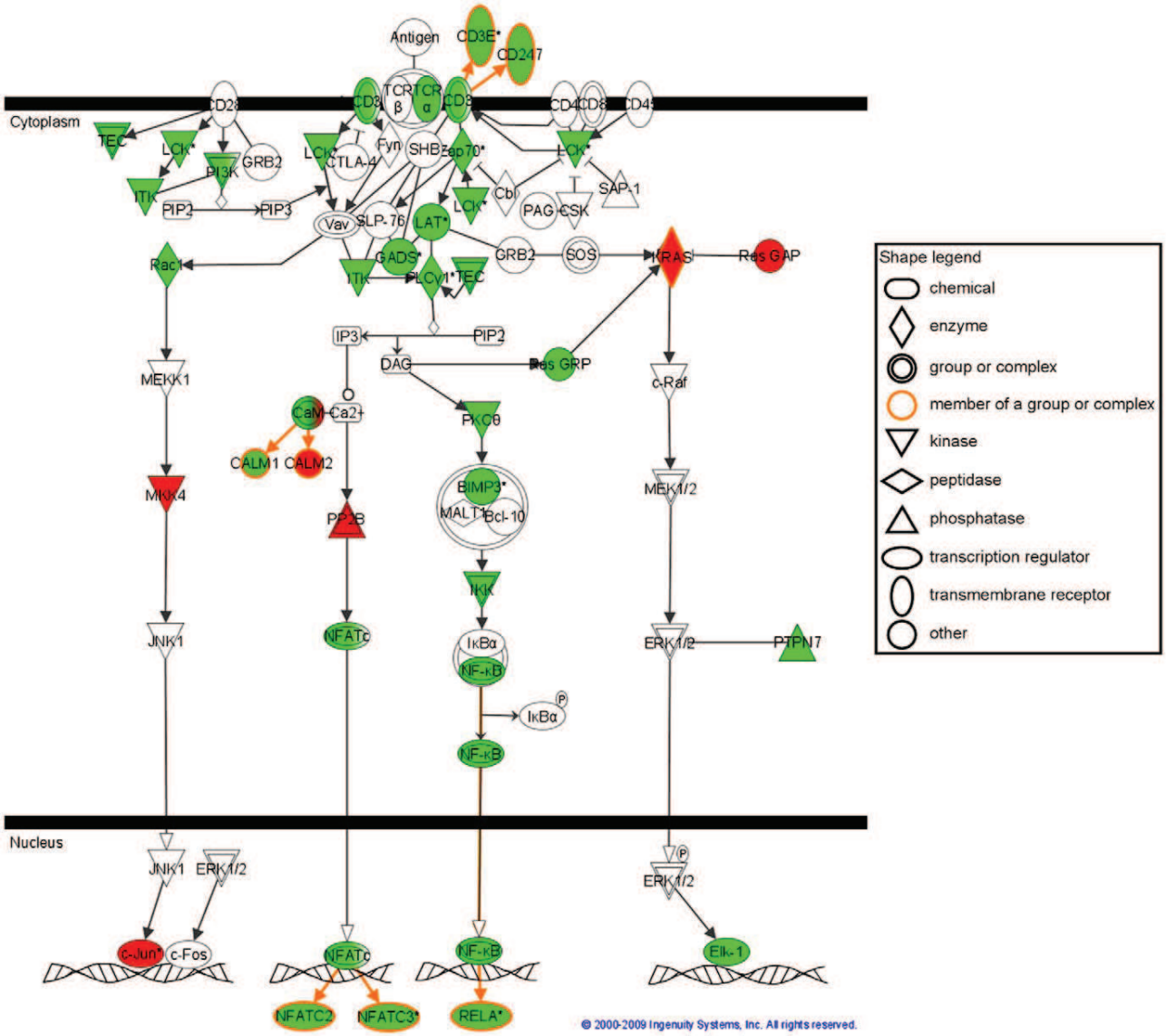

Figure 3. T Cell Receptor Signaling canonical pathway from Ingenuity Pathway Analysis. Genes in green and red are respectively under- and over-expressed in the DSS-gene signature. Genes in white are other genes present in the canonical pathway but absent from the DSS-gene signature. DSS: Dengue Shock Syndrome.
doi:10.1371/journal.pone.0011671.g003

myeloperoxydase (MPO), neutrophil RNASE2, RNASE3, cathepsin G and neutrophil elastase (ELANE). Transcripts encoding the potent pro-inflammatory calgranulins proteins S100A8/9 and S100A12, characteristic of granulocyte neutrophil activity [51] and involved in a diversity of inflammatory diseases [56], as well as the granulocyte-related metalloprotease MMP8 are also over-expressed. Increased abundance of those transcripts cannot be explained by increase in granulocyte count, since DSS patients have lower relative granulocyte counts than DF and DHF counterparts (median values/DF: 3900; DHF: 3950; DSS: 2500; p-value: 0.03; Kruskal Wallis test), thus reflecting more likely cellular activation. Altogether those results show that a transcriptional pattern of innate defense genes is activated in the whole blood of DSS children.

The second pro-inflammatory gene pattern identified is typical of altered homeostasis of cholesterol in monocytes/macrophages that characterises inflammatory lipid-laden monocytes/macrophages (lipid-laden Mo/Mac), a subtype of foam cells initiating vascular lesions in metabolic inflammatory diseases [57–59] (Table 5; non exhaustive list; individual p-values available in Table S2). Since the *PPARG* gene, which encodes a nuclear-lipid receptor involved in lipid signaling and lipid-homeostasis in inflammatory lipid-laden Mo/Mac [65], has a very strong association with the dengue disease phenotype, we searched whether other genes involved in cholesterol homeostasis in Mo/Mac had altered expression in the DSS-gene signature. Remarkably, we found a large lipid-laden Mo/Mac-related gene expression pattern characterized in particular by a higher abundance of transcripts encoding the key scavenger receptors of modified low density lipoproteins OLR-1, CD36 and MSR1, but a decreased abundance of transcripts encoding critical cholesterol transporters such as the NPC1 [66] or the ABCA1-

Table 2. T lymphocytes and NK cells-related genes present in the DSS-gene signature.

Function	Genes	P-value	Var.[a] (%)
Th1 differentiation	RUNX3, STAT4, TBX21	<0.00001 to 0.00242	25 to 42
Th2 differentiation	GATA3, STAT5A	0.00003 to 0.00225	17 to 32
Cytotoxic T lymphocyte functions	CTSW, PRF1	0.00005 to 0.00231	21 to 33
T lymphocyte activation	IL2RB, IL2RG	0.00014 to 0.00039	29 to 35
Cooperation with antigen-presenting cells	CD40LG	0.00105	21
Recruitment and interaction of T lymphocytes with endothelium	ITGAL, XCL1, XCL2	<0.00001 to 0.00214	20 to 33
Inhibitory NK cell receptors	KLRD1	0.00001	31
Activating NK cell receptors	NCR1, NCR3, CD160	<0.00001 to 0.00069	28 to 39
Cytotoxic molecules	GZMM	<0.00001	32
Receptors for NK cells homing to peripheral tissues	S1PR5	<0.00001	48
Differentiation factors of NK cells	FLT3LG, **IL15**, **IL17C**, KITLG	0.00088 to 0.00774	13 to 21
Suppression of T lymphocytes and NK cells response	**PTGES**, **VSIG4**	<0.00001	60 to 63
NFκB-related genes	**IRAK3**, TNIK, RELA, NFKBIB, TRAF1, TRAF2, TRAF6	0.00001 to 0.00506	9 to 31

HUGO gene names are indicated. When genes were represented by several clones on the microarray, p-value and variance medians were calculated. Genes in regular and bold are respectively under- and over-expressed in dengue shock syndrome patients.
[a]percentage of variance associated to disease phenotype.
doi:10.1371/journal.pone.0011671.t002

like ABCA10 [86], which regulate the efflux of modified cholesterol from Mo/Mac. Other lipid-laden cells-related genes also have altered expression in the DSS-gene signature. In particular, the *PPARA* gene that negatively regulates the formation of lipid-laden Mo/Mac [87] has decreased abundance in DSS patients. At the opposite, transcripts encoding the chitinase 1, a marker of pro-inflammatory lipid-laden Mo/Mac [73], and the FABP4, SOCS6, RETN and IRS2 proteins involved in lipid-laden Mo/Mac-induced insulin-resistance and compensatory response [68–72], have all increased abundance, also strongly supporting a biological signature of foam cells. Interestingly, the *PCSK9* transcript, which encodes a secreted protein that decreases the recycling of LDL to the liver by inducing the degradation of liver LDL receptors [74], is also over-expressed in the DSS signature and highly associated with the disease phenotype.

Thus, a gene expression pattern similar to that characterizing lipid-laden monocytes, is activated in the whole blood cells of DSS children at the time of cardiovascular decompensation.

The third pro-inflammatory gene pattern associated with the DSS-gene signature is characteristic of the metabolic pro-inflammatory arachidonic-acid pathway, one of the lipid metabolic pathways identified through IPA. As shown in table 5, the gene encoding the upstream cytosolic phospholipase

Table 3. Anti-inflammatory, tissue remodeling and repair genes present in the DSS-gene signature.

Function	Gene Symbol	P-value	Var.[a] (%)
Anti-inflammatory genes			
immunoregulatory molecules	**IL10**	0.00430	20
anti-proteases	**SERPINB2**, **SERPINB8**, **SERPINB10**, **SLPI**	<0.00001 to 0.00081	19 to 49
metalloproteinase inhibitor	**TIMP1**	0.00183	19
decoy receptor	**IL1R2**	0.00077	30
free-heme scavenger molecules	**CD163**, **HP**, **HMOX1**	<0.00001 to 0.00064	26 to 46
complement regulatory molecules	**CD55**, **VSIG4**	<0.00001 to 0.00096	24 to 60
Tissue remodeling and repair genes			
metallopeptidase	**MMP9**	0.00001	33
extracellular matrix components	**COL1A2**[b], **COL8A2**[b], **COL14A1**[b], **COL17A1**[b], **FN1**[b], **SDC1**[b], **VCAN**[b]	<0.00001 to 0.00309	18 to 34
pro-angiogenic factors	**ANG**, **VEGFA**	0.00004 to 0.00236	25 to 30
others	**ARG1**, NOS3	<0.00001 to 0.00054	18 to 44

HUGO gene names are indicated. When genes were represented by several clones on the microarray, p-value and variance medians were calculated. Genes in regular and bold are respectively under- and over-expressed in dengue shock syndrome patients.
[a]percentage of variance associated to disease phenotype.
[b]Danger-associated molecular pattern (DAMP) activity.
doi:10.1371/journal.pone.0011671.t003

Table 4. Pro-inflammatory innate immunity-related genes present in the DSS-gene signature.

Function	Gene Symbol	P-value	Var.[a] (%)	Main cellular origin	Ref
microbicidal peptides	**DEFA1**[b], **DEFA3**[b], **DEFA4**[b]	<0.00001 to 0.00007	0.25 to 0.44	PMN neutro, EpC	[49,50]
	CAMP[b]	<0.00001	0.34	PMN neutro, Mo, mast cells, EpC	[49,50]
	LTF[b]	<0.00001	0.41	PMN neutro, inflammed EpC	[50]
calgranulin proteins	**S100A8**[b], **S100A9**[b]	<0.00001 to 0.00014	0.18 to 0.38	PMN neutro, Mo/Mac	[51,52]
	S100A12[b]	<0.00001	0.33	PMN neutro	[51]
granulocyte enzymes	**RNASE2**[b]	0.00017	0.25	Mo/Mac, Eo, EpC, PMN neutro	[53]
	MPO[b]	0.00024	0.25	PMN neutro, Mo, subtypes of tissue Mac	[50]
	RNASE3[b]	<0.00001	0.29	Eo, Mo, PMN neutro	[54]
	MMP8	<0.00001	0.49	PMN neutro	[50]
	CTSG	<0.00001	0.36	PMN neutro	[50]
	ELANE	<0.00001	0.39	PMN neutro	[50]
pro-inflammatory cytokines and related molecules	**IL18**	0.00052	0.21	Kupffer cells, activated Mac, Mo, DC, EpC	[55]
	IL18BP	0.00710	0.20	T cells, peripheral blood leukocytes, EC	[55]

HUGO gene names are indicated. When genes were represented by several clones on the microarray, p-value and variance medians were calculated. Genes in regular and bold are respectively under- and over-expressed in dengue shock syndrome patients. DC, dendritic cell; EC, endothelial cell; Eo, eosinophil; EpC, epithelial cell; Mac, macrophage, Mo, monocyte; PMN neutro, polymorphonuclear neutrophil; RAGE, receptor for advanced glycation end products.

[a]percentage of variance associated to disease phenotype.

[b]Danger-associated molecular pattern (DAMP) activity.

doi:10.1371/journal.pone.0011671.t004

PLA2G4A, which is the initial rate-limiting enzyme that cleaves membrane phospholipids [78], is over-expressed. Similarly, most downstream key enzymes from the COX-2 and 5-LOX sub-pathways involved in the final synthesis, conversion and transport of inflammatory eicosanoids lipid mediators, are over-expressed. In particular, the transcript encoding the inducible microsomal prostaglandin E synthase PTGES that catalyzes the conversion of prostaglandin PGH2 to PGE2 in the COX-2 sub-pathway, and thought to play a pathogenic role in a number of inflammatory processes [88] is significantly increased and has the highest statistical association with the disease phenotype (62% of gene variance explained by the disease phenotype according to multi-way ANOVA). At the opposite the *PTGDS* transcript, which encodes the anti-inflammatory prostaglandin D2 synthase, has decreased abundance, a finding already reported in metabolic inflammatory processes [89]. Increased abundance of the transcript encoding the LTA4H enzyme that converts the LTA_4 leukotriene to LTB_4 reflects the activation of the 5-LOX sub-pathway. Finally, transcripts encoding the oxidative enzymes ALOX15B lipoxygenase [84] and cytochrome P450 epoxygenase family members [85], involved in the arachidonic acid metabolic pathway, are also significantly increased in the DSS-gene signature, also reflecting activation of those sub-pathways during DSS.

Thus, a transcriptional signature related to the lipid-related metabolic arachidonic acid pathway is activated in the whole blood cells of DSS children at the time of cardiovascular decompensation.

Discussion

Numerous studies have addressed the pathophysiology of DSS, the more frequent and severe complication of dengue infections. Despite important findings, only partial understanding of the cellular and molecular processes that may support this life-threatening syndrome has been obtained, and we still lack a comprehensive overview of the complete figure of alterations that contribute to – or reflect – the setting-up of the shock syndrome. This could allow the improvement of patients' management and treatment, a major challenge for clinicians. We designed a study aimed at analysing the quasi-global transcriptome of whole blood cells from dengue paediatric patients, looking at every modification that could make sense to the understanding of the pathogenic process. The capacity of such an exhaustive approach to identify relevant host responses, of which unsuspected pathways has been demonstrated in other systemic inflammatory syndromes such as human sepsis or post-trauma sterile SIRS [23–25]. We compared the transcriptome of blood cells from DSS paediatric patients at time of shock to those of children classified as DF or DHF grades I/II [29] matched for important variables such as age, gender, immune status towards dengue infection (primary or secondary infection) and time of disease evolution after onset of fever. Our study has produced significant results, further discussed in the context of DSS pathophysiology.

First, we identify a transcriptional signature of the DSS, differentiating DSS from the other forms of dengue infection and characterizing DSS as a unique and specific entity. Giving particular attention to study design and statistical analysis, we identify a large and robust gene expression profile of 2959 genes that discriminates DSS paediatric patients from other dengue patients, DF or DHF, who did not progress to shock, whatever the supportive treatment they received. Importantly, DSS children clustered together whatever they were considered as having primary or secondary dengue infection, while secondary infections represented the majority of DF, DHF and DSS children recruited (see table S1), as expected in hyper-endemic areas. The robustness of the DSS-associated gene signature was established by showing that the disease phenotype variable significantly affected expression levels of all the genes identified (multi-way ANOVA) and

Table 5. Pro-inflammatory lipid-related genes present in the DSS-gene signature.

Function	Gene Symbol	P-value	Var.[a] (%)	Disease	Ref
Lipid-laden Mo/Mac-related genes					
scavenger receptors of modified LDL in Mo/Mac	**OLR1**, **CD36**, **MSR1**	<0.00001 to 0.00013	0.21 to 0.32	metabolic diseases	[60–64]
lipid nuclear receptor/signalisation by lipids	**PPARG**, PPARA	0.00007 to 0.00732	0.21 to 0.34	metabolic diseases	[65]
efflux of modified cholesterol from Mo/Mac	NPC1	0.00005	0.32	Niemann-Pick disease, atherosclerosis	[66,67]
	ABCA10	0.00016	0.14	none	
migrating Mo/resident Mac chemokine receptors	**CCR2**, CX3CR1	0.00001 to 0.00099	0.22 to 0.40	atherosclerosis	[57]
other lipid-laden-related Mo/Mac genes	**FABP4**, **SOCS6**, **RETN**, **IRS2**	<0.00001 to 0.00092	0.20 to 0.26	metabolic diseases	[68–72]
	CHIT1	<0.00001	0.48	Gaucher's disease, atherosclerosis	[73]
	PCSK9	0.00001	0.42	familial hypercholesterolemia	[74]
	SPP1	<0.00001	0.49	metabolic and inflammatory diseases	[75,76]
anti-oxydant enzymes	LCAT, PAFAH2	0.00196 to 0.00461	21 to 26	metabolic diseases	[77]
Arachidonic acid pathway-related genes					
phospholipase	**PLA2G4A**	0.00003	0.21	rheumatoid arthritis	[78]
eicosanoid synthesis enzymes	**PTGES**, **LTA4H**, PTGDS, **TBXAS1**, PTGDR	<0.00001 to 0.00123	0.22 to 0.63	metabolic and inflammatory diseases, asthma, cancer	[79–82]
leukotrienes convertion enzyme	**MGST2**	0.00003	0.32	none	
leukotriene transporter	**SLCO2B1**	0.00010	0.31	asthma	[83]
lipid oxidation	**ALOX15B**	0.00011	0.33	atherosclerosis	[84]
cytochrome P450 superfamily enzymes	**CYP1B1**, CYP2U1, **CYP51A1**	<0.00001 to 0.00686	10 to 32	Vascular inflammation	[85]

HUGO gene names are indicated. When genes were represented by several clones on the microarray, p-value and variance medians were calculated. Genes in regular and bold are respectively under- and over-expressed in dengue shock syndrome patients.
[a]percentage of variance associated to disease phenotype.
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demonstrating the classifying capability of this gene signature using unsupervised hierarchical clustering and SVM/leave-one-out methods [35,39,40]. Based on unsupervised hierarchical clustering, DHF grades I/II patients' expression profiles appear very close or indistinguishable from those of DF patients at the same time of disease evolution, while they group into two heterogeneous sub-groups (1 and 2b, Figure 1), of which significance should be investigated. Altogether, the present results highlight the inadequacy of the 1997 WHO classification of dengue clinical forms [19–21], that considers DF and DHF grades I/II as two separate disease phenotypes, and support the recently proposed classification [90].

Two important questions arise about the DSS-associated transcriptional profile: are the observed modifications of genes expression the cause or the consequence of the pathology, and could these modifications have a predictive value? We cannot definitively answer these questions from the present study, in part because blood samples were collected at the onset of shock (14 out of the 19 DSS patients) or after (5 patients). Functional study of each individual pathway will be required to fully understand the role of each gene in a complex network of molecular interactions.

The ability of some genes transcripts or genes products to accurately predict progression to DSS should be evaluated by multivariate regression models [91] using blood samples collected before the onset of shock, while this proves to be difficult in the context of dengue outbreaks [28]. In the present study, we chose to focus on those of the identified molecular mechanisms that made the more sense to DSS pathophysiology and systemic vascular dysfunction, referring to recent findings on the role of innate immunity in systemic inflammatory processes leading to shock, multi-organ dysfunction syndromes or other pejorative clinical outcomes.

Second, while present results confirm some putative DSS-related biomarkers, it also reveals unreported alterations that make sense to hypovolemic shock pathophysiology. This reinforces the ability of a global and "open mind" approach to identify molecular processes relevant to the studied pathology. Blood cells transcriptional profiles clearly reveal alterations of different immune responses and the activation of a large pro-inflammatory response. A significant proportion of genes of which expression is modified are related to host innate immunity, lymphocyte functions and lipid metabolism in particular. This genome-wide expression analysis also confirms the over-expression of individual biomarkers previously associated with severe dengue, such as the acute phase pentraxin-related protein PTX3, the pro-inflammatory IL-18 cytokine or the anti-inflammatory IL-10 cytokine (Table S2) [11,12,15], providing a more comprehensive overview of their implication in the pathophysiology of DSS.

Our results differ however from those reported by Long *et al* in a genome-wide expression profiling study comparing DSS children with uncomplicated paediatric patients [28]. This study concluded on a global "benign" and "muted" immune transcriptional response but a decreased expression of genes involved in IL-10 and IFN type I-related pathways in DSS children blood cells [28].

Differences in study design, size of cohorts and time of blood sampling from patients in the course of dengue disease may explain these differences. Indeed, in our study, two DSS children had gene expression profiles close to those of uncomplicated DF and DHF, and clustered within the DF/DHF cluster. Both proved to be the children from whom blood was sampled three days after the onset of shock, while the three DSS children sampled two days after shock onset still exhibited a typical DSS gene expression profile. This suggests that a shift from a "severe" to an "uncomplicated" transcriptional profile may occur within a very short time, and could explain the "uncomplicated" and benign gene immune transcriptional responses reported by Long *et al* [28]. Differences in strategies and methods used to filter genes differentially expressed between patients' groups could also explain the finding that few IFN type I-related genes but a large diversity of other pathways were identified in the present study compared to other transcriptomic studies of DHF or DSS patients. Here, genes were selected considering only their statistical significance and their association with the disease phenotype. Differently from others [27,28,42], no "fold change" cut-off filter was applied since this non-statistically-motivated criteria selects preferentially genes prompt to high variations such as the IFN type I-induced genes [92,93], thus excluding from subsequent bio-informatic analysis a diversity of transcripts exhibiting more subtle variations but strong associations and biological relevance with the considered disease phenotype.

Third, unsuspected mechanisms identified in DSS patients could contribute importantly to the pathophysiology of this severe syndrome, as supported by similarities between those DSS-related alterations and other critical syndromes. Interestingly, a number of immune, repair-remodeling and metabolic-related related pathways are simultaneously altered in the blood cells of DSS children at the onset of shock. In particular, T and NK lymphocyte transcriptional responses are globally impaired while genes implicated in compensatory anti-inflammatory and repair/remodeling responses and in innate immune responses are over-expressed. This highlights the complexity of biological responses at the time of dengue shock syndrome, and points out similarities between DSS and other critical syndromes such as severe sepsis, or post-trauma SIRS that are similarly characterized by depressed T lymphocyte responses but over-expressed innate immunity [94,95].

Reduced abundance of a number of T-lymphocyte related transcripts at the time of DSS may reflect a feed-back mechanism aimed at limiting an initial early T lymphocyte activation, reported to occur in patients who further progress to severe dengue [96,97]. Such a negative feed-back may be sustained by the over-expression of a diversity of anti-inflammatory transcripts in DSS patients' blood cells at the time of shock. In particular, the two potent immunomodulating factors prostaglandin E synthase and VSIG4, which dampen both T and NK lymphocyte responses [43–45] and have both a strong statistical association with the DSS phenotype, could have such a negative effect. Based on those observations and previous clinical reports, the benefit of corticotherapy in DSS patients might thus be questioned [98,99].

Over-expression in the blood of DSS children of several repair and remodeling genes encoding extracellular matrix proteins, vasoactive mediators and matrix metalloproteases such as the MMP9, likely reflects a compensatory response to inflammatory insults, and a number of those genes products are now considered putative biomarkers in systemic inflammatory syndromes such as severe sepsis [100]. Most proteins encoded by those genes are indeed secreted by activated immune cells such as monocytes/macrophages. They may have adverse effects towards the vascular endothelium when produced in excess, since they may increase immobilization of inflammatory mediators at the surface of endothelial cells [101], permeability of capillaries [102], or induce direct damage to endothelial tissues [103]. Recently, one of them, MMP9 has been proposed as a putative candidate in the occurrence of plasma leakage during dengue infection [47].

While previous transcriptional studies failed to identify pro-inflammatory gene patterns in the blood cells of DSS patients [27,28,42], our study is the first one to report that a diversity of pro-inflammatory transcriptional responses at the interface of innate immunity, inflammation and host lipid metabolism are activated at the time of cardiovascular failure. Since those mechanisms are considered pathogenic in other systemic inflammatory diseases where systemic vascular dysfunction does occur, we suggest that they may altogether contribute to DSS pathophysiology.

Activation of a pro-inflammatory defence gene pattern in DSS patients' blood cells (Table 4) has relevance to the pathophysiology of systemic vascular dysfunction, since most microbicidal peptides and enzymes have recognized pro-inflammatory and pathogenic effects towards vascular endothelial tissues [50]. Among them, the neutrophil microbicidal peptides alpha defensins and the highly pro-inflammatory calgranulins proteins S100A8/A9 and S100A12 are now considered putative pathogenic factors in sepsis, cardiovascular diseases, rheumatoid arthritis or atherosclerosis [51,56,104]. While neutrophils are considered the main source of those defence molecules, this cellular origin cannot be established from the present study due to the cellular complexity of unfractionnated whole blood samples and to the possibility that other circulating cell types may express a neutrophil-like inflammatory repertoire under pathologic conditions [105]. A putative neutrophil origin of this gene expression pattern is however supported by the over-expression in DSS patients blood cells of transcripts encoding other neutrophil-related molecules such as the MMP8 matrix metalloprotease, CEACAM-6, CEACAM-8 and CD99L2 adhesion molecules (Tables 4 and S2) involved in the recruitment of neutrophils to vascular endothelia. Functional studies should confirm whether those first line-defence immune cells which produce an array of pro-inflammatory mediators highly damaging to host tissues and vascular endothelia [50,106], and poorly regarded in dengue [107], definitively play a role in DSS pathophysiology.

Alteration of a gene pattern related to homeostasis of cholesterol in monocytes/macrophages (Mo/Mac) in the blood cells of DSS children (Table 5) was an unexpected finding, while it should be considered regarding recent knowledge on the role of monocytes as a pivotal link between inflammation, innate immunity and host lipid metabolism [108,109]. Indeed, under physiological conditions, monocytes maintain cholesterol homeostasis by clearing modified LDL such as oxidized LDL (ox-LDL) from plasma. Under pathological conditions, balance between uptake and efflux of those modified cholesterol molecules may be altered [59,60,62,66,67,86], resulting in the intracellular accumulation of modified cholesterol. This turns classical monocytes towards a pro-inflammatory phenotype, lipid-laden monocytes/macrophages (Mo/Mac), a sub-type of pro-inflammatory immune cells initially identified in vascular lesions of chronic inflammatory metabolic diseases [110].

Recent knowledge has shown that those atypical monocytes produce a large array of pro-inflammatory mediators such as ROS, metalloproteases, eicosanoids, and pro-inflammatory adipokines, making these cells potent contributors to vascular damages, systemic inflammation and major metabolic changes

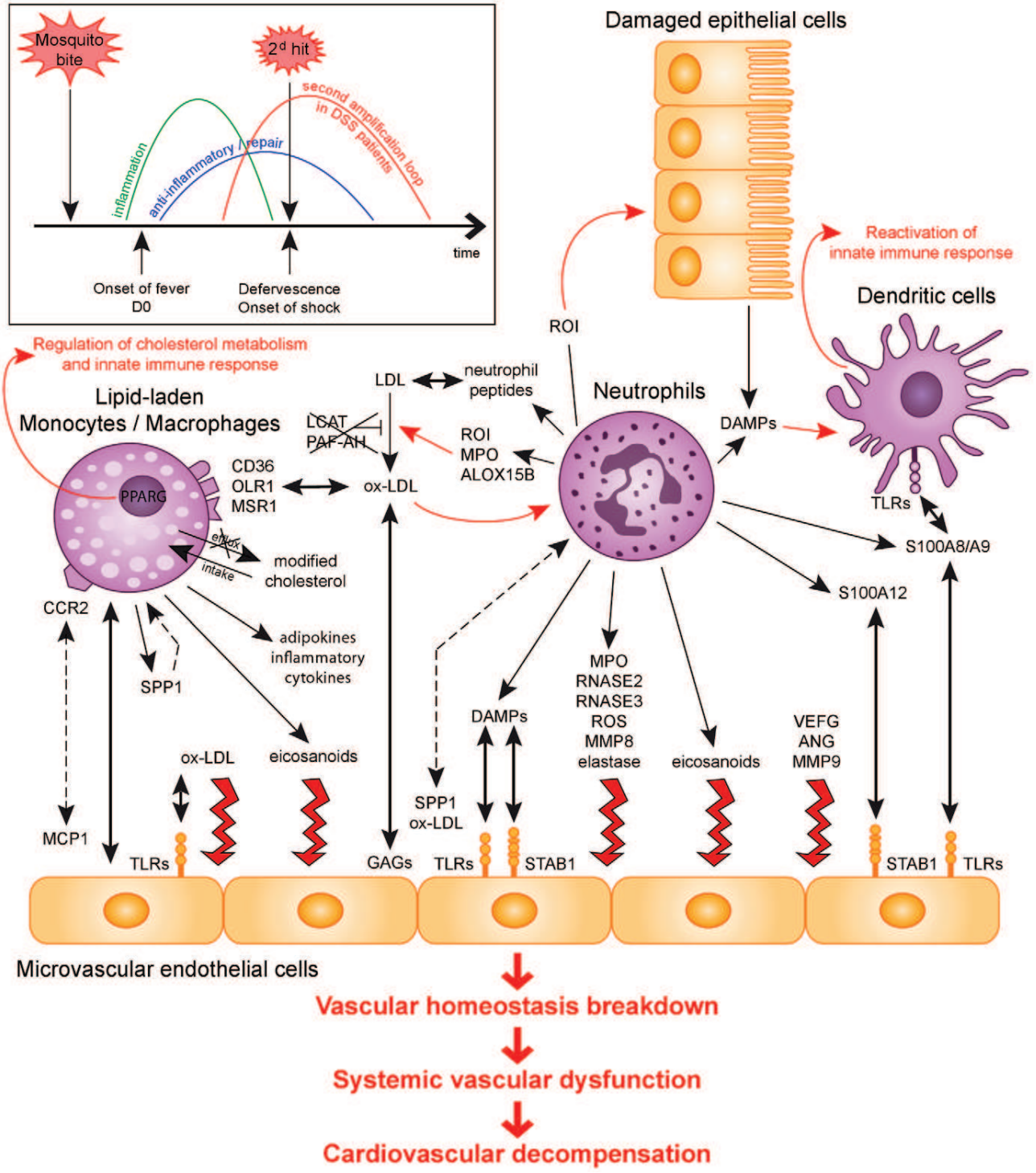

Figure 4. Hypothesis of a second inflammatory amplification loop in dengue shock syndrome. After induction of a first inflammatory and anti-viral response to dengue virus, disease resolution generally occurs around time of defervescence for most dengue-infected patients. Some patients however progress towards a life-threatening dengue shock syndrome. Results obtained in this study suggest that in those patients, a second inflammatory amplification loop, which involves a diversity of pro-inflammatory responses related to innate immunity, occurs and leads to a major inflammatory systemic syndrome and to vascular homeostasis breakdown. The putative role of different markers identified in vascular endothelial dysfunction is indicated. Thin black arrow, release of; Bold black arrow, interaction between; Punctuated black arrow, chemotactic effect; Thin red arrow, biological activity; Bold red arrow, direct activity on endothelium. DAMPs, danger-associated molecular pattern; GAG, glycosaminoglycane; ROI, reactive oxygen intermediates; TLR, Toll-like receptor.
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such as insulin-resistance [109,111], which altogether characterize systemic inflammatory syndromes such as DSS or severe sepsis. While the existence of functional lipid-laden Mo/Mac during DSS should be established by functional studies, such a molecular mechanism could explain decrease of circulating sub-fractions and total cholesterol previously reported in DSS [112–115] and in other critically ill patients where low cholesterol levels are associated with poor clinical outcome [116,117].

Altered homeostasis of cholesterol in blood cells from DSS patients could also favour replication of dengue viruses into host cells [118], thus contributing to increased viremia in patients with severe dengue infection [119,120], while this could not be evaluated in this study since part of patients had undetectable viremia at the time of blood sampling.

The factors contributing to altered homeostasis of cholesterol in the blood cells of DSS children at time of shock are numerous. Increased lipid peroxydation activity [113,114] insufficiently compensated by anti-oxydant mechanisms [121], as supported by related altered gene patterns identified in this study (Table 5), may result in high levels of circulating ox-LDL contributing to altered cholesterol metabolism. Differences in nutritional status [122–124] or host genetics may also contribute to altered homeostasis of cholesterol gene pattern in the blood cells of DSS patients. Interestingly, transcripts encoding molecules considered candidates to diseases characterized by impairment of cholesterol homeostasis such as *NPC1*, *PCSK9* and *PPARG* [66,67,74], have significant altered abundance in the blood cells of DSS children (Table 5). Further investigations should consider possible associations between DSS and allelic variants of such genes. Whatever the determinants of cholesterol metabolism alterations in DSS patients, our results reinforce interest in considering sub-fractions and total cholesterol as putative biomarkers of DSS [115]. They also suggest that drugs used to treat metabolic disorders such as atherosclerosis should deserve further attention for the control of such a pro-inflammatory process in dengue-infected patients, now proposed for other critical illnesses [117].

Transcriptional activation of the lipid-related arachidonic acid pathway in the whole blood cells of DSS children at the time of shock was another pro-inflammatory mechanism relevant to the pathophysiology of DSS [81]. Activation of this lipid metabolic pathway in innate cells such as neutrophils or lipid-laden monocytes during inflammatory process or infection [125], results in the production of eicosanoid lipid mediators, that are not only physiological regulators of vascular tone and permeability [81] but also potent pro-inflammatory mediators involved in a number of pathologies such as asthma [81]. Interestingly, formation of lipid bodies where eicosanoid synthesis takes place, can be induced by ox-LDL through activation of the PPARγ nuclear-lipid receptor [126], thus suggesting a direct link between the three pro-inflammatory pathways identified in DSS children and a contribution of arachidonic pathway-related inflammatory lipids and oxidative enzymes to the systemic vascular dysfunction leading to DSS.

Fourth, DAMPs and TLRs could be a link from primary to secondary inflammation, leading to DSS. Occurrence of DSS in only some patients at the late phase of infection is likely due to an inadequate control or an amplification of the primary inflammatory response aimed at fighting infection. The pro-inflammatory molecular responses activated in the blood cells of DSS children at time of shock involve a diversity of innate immune mediators that may amplify a first-line inflammatory response mediated by TNF, IL-6 or IL-1, thus contributing to a secondary inflammatory loop. Indeed, a number of repair/remodeling and of defence gene products over-expressed in DSS patients blood cells are considered endogenous danger signals or Danger-Associated Molecular Patterns (DAMPs) (Tables 3 and 4; molecules with DAMP activity are indicated) capable to trigger secondary systemic inflammatory responses through direct interaction with surface or intracellular receptors such as TLRs or NODs expressed in endothelial or innate immune cells [127]. DAMPs include a diversity of molecules without structural similarity either actively produced by immune cells in the context of an infection or passively secreted by damaged tissues [128,129], now considered key inducers of secondary systemic inflammation in a number of acute inflammatory syndromes [130,131] or chronic diseases [132].

Amplification of inflammation during DSS through direct signalling by molecules harbouring DAMP activity via TLRs, is also supported by the increased abundance of DAMP-induced transcripts as those encoding the pro-inflammatory IL-18 cytokine or the NLRC4/CARD12 intracellular sensor [55]. Interestingly, association of allelic polymorphisms of TLR4 with DSS suggested by De Kruif and colleagues [46] suggests that differential signalling through TLRs may contribute to the severity of dengue disease, as suspected for other pathologies [133]. Accordingly, anti-inflammatory drugs targeting Toll-like receptors are now under development for a number of inflammatory pathologies where innate immunity and TLRs play a central pathogenic role [134].

DSS pathophysiology: a secondary inflammatory loop hypothesis

To summarize, we report the identification of a specific gene expression profile in the blood cells of DSS children at time of shock, characterizing DSS as a unique entity at the transcriptional level whatever the immunological status of children regarding primary or secondary infection. Major immunological alterations identified at the time of shock are characterized by an altered balance between depressed T lymphocyte responses and exacerbated compensatory and pro-inflammatory innate immune responses that may, finally, be detrimental to the host [135–137], while functional studies should confirm the contribution of those molecular mechanisms to DSS pathophysiology.

Based on recent knowledge on molecular mechanisms altered in other systemic inflammatory diseases, DSS may result from a complex pro-inflammatory network involving a diversity of innate immune effectors sustaining a secondary systemic inflammatory loop, leading in turn to vascular homeostasis breakdown and systemic microcirculatory failure characterizing DSS (Figure 4).

We suggest that drugs available to treat metabolic and other systemic chronic inflammatory diseases could be considered for the treatment of dengue-infected patients before shock occurs, and that a number of bio-markers found altered in DSS patients blood cells should be evaluated. as putative predictive markers of progression to DSS.

Supporting Information

Figure S1 Validation of microarray results by RT-PCR. Pearson's correlation was calculated between microarray expression signals (horizontal axis) and Delta Ct values from real-time PCR (vertical axis) for nine genes highly associated to dengue shock syndrome. ** Correlation is significant at 0.01.
Found at: doi:10.1371/journal.pone.0011671.s001 (4.94 MB TIF)

Table S1 Clinical and biological characteristics of each DF, DHF and DSS patient
Found at: doi:10.1371/journal.pone.0011671.s002 (0.04 MB XLS)

Table S2 List of the 3515 clones corresponding to the 2959 genes differentially expressed between DF, DHF and DSS

patients, identified using the multi-way ANOVA at a false discovery rate of 10. Clones corresponding to the 2959 genes are listed according to their association to DSS, the first one being the gene of which expression level variance is the most influenced by the clinical phenotype. HUGO gene names are indicated. The variation is the one related to the DSS group relatively to DF and DHF. ANOVA, analysis of variance; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; NA, not available. a percentage of variance associated to disease phenotype.

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Author Contributions

Conceived and designed the experiments: SD CS VD PB PCP. Performed the experiments: SD CS PCP. Analyzed the data: SD CS AB PR PCP. Contributed reagents/materials/analysis tools: VD SO PTL NC SN PB PCP. Wrote the paper: SD PCP. Contributed to obtainment of funding: HT.

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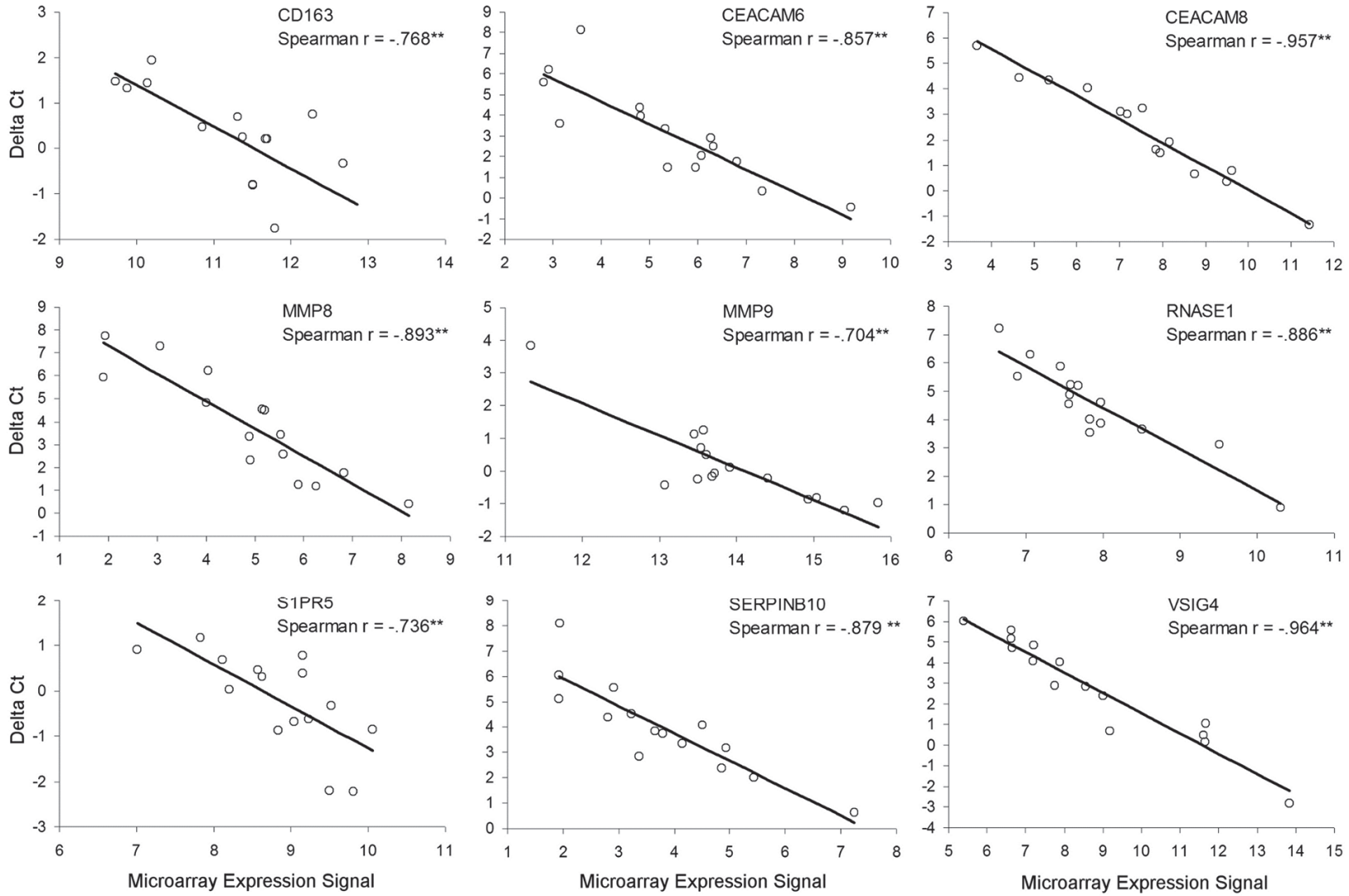

Figure S1 Validation of microarray results by RT-PCR

Table S1. Clinical and biological characteristics of each DF, DHF and DSS patient

	PL002	PL004	PL005	PL007	PL009	PL017	PL020	PL022	PL024	PL025	PL026	PL027	PL028
Patient characteristics													
severity	DF	DF	DSS	DSS	DHF	DSS	DF	DF	DSS	DHF	DSS	DF	DHF
gender	male	female	male	male	female	male	male	male	female	female	female	female	male
age, years	7	3	11	15	13	6	9	10	9	7	9	9	8
weight, kg	14	13	23	38	35	15	20	20	20	16	26	20	17
day of hospital admission after onset of fever	1	0	4	4	3	3	4	2	5	2	4	1	1
day of shock after onset of fever	N.A.	N.A.	4	?	N.A.	4	N.A.	N.A.	5	N.A.	4	N.A.	N.A.
day of blood sampling after onset of fever	6	5	7	4	4	6	5	6	5	5	5	4	5
Dengue status													
viral serotype	DEN-2	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-2	?	DEN-3
immunological status	secondary	secondary	secondary	primary	primary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary
Clinical manifestations													
Tourniquet test	neg	pos	pos	pos	neg	pos	pos	pos	?	pos	neg	neg	pos
hepatomegaly	neg	pos	pos	pos	neg	pos	pos	neg	pos	pos	pos	neg	pos
gastro-intestinal tract bleeding	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
gingival bleeding	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
Blood pressure													
heart frequency, pulse per minute	130	110	100	90	100	130	132	106	0	112	0	96	112
pulse pressure, mm Hg	40	30	20	30	30	30	40	50	10	30	10	30	30
Haematological parameters													
platelet count	?	164000	91000	86000	73000	?	48000	251000	31000	30000	12000	?	?
hematocrit, %	?	35	36	38	36	?	35	38	40	50	41	?	?
hemoconcentration (hematocrit >20%)	neg	neg	pos	neg	neg	pos	neg	neg	pos	pos	pos	neg	neg
white blood cells, number/mm3	?	6200	3100	5100	4100	?	4200	8000	25000	?	3300	?	?
neutrophils, number/mm3	?	2900	2201	2448	2747	?	2300	5300	10200	?	1600	?	?
lymphocytes, number/mm3	?	2400	620	2142	984	?	1400	1700	5200	?	1400	?	?
Supportive medical care													
oxygen supplementation	neg	neg	pos	neg	neg	pos	neg	neg	pos	neg	pos	neg	neg
perfusion of colloid (dextran 40)	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos (bolus)	pos	pos
perfusion of human plasma	neg	neg	neg	neg	neg	pos	neg	neg	pos	neg	neg	neg	neg
day of perfusion of plasma after onset of fever	N.A.	N.A.	N.A.	N.A.	N.A.	4	N.A.	N.A.	5	N.A.	N.A.	N.A.	N.A.

N.A., not applicable ; Neg, negative ; Pos, positive ; ?, unknown

Table S1. Clinical and biological characterist

	PL029	PL033	PL037	PL038	PL039	PL043	PL045	PL046	PL047	PL048	PL049	PL055	PL056
Patient characteristics													
severity	DSS	DSS	DHF	DF	DSS	DSS	DHF	DSS	DSS	DSS	DF	DSS	DHF
gender	female	female	female	female	male	female	female	female	female	male	female	female	female
age, years	4	8	8	7	10	4	7	8	8	7	8	13	6
weight, kg	12	23	18	15	23	10.5	15	19	17	15	18.5	23	15
day of hospital admission after onset of fever	4	0	3	1	4	6	3	4	4	3	1	3	2
day of shock after onset of fever	4	4	N.A.	N.A.	4	6	N.A.	4	4	3	N.A.	3	N.A.
day of blood sampling after onset of fever	4	6	7	5	5	7	4	5	6	4	5	4	6
Dengue status													
viral serotype	DEN-3	?	DEN-1	DEN-1	?	?	DEN-3	DEN-3	?	DEN-3	DEN-2	?	DEN-3
immunological status	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary
Clinical manifestations													
Tourniquet test	?	pos	pos	neg	pos	neg	pos	pos	?	?	pos	?	pos
hepatomegaly	pos	neg	pos	neg	pos	pos	neg	pos	pos	pos	neg	neg	neg
gastro-intestinal tract bleeding	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
gingival bleeding	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Blood pressure													
heart frequency, pulse per minute	0	120	100	120	0	0	120	120	120	0	?	86	112
pulse pressure, mm Hg	15	50	20	50	20	15	40	20	40	10	30	20	40
Haematological parameters													
platelet count	39000	187000	165000	395000	21000	74000	84000	34000	29000	13000	190000	47000	148000
hematocrit, %	38	35.8	38.1	36.9	46.8	36.8	37.5	39.7	45.5	45	39	44	38.1
hemoconcentration (hematocrit >20%)	neg	pos	neg	neg	pos	pos	neg	pos	pos	pos	neg	pos	neg
white blood cells, number/mm3	10200	13200	6600	10900	9300	7200	6400	9200	8200	4400	8100	6900	6500
neutrophils, number/mm3	5600	11600	4200	8300	3400	1900	6400	4700	2600	2200	5500	2200	4000
lymphocytes, number/mm3	4600	1600	700	1500	2800	4200	3900	2800	3400	1500	2100	2500	1800
Supportive medical care													
oxygen supplementation	pos	pos	neg	neg	pos	pos	neg	neg	neg	pos	neg	pos	neg
perfusion of colloid (dextran 40)	pos (bolus)	pos (bolus)	pos	pos	pos (bolus)	pos (bolus)	pos	pos (bolus)	pos (bolus)	pos	pos	pos (bolus)	pos
perfusion of human plasma	neg	pos	neg	neg	pos	neg	neg	neg	neg	pos	neg	pos	neg
day of perfusion of plasma after onset of fever	N.A.	5	N.A.	N.A.	4	N.A.	N.A.	N.A.	N.A.	3	N.A.	?	N.A.

N.A., not applicable ; Neg, negative ; Pos, positi

Table S1. Clinical and biological characterist

	PL058	PL059	PL060	PL063	PL064	PL065	PL068	PL070	PL074	PL075	PL076	PL078	PL080
Patient characteristics													
severity	DHF	DHF	DSS	DF	DF	DHF	DF	DHF	DF	DSS	DF	DF	DHF
gender	female	female	male	female	female	male	female	female	male	female	female	male	male
age, years	14	4	7	9	7	4	4	3	3	8	8	3	6
weight, kg	29	12	14	20	17	15	12	11.5	12.5	16.6	19	11	14
day of hospital admission after onset of fever	2	2	5	1	4	3	2	3	2	3	3	2	3
day of shock after onset of fever	N.A.	N.A.	6	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	3	N.A.	N.A.	N.A.
day of blood sampling after onset of fever	5	4	6	6	4	7	3	4	3	4	5	7	4
Dengue status													
viral serotype	DEN-4	DEN-3	?	DEN-3	DEN-3	DEN-3	DEN-3	DEN-3	DEN-1	DEN-3	DEN-1	DEN-3	DEN-2
immunological status	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	primary	secondary
Clinical manifestations													
Tourniquet test	neg	pos	pos	pos	pos	pos	neg	neg	neg	neg	pos	pos	?
hepatomegaly	neg	neg	pos	neg	pos	pos	neg	pos	neg	pos	neg	neg	pos
gastro-intestinal tract bleeding	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
gingival bleeding	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Blood pressure													
heart frequency, pulse per minute	120	130	100	136	100	120	124	120	120	0	100	?	120
pulse pressure, mm Hg	30	40	20	40	50	60	30	30	40	10	40	50	30
Haematological parameters													
platelet count	153000	30000	35000	248000	57000	?	503000	124000	253000	21000	322000	210000	33000
hematocrit, %	42	39.5	67.4	39.8	33.1	42	36.9	41.2	36.1	44.8	29.1	28.7	48.5
hemoconcentration (hematocrit >20%)	neg	pos	pos	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
white blood cells, number/mm3	6200	12000	6000	6600	18600	?	13300	6300	3300	4800	9900	6600	7400
neutrophils, number/mm3	3900	6400	3800	3900	7600	?	8700	3900	1100	2500	8400	3700	3500
lymphocytes, number/mm3	1500	5400	2200	1600	5200	?	2700	1600	1600	1600	700	2100	2100
Supportive medical care													
oxygen supplementation	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg
perfusion of colloid (dextran 40)	pos	pos	pos (bolus)	pos	pos	pos	pos	pos	pos	pos (bolus)	pos	pos	pos
perfusion of human plasma	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg
day of perfusion of plasma after onset of fever	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	3	N.A.	N.A.	N.A.

N.A., not applicable ; Neg, negative ; Pos, positi

Table S1. Clinical and biological characterist

	PL082	PL089	PL091	PL094	PL095	PL098	PL101	PL107	PL114
Patient characteristics									
severity	DHF	DSS	DSS	DF	DSS	DHF	DSS	DF	DF
gender	male	female	male	male	female	female	female	female	male
age, years	5	7	8	10	7	6	9	14	4
weight, kg	14	20.5	24	26	14	18	17	40	10
day of hospital admission after onset of fever	2	4	6	2	4	2	2	3	4
day of shock after onset of fever	N.A.	4	6	N.A.	4	N.A.	2	N.A.	N.A.
day of blood sampling after onset of fever	3	5	6	6	5	6	5	5	7
Dengue status									
viral serotype	DEN-3	?	DEN-1	DEN-4	DEN-3	DEN-3	DEN-3	DEN-3	DEN-1
immunological status	secondary	secondary	secondary	secondary	secondary	secondary	secondary	secondary	primary
Clinical manifestations									
Tourniquet test	neg	neg	?	pos	neg	neg	neg	neg	neg
hepatomegaly	neg	pos	pos	neg	pos	neg	pos	neg	neg
gastro-intestinal tract bleeding	neg	pos	pos	neg	neg	neg	neg	neg	neg
gingival bleeding	neg	neg	neg	neg	neg	neg	neg	neg	neg
Blood pressure									
heart frequency, pulse per minute	128	0	0	116	0	100	76	102	100
pulse pressure, mm Hg	40	10	10	40	0	30	40	40	30
Haematological parameters									
platelet count	190000	19000	25000	?	26000	49000	?	198000	260000
hematocrit, %	40	40.8	45	42	45.2	38.6	55	39.1	36
hemoconcentration (hematocrit >20%)	neg	pos	pos	pos	pos	neg	pos	neg	neg
white blood cells, number/mm3	14000	4900	4100	?	9500	4500	?	5500	3900
neutrophils, number/mm3	10900	2100	2500	?	3800	1800	?	3700	2200
lymphocytes, number/mm3	1900	1200	1400	?	3200	1800	?	1200	1300
Supportive medical care									
oxygen supplementation	neg	pos	pos	neg	pos	neg	pos	neg	neg
perfusion of colloid (dextran 40)	pos	pos (bolus)	pos (bolus)	pos	pos (bolus)	pos	pos (bolus)	pos	pos
perfusion of human plasma	neg	pos	neg	neg	neg	neg	neg	neg	neg
day of perfusion of plasma after onset of fever	N.A.	4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

N.A., not applicable ; Neg, negative ; Pos, positi

CONCLUDING REMARKS

All the work accomplished in this thesis came from data collected in NDCP mainly since the introduction of virological surveillance in 2001 and also within in the DENFRAME project which was conducted between 2006 and 2007. The results produced in this thesis covered different aspects of dengue infection and are one of the few works on dengue that have been performed in Cambodia. The main findings would be able to contribute as a piece of information and knowledge on dengue in term of epidemiology, virus evolution, clinical presentation and factors contributing to disease severity. The main objective of this work was therefore to address the problem of dengue in a comprehensive and integrative way and to provide the first global set of data since the introduction of dengue surveillance in Cambodia and based on reliable sources of data.

The results reported in this work have shown that Cambodian children become exposed to DENV at the very early age (<1 year) and this age group along with children between 4-6 years are the most affected. This finding could have a double impact on prevention programs: (1) the first dose of potential future dengue vaccine should aim at children at the age when they become susceptible to dengue infection. Previous studies have shown that dengue disease in infants less than six months old is infrequent indicating that passively transferred maternal antibodies can protect the infant (Chau et al., 2009; Pengsaa et al., 2006); (2) secondly, the high incidence of dengue in children less than 6 years old indicates that the transmission occur locally and more importantly around households where intervention measure should be emphasized.

Although dengue vector control interventions (based on temephos) have been introduced since 2002, the incidence of dengue remains unchanged in majority of studied districts (162/185). With a highly underreported dengue incidence by NDCP (3.9 to 29 -fold lower than found in a capture-recapture study by Vong et al. (Vong et al., 2011)), dengue continues to be a huge public health problem in Cambodia and affects in majority population residing in rural area with limited income. Other alternative intervention program should be examined such as *Wolbachia* which has been proved to be very successful (Hoffmann et al., 2011; Jeffery et al., 2009; McMeniman and O'Neill, 2010). Biological control using *Bacillus thuringiensis israelensis* (BTI) has also proven very efficient in mosquito control worldwide over the last 30 years. This environmentally-friendly product is extensively used in America and is the only vector control product now authorized in Europe. A

large diversity of formulations, already registered and commercially available exist and this approach should also be investigated (Després et al., 2011; Lacey, 2007)

All DENV serotypes are circulating each year with a predominance shift between DENV-2 and DENV-3 observed since 1998. Although there are limited virological data before 2001, it seems that the 2 large epidemics in 1998 and 2007 were all caused by DENV-3 coinciding with the serotype change. Whether DENV-3 is more virulent than other serotypes is unclear and further genomic or experimental studies need to be done.

With the precious collection of DENV strains collected in various sentinel sites which participated in NDCP and with the help of bioinformatic tools, we were able to study the evolutionary process of DENV and trace back history in the past 12 years. Based on phylogenetic analysis, different genotypes or lineages of DENV (genotype 1 for DENV-1, Asian/American and Asian 1 for DENV-2, genotype II for DENV-3 and genotype 1 for DENV-4) have been found in circulation and replacement and extinction events have been observed in the 4 serotypes. The up-to-date data on genotypes or lineages in circulation might help in decision of strains to include in vaccine production and in anti-viral drugs strategy when available. Tracing back time to the most common ancestor, it has been shown that DENVs in Cambodia have quite recent ancestors and probably with a Thai origin. Intriguingly, it was frequently observed that Cambodian DENVs clustered with either Thai or Vietnamese viruses depending on serotype. It is very indicative of an important human population and/or mosquitoes' movements between these 3 countries. This could be useful information that should be taken into consideration in prevention program.

Our result could draw some hypothesis that may assist in shaping the evolutionary dynamics of our DENV strains: introduction of a probably better fitness new genotype (Asian 1) which became predominant serotype in 2004, stochastic process caused by large scale flood in 2000 and consecutive drought afterward and herd cross-immunity protection induced by big outbreak mainly caused by DENV-3 in 2006-2007. Although we could not infer with certainty what had really happened, but we were able to demonstrate that we could trace back the history and provide plausible hypothesis with simple blood sample and bioinformatic tools.

Lastly, it has been demonstrated that recombination or positive selection most likely were not involved in the evolutionary process of DENV in Cambodia.

This work is one of the few studies that provide thorough description of the broad spectrum of dengue disease including unapparent DENV infection in Latin America and in Southeast Asia.

Among patients presenting dengue-like illness, two thirds of patients from Latin America and one third from Southeast Asia were not related to DENV. This figure underscores the importance to provide new early diagnostic tools especially in dengue highly endemic countries to rule out other undifferentiated fever etiologies like malaria, rickettsiosis, typhoid fever, leptospirosis or yellow fever and other hemorrhagic fever diseases. The study has also highlighted that some routine biological figures such as low platelets, neutrophils and high haematocrit and liver enzyme level could be helpful to differentiate dengue from others non-dengue diseases. Another interesting aspect of this study was the similarity in laboratory findings (except lymphocyte counts and positive NS1 antigen detection) between symptomatic and unapparent dengue-infected subjects. Again, this observation is in concordance with that in Chapter 2 and supports the significance of further work on the DENV phenotype and genetic differences and host genetic predisposition. DENV virulence need to be further studied and will be discussed in the perspective chapter of this manuscript.

DENV NS1 antigen has been the center of interest in diagnostic test development as well as in pathogenesis of severe dengue. In diagnostic, NS1 antigen capture test was reported to have high sensitivity and specificity. However, in our hand, its overall sensitivity was moderate and varied greatly in relation to day after fever onset, virus serotype, primary or secondary infection, dengue severity and level of viraemia. Nonetheless, the significantly high sensitivity (similar to that of genome detection) of the NS1 and IgM combination is an encouraging result. These two diagnostic tools are easy to perform and require less sophisticated equipment. Additionally, a rapid diagnostic test using both NS1 and IgM detection could provide early dengue diagnosis that can be performed as a bedside point-of-care diagnostic tool. This could have a huge impact in dengue patient's management. Clinician can timely initiate appropriate treatment and reduce unnecessary antibiotic's prescription. Commercial forms of this combined rapid test have been developed and evaluated by only a couple of studies but preliminary results are promising.

In asymptomatic cases, NS1 antigen capture was less sensitive compared to PCR method. Intriguingly, NS1 antigen and viraemia level in some of these cases were as high as those in symptomatic patients. This contradictory finding to previous dogma shown that high viraemia does not always associate with more severe dengue and supports the hypothesis of the contribution of virulence phenotype of DENV and/or host genetic predisposition or immune response. Better characterization of DENV virulence requires further study and will be discussed in perspective chapter of this thesis. Along the same line, our results showed that low level of viral

load and NS1 antigen at day 4 to 8 after onset of fever increased the likelihood of developing a severe form of the dengue infection. In dengue, day 4-5 after onset of fever correspond to the critical phase when patients progress to either full recovery or to severity. The semi-quantitative ELISA NS1 antigen capture could be used as dengue diagnosis as well as prognostic marker of progression to severity. The early rapid decrease of NS1 level during critical phase can drive clinician attention to closely monitor potentially severe patients.

DHF/DSS are severe complication forms of dengue infection and are the main cause of mortality in dengue due to lack of specific antiviral treatment and vaccines. A genome-wide expression study has illustrated some significant expression profiles of genes that were specific to DSS. Over 2950 genes have been identified and this DSS-associated transcriptional profile leads to the major questions of whether the gene expression profiles are the cause or consequence (drug target) of the pathology and whether they have a predictive value (prognostic marker). The four main findings in this work are: (1) the gene signature expression showed that DF and DHF grades I/II are not two separate disease phenotypes and this result supports the new WHO 2009 dengue cases classification. Dengue disease should be considered as a dynamic disease (not a clear distinct entity); (2) some putative biomarkers related to DSS were identified such as activation of pro-inflammatory response involving over-expression of PTX3, IL-18 IL-10 cytokines. However, this severe transcriptional profile may return to normal or uncomplicated within a very short time underscoring the benign (not destructive) nature of pro-inflammatory response in dengue; (3) a number of immune cells such as T and NK lymphocytes were impaired while gene associated with anti-inflammatory and repair/modeling were over-expressed. (4) DAMPs and Toll-like Receptors (TLR) were attributed in DSS via secondary systemic inflammatory loop, leading to vascular leakage.

With all these results, we can suggest that available or future drugs that treat metabolic and other systemic chronic inflammatory diseases should be considered for early treatment of dengue-infected patients before shock occurs. A number of bio-markers associated to DSS should be evaluated as putative predictive markers of progression to DSS.

PERSPECTIVE

The results reported in this thesis have opened several ways for future investigations to be carried out in the Virology Unit at Institut Pasteur in Cambodia (IPC). Dengue is a major issue in term of public health as well as economic burden in Cambodia, a highly dengue endemic country. IPC as the National Reference Center for Arboviruses and an expert laboratory for WHO/TDR has close collaborations with NDCP, local hospitals and other national institutions. This strong network connection and a recognized expertise have contributed to obtain new grants for 3 projects that have already begun or that are about to be initiated. I will be involved as co-PI in all the studies that will be carried out at IPC. The future works will put particular interest in identifying prognostic markers of severe dengue infection including the role of NS1 antigen, host genetic predisposition, viral phenotype and genetic diversity of DENV in human (experiencing apparent and unapparent dengue infection) and in mosquitoes.

1. Transversal Program of Research (PTR): Host-viral interaction in the outcome of dengue infection

The PTR began in mid-2011 and will be completed in late 2012 or early 2013. This project does not require new patient recruitment but relies uniquely on well characterized biobank samples collected during previous European project (DENFRAME).

It is estimated that the majority of infections with DENV (50-90%) remain asymptomatic (Kyle and Harris, 2008). To date, the reasons why some patients develop clinically apparent forms or death are unknown. The major hypothesis behind this question might involve host genetic factors, virological factors and most likely a combination of these two factors. We propose to study both virological and host factors as well as their interactions and their effects on the severity of dengue. Our methodological approach is to analyze the sequences of the DENV using deep sequencing method using Illumina technology (at sub-populations/quasispecies level) isolated from patients with different clinical expressions of disease, from asymptomatic to fatal circulatory shock. The sequences are generated in Institut Pasteur in Paris (IPP) from the samples that we have provided and we will collaborate to the analysis to study the role of minor virus populations in the pathophysiology. The second part of the project is to analyze the expression of the entire genome of dengue patients as well as asymptomatic individuals living in the same household. Our collaborator at IPP will use Affymetrix chips for the analysis of human mRNAs in order to compare

the gene expression in matched individuals living in the same household (same exposure factors) having biological relationship but with very different clinical expressions (asymptomatic versus apparent clinical form).

The Illumina sequencer should generate about 15,000 virus genomes per sample, allowing thus the identification of variants present even in small copy numbers. The comparison of the distribution of mutations observed between viruses obtained from asymptomatic patients and those isolated from patients who have clinically apparent forms can identify point mutations that are either associated with the severity of the disease or are the result of differences in host response due to different genetic predisposition. The mutations identified in this way must then be reintroduced into the infectious cDNA to generate virus stocks that allow for phenotypic characterization *in vitro* (cell culture) or *in vivo* (mosquitoes). Along the same line, the phenotypic characterization of different DENV isolates will help to validate and to reproduce on a larger scale and in Cambodia the results that we published with Tuiskunen A. (using virus strains from Cambodia) showing that DENV isolated from patients with different clinical forms could be phenotypically and genetically characterized *in vitro* as well as in experimental studies in mice (Tuiskunen et al., 2011a; Tuiskunen et al., 2011b). The mutations identified could be then eventually be used as potential targets for antiviral drugs or for producing attenuated vaccine strains. The "Transcriptome and Epigenome" platform will be in charge, with the geneticists from “viral pathogenesis unit”, to perform analysis of human mRNA obtained from symptomatic and asymptomatic patients. The analysis would allow the identification of host genetic factors involved in disease severity. Once the analysis of virological and human genomic data completed, the results will be confirmed by us in a study in Cambodia of the corresponding proteins expressed in the serum of patients with a Luminex platform. This could also lead to the identification of blood markers of severity and possible new therapeutic targets.

The knowledge on quasispecies analysis obtained in this project will complement the work that will be done in another European project (DENFREE) which is described below.

2. European project DENFREE

This program has been accepted for funding by the European Union for a period of five years starting from 1 January 2012. IPC assures coordination in one of 8 work-packages that will be carried out in Cambodia but our laboratory is strongly involved in most of the work-packages.

The main objective of this multidisciplinary project is to identify the key factors involved in dengue transmission in order to develop new tools and strategic approaches for better control of the disease. In particular, it is expected to generate models to estimate the potential risk of DENV spreading in areas not yet affected, but where the vector exists, such as Europe. This requires the development of powerful diagnostic tools and easy to use for field studies on human and mosquitoes populations. It also requires better surveillance and the ability to diagnose asymptomatic cases that could be a reservoir for the virus. However, the exact role of asymptomatic subjects is unknown and it is not known, for example, whether they are able to effectively infect mosquitoes. To answer the main question, several approaches will be conducted:

1) **Development of new tools for better diagnosis and viral surveillance**
   - Development of ultra-sensitive diagnosis to detect virus in biological samples.
   - Development of a rapid “dipstick” test to detect anti-DENV antibodies.
   - Development of a multi-epitope antigen array “chip” to correlate the clinical outcome and presence of neutralizing antibody to DENV induced immune responses.
   - Field testing of patented mosquito trap for surveillance
2) **Prediction and Prevention of spread of dengue in context of climate change**
   - Studies of various scenarios for DENV propagation in endemic areas in response to climate change
   - Seasonal forecasts for the disease in target regions
   - Micro-scale risk factor analyses
   - DENV transmission dynamic models
   - Identification of viral genetic markers determining susceptibility to European vectors
3) **Viral and host factors contributing to pathogenesis/predictors of disease severity**
   - Identification of human susceptibility genes determining outcome of dengue infection
   - Identification of viral factors associated with outcome of infection
   - Identification of bio-markers of host-viral interaction associated with the outcome of infection
4) **Prepare for further development of new vaccines, antiviral compounds and more targeted treatment schemes.**
   - Development of a panel of monoclonal antibodies for vaccine candidates
   - Test for pharmacokinetics, toxicities, control of viral replication and immunological consequences of a potential anti-DENV agent.

The project DENFREE revolves around a total of 8 work packages (WPs). Our unit at IPC works with 6 WPs detailed below:

- **WP 1:** This work package encompasses a multi-centric study of fine-scale dengue epidemiology across sites of differing endemicity and extent of urbanization. Symptomatic index dengue case identified will be used and tacked to enable house-hold level epidemiological study required for asymptomatic cases detection. House-specific factors, co-habitants, mosquitoes, geo-spatial and environmental parameters will be measured. The objectives in which we participate will be: 1) to characterize local DENV transmission patterns through cluster analyses of dengue index cases, 2) to identify asymptomatic infections for mosquito transmission studies (in WP5), 3) To establish empirical mosquito, human density and geo-spatial data for use in fine-scale and agent-based simulation models (in WP4), and 4) to establish a biobank of biological samples from patients, household members and mosquito vectors for further studies in other WPs, 5) to test novel diagnostic and prognostic tools developed by WP 2 in the field. All these tasks will be performed in the field sites and I will be the co-PI of the research conducted in Cambodia.
- **WP 2:** This WP will focus on development of new tools such as the development of a rapid "dipstick" test to detect anti-DENV antibodies, development of a multi-epitope antigen array to correlate the clinical outcome and presence of neutralizing antibody to DENV-induced immune responses and development of an ultra-sensitive tool to detect virus in biological samples. Our participation will focus on the evaluation of new tests in the field and on well characterized samples in our biobank and collaboration in the development of the immunoassay chip.
- **WP 3:** Our contribution in this WP is to provide biological and entomological data needed to build models by experts in this field for epidemiological model of dengue for future implementation of a seasonal climate prediction system.
- **WP 5:** This work package will specifically examine the role of mosquito vectors in dengue emergence, with special emphasis on the European context, as well as to evaluate an innovative vector control tool which could be applied to reduce risk of dengue infection at the household level. The Virology Unit, with team of entomologists, will help measure the role of asymptomatic individuals in the transmission of dengue by mosquitoes, to identify

the genetic polymorphism associated with a higher capacity of transmission in European populations of mosquitoes, and to evaluate new tools for surveillance and vector control.

- **WP 6:** This WP will be coordinated by Virology Unit at IPC and aims to understand the evolution of DENV during two consecutive epidemics, to study the evolution variations in vector and in human host, and to determine if specific mutations and quasi-species are selected in mosquito and humans. To address these issues, we will analyze the quasi-species found in asymptomatic subjects and compare them with those of patients expressing various clinical forms of the disease within the same family (biological link), will study the evolution of sub-viral populations found in mosquitoes during epidemics and during inter-epidemic periods, try to identify genetic polymorphism associated with a better transmission in populations of European vectors infected experimentally, characterize the possible role of minority viral subpopulations in the pathogenesis and attenuation of virulence in vitro and *in vivo* and analyze the spatial and temporal evolution of the virus circulating in humans and mosquitoes.
- **WP 7:** This WP will try to identify major immunological determinants influencing the course of the disease. We will contribute to the isolation of new monoclonal antibodies from the patients' blood using cell sorter from fresh peripheral blood mononuclear cells of patients (more than 200 will be needed) and to the characterization of the immunological response in asymptomatic subjects (T response, cytokine profile, etc.). Our work will then allow teams from Imperial College to study cross-reactivity of antibodies, to map the epitopes of DENV, and to perform some functional studies.

3. Research project on predictive markers of severity in dengue infection

This project is part a larger collaborative program on dengue that is being established between Biomérieux (BM) and Institut Pasteur. This project is funded by BM who is interested in studying the prognostic markers that can predict the evolution of infection to severe disease which require patient's hospitalization for optimal therapeutic management. This project was launched since September 2011 and is due to end in its first phase in 2013.

A specific cohort, followed over 3 years, comprising 120 hospitalized patients admitted for various forms of dengue (from DF to DHF/DSS) will be recruited. Well characterized biological samples (plasma, saliva and urine) and clinical data will be collected. Firstly, we will study the cellular and humoral immune response of sequential sampling to address several research topics of interest to

our unit including study of NK and DC response in DF and DHF/DSS patients, measure the kinetics of NS1 protein and the virus or its RNA in saliva and urine, and determine urinary markers of severity associated with disruption of the endothelial barrier. Secondly, the well characterized plasma bank will be used for downstream studies such as proteomic screening and NS1 ligands characterization aiming at the identification of potent markers of disease severity that will allow a reliable prognosis within the first days of hospitalization. The proteomic and NS1 ligands analysis will not be conducted in Cambodia. Briefly, the proteomic screening will characterize the protein differences DF and DHF/DSS sera at defervescence period (critical phase) and 3 mains proteins will be studied: plasma proteins (antibodies excepted), the antibodies, and the virion proteins and their interactions with the host. The identification and characterization of NS1 aims to identify proteins that bind specifically to the DENV NS1 protein in vitro and to further characterize the nature of the NS1-protein complex in solution which is believed to play a critical role in DENV pathogenesis. Relevant interactions will then be analyzed in patient sera and the prognostic value of candidate protein complexes assessed on large panels of biobank collected at IPC.

RESUME EN FRANÇAIS

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Pour obtenir le grade de
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LA DENGUE AU CAMBODGE:

EPIDEMIOLOGIE, EPIDEMIOLOGIE MOLECULAIRE, PRESENTATIONS CLINIQUES, DIAGNOSTIC DE LABORATOIRE ET MARQUEURS DE LA SEVERITE

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sous la direction de Dr Philippe BUCHY et Dr Thierry DUPRESSOIR

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INTRODUCTION

Le virus de la dengue (DENV) appartient au genre *Flavivirus* dans la famille des *Flaviviridae* qui compte également deux autre genres : *Pestivirus* et *Hepacivirus* (Calisher and Gould, 2003). L'infection des individus par un des 4 sérotypes de DENV cause la maladie de la dengue. Le virus est transmis par la piqûre de moustiques diurnes infectés. *Aedes aegypti* est considéré comme le vecteur principal de la dengue et se retrouve en abondance en milieu urbain et péri-urbain des pays d'endémie. *Aedes albopictus*, un vecteur secondaire, se trouve plutôt dans le milieu rural et péri-urbain et a été incriminé dans le passage du cycle selvatique au cycle endémique de la dengue en Asie (Rudnick, 1986).

L'infection par le virus de la dengue se présente sous forme asymptomatique dans la majorité des cas (50-90%) (Kyle and Harris, 2008). Cependant, l'expression clinique de l'infection peut être très variable allant d'une simple fièvre non spécifique (DF) aux formes sévères avec des hémorragies (DHF) ou accompagnée d'un syndrome de choc (DSS) qui peut mettre en jeu le pronostic vital (WHO, 2009). A l'échelle mondiale, la dengue est l'arbovirose la plus répandue avec environ 55% de la population humaine exposée au virus et plus de 100 pays affectés dans le monde (WHO, 2009). Selon une estimation de l'Organisation Mondiale de la Santé (OMS), environ 50 millions de cas d'infections pourraient se produire chaque année avec 500.000 cas de formes sévères causant le décès du patient dans au moins 2,5% des cas (WHO, 2009). La forme hémorragique de la dengue a été décrite pour la première fois dans les années 1950, lors d'épidémies aux Philippines et en Thaïlande (Hammon et al., 1960; Quintos et al., 1954). Elle est aujourd'hui trouvée dans presque tous les pays d'Asie et d'Amérique du Sud où elle est une cause importante d'hospitalisation ; ainsi, la dengue est considérée comme une maladie émergente en plein développement et pose un grand problème de santé publique mondiale.

Jusqu'à présent, il n'existe ni traitement spécifique ni vaccin contre la dengue. Dans la pratique quotidienne, dans les pays en voie de développement, les médecins font face à deux grands problèmes dans la prise en charge des cas de dengue. Tout d'abord, ils expriment le besoin d'un test de diagnostic fiable, rapide et peu coûteux permettant de détecter l'infection dans la phase précoce de la maladie. Ensuite, ils déplorent le manque d'outils ou de marqueurs biologiques permettant de prédire la progression de la dengue classique vers une forme sévère. En effet, de tels outils aideraient les cliniciens à améliorer la prise en charge clinique des cas de dengue, à réduire l'utilisation inutile d'antibiotiques et à limiter l'hospitalisation des patients atteints d'une dengue bénigne. Ces outils sont essentiels dans les pays à ressources limitées et à forte incidence

de la dengue comme le Cambodge mais aussi dans d'autres pays en Asie du Sud-Est et en Amérique du Sud.

La dengue a été détectée pour la première fois au Cambodge en 1963 (Chastel, 1963) et ce n'est qu'en 1980 que le programme national de surveillance de la dengue a été mis en place. En 2000, l'Institut Pasteur du Cambodge (IPC) a débuté sa collaboration avec le programme national de lutte contre la dengue (PNLD) en proposant un volet de surveillance virologique des arboviroses. Malgré plus de 30 ans d'existence du PNLD et bien que la dengue soit hyper-endémique et l'une des premières causes de morbidité et de mortalité chez des enfants cambodgiens, de nombreux éléments devaient encore être étudiés : amplitude des épidémies, profils de transmission, impact du programme national sur la maladie, dynamique d'évolution virale, virulence des souches circulantes, etc.

Ce travail de thèse utilise des données cliniques et virologiques du PNLD et du projet DENFRAME, programme de recherche s'appuyant sur un consortium de 13 institutions avec comme objectif de développer des outils de diagnostic et de nouvelles approches thérapeutiques afin d'améliorer la prise en charge de la dengue (www.denframe.org).

CHAPITRE 1 : EPIDEMIOLOGIE DE LA DENGUE AU CAMBODGE

Contexte et objectifs de l'étude

Malgré une création déjà ancienne remontant à 1980, la surveillance nationale n'a été opérationnelle qu'à partir des années 1990. La déclaration des cas de dengue est obligatoire au Cambodge et repose principalement sur une définition clinique qui s'applique à des enfants nécessitant une hospitalisation. En 2000, le système de surveillance a été renforcé par la mise en place d'une surveillance hospitalière active dans 5 sites sentinelles (hôpitaux nationaux) répartis dans tout le Cambodge. Parallèlement, une surveillance virologique et sérologique a été proposée. Ce Centre National de Référence analyse des prélèvements randomisés et recueillis à l'admission et à la sortie de l'hôpital chez environ 10 % des patients hospitalisés pour une suspicion de dengue. Les données démographiques et cliniques sont également recueillies sur un formulaire simple contenant des informations essentielles à la classification des cas de dengue (selon la classification de l'OMS 1997). De plus de la surveillance active, des mesures de contrôle vectoriel ont été introduites avec une distribution biannuelle de larvicides en ciblant les endroits où existe une forte densité humaine.

En se basant sur ces données, l'objectif de ce travail était d'évaluer sur une période de 28 ans la dynamique et le profil de transmission de la dengue et d'évaluer l'impact du programme de lutte anti-vectorielle mis en place depuis 2001 sur l'incidence de la dengue. En outre, ce travail devait permettre de mettre à la disposition des autorités sanitaires nationales des données de surveillance qui pourraient être utilisées comme éléments d'information auxquels se référer.

Résultats et conclusions

Entre 1980 et 2008, plus de 194000 cas suspects de dengue ont été rapportés au PNLD. La dynamique de transmission est caractérisée par une endémicité, une transmission qui perdure toute l'année avec une période épidémique durant la saison des pluies (entre mai et novembre). Deux épidémies majeures ont été rapportées en 1998 et en 2007. Les incidences ajustées à l'âge étaient de 0,7 à 3,0 pour 1000 habitants et les taux de mortalités variaient entre 0,7 et 1,7% des cas. La proportion des cas graves (DHF/DSS) était en moyenne de 45%. Il est à noter que les épidémies de 2006 (60,6%) et 2007 (54,2%) étaient caractérisées par une proportion remarquablement élevée de formes graves causées principalement par le sérotype DENV-3.

La dengue touche particulièrement deux tranches d'âge : les moins de 1 an et les enfants âgés de 4 à 7 ans. Ce résultat souligne l'importance de la transmission péri-domestique des enfants à l'âge

préscolaire. Par comparaison avec des pays voisins, la dengue survient chez des enfants plus âgés en Thaïlande (11 – 14 ans) (Kongsomboon et al., 2004) et âgés entre 7 et 14 ans au Viet Nam (Thai et al., 2011). En Indonésie, Malaisie et à Singapour, la dengue touche principalement les adultes (Ooi et al., 2006; Porter et al., 2005). Nos résultats pourraient néanmoins être biaisés par le fait que l'âge des patients inclus dans la surveillance est limité à 16 ans.

Sur un nombre moyen de 715 échantillons sanguins prélevés chaque année chez des patients suspects de dengue, 87.8 % d'entre eux étaient positifs en sérologie et 70% par RT-PCR pour l'ARN viral. Ces résultats montrent que des médecins ont une bonne connaissance de cette maladie endémique. Bien que les 4 sérotypes du virus co-circulent chaque année en même temps, les sérotypes DENV-2 ou DENV-3 se sont toujours avérés prédominants avec un changement de sérotype tous les 3-4 ans. Il apparait que ces changements de prédominance de sérotypes ont coïncidé avec la survenue d'épidémies majeures (1998 et 2007). Ce phénomène a été également observé en Thaïlande où le sérotype DENV-3 a été responsable des épidémies majeures de 1987 et 1998 (Nisalak et al., 2003).

La lutte anti-vectorielle, qui utilise le larvicide organo-phosphoré téméphos, ainsi que des campagnes d'informations via les médias ont eu un peu d'impact sur l'incidence de la dengue depuis leur mise en œuvre en 2001. Ceci montre qu'il est temps de change la mesure de lutte par des alternatives plus performantes et plus efficaces comme *Bacillus thuringiensis israelensis ou Wobachia* qui a été prouvés efficace contre des moustiques.

Les dynamiques de circulation des 4 sérotypes de dengue observées dans cette étude soulèvent des questions sur les facteurs présidant à ces fluctuations. Grâce à l'exploitation de la bibliothèque d'échantillons collectés dans le PNLD, le travail décrit dans le chapitre 2 permet d'explorer ce profil d'évolution à l'aide d'outils bioinformatiques.

CHAPITRE 2 : EPIDEMIOLOGIE MOLECULAIRE DE LA DENGUE

Contexte et objectif de l'étude

Les données virologiques à long terme montrent que la prévalence des 4 DENV oscille d'une épidémie à l'autre et que seuls DENV-2 et DENV-3 sont alternativement des sérotypes prédominants. Les prévalences de DENV-1 et DENV-4 sont faibles avec de petits pics d'incidence coïncidant avec la période de changement de sérotypes prédominants (Figure 19 ; page 61).

Avec les progrès du séquençage et l'amélioration des performances des outils bioinformatiques, de nombreuses études ont tenté de mieux comprendre la dynamique d'évolution des DENV en utilisant des données épidémiologiques et moléculaires. Plusieurs hypothèses ont été proposées pour expliquer ces fluctuations : (1) l'existence d'une sélection positive (Twiddy et al., 2002a; Twiddy et al., 2002b), (2) celle d'une sélection naturelle induite par la différence de « fitness » (Vu et al., 2010), (3) la survenue d'évènements stochastiques (Sittisombut et al., 1997; Wittke et al., 2002), (4) les effets de la protection par une immunité croisée dans la population humaine (Adams et al., 2006; Wearing and Rohani, 2006) ou (5) des évènements de recombinaison (Holmes et al., 1999; Tolou et al., 2001).

Malgré la découverte du DENV il y a déjà presque 50 ans, on sait encore relativement peu de choses sur l'évolution moléculaire et l'épidémiologie du virus au Cambodge. En utilisant des souches de DENV du PNLD et de l'IPC, nous présentons la première étude qui s'intéresse à l'évolution moléculaire des 4 sérotypes de DENV au Cambodge sur une période de 12 ans. Dans cette étude, nous voulons expliquer comment les DENV ont évolué, pourquoi il y a eu des changements de sérotypes prédominants tous les 3 ou 4 ans, et vérifier si nos DENV ont subi des pressions de sélection ou des évènements stochastiques ou encore de recombinaison génétique.

Résultats et conclusion

L'analyse phylogénétique a permis d'identifier les génotypes de DENV circulant au Cambodge : génotype I pour DENV-1, génotype « Asiatique/Américain » et « Asiatique 1 » pour DENV-2, génotype II pour DENV-3 et génotype 1 pour DENV-4. Ces génotypes sont proches de ceux qui circulent dans les pays voisins (Klungthong et al., 2004; Schreiber et al., 2009; Vu et al., 2010; Zhang et al., 2005). Les souches cambodgiennes étaient proches ou même pour certaines souches pratiquement identiques à celles isolées en Thaïlande ou au Vietnam. De plus, des souches thaïlandaises se positionnent à la base des branches des arbres phylogénétiques de chaque sérotype indiquant probablement que nos souches cambodgiennes trouvent leur origine en

Thaïlande. Ce résultat indique également que les virus ont abondemment circulé entre les trois pays, probablement en raison des mouvements de population cherchant du travail en Thaïlande ou au Vietnam (CDRI, 2007).

Deux génotypes différents de DENV-2 étaient en circulation. Le génotype Asiatique 1 a été isolé pour la première fois en 2002 et a complètement remplacé le génotype Asiatique/Américain en 2005. Ce phénomène a été également observé au Vietnam et ceci a été attribué au fait que les souches du génotype Asiatique 1 avaient une meilleure « fitness » génétique que celles du génotype Asiatique/Américain (Vu et al., 2010). Au Cambodge, l'introduction de ce génotype Asiatique 1 a probablement été à l'origine de la forte incidence de DENV-2 observée en 2004.

Notre analyse phylogénétique a montré également qu'il y a une variation importante en fonction du temps et que diverses lignées apparaissent, persistent et disparaissent. Nos résultats suggèrent l'existence de « goulets d'étranglement » au moins sur deux périodes différentes qui ont assisté à l'extinction et à l'émergence de lignées. D'une part, il y a eu une synchronisation d'apparitions de nouvelles lignées dans les 4 sérotypes de DENV en 2003. Cet évènement particulier pourrait s'expliquer par la survenue de phénomènes naturels comme les inondations ou les sécheresses. En effet, en 2000-2001, une grande inondation a ravagé une majorité des provinces le long du Mékong et au tour du lac Tonlé Sap, suivie par une sécheresse en 2003 (MAO, 2005). Ces catastrophes naturelles ont probablement réduit le nombre des gîtes larvaires et par conséquence la densité des moustiques. D'autre part, l'analyse « Bayesian skyline plot » a démontré que la diversité génétique des DENV1, DENV-2 et DENV-4 a brutalement chuté entre 2006 et 2007 et que c'était le phénomène inverse pour DENV-3. Cette observation pourrait être expliquée par une importante pression immunologique due à une réaction croisée induite par la prévalence dominante de DENV-3 dans une majorité de la population. Enfin, ni les phénomènes de recombinaison et ni la sélection positive ne semblent être intervenus dans le processus d'évolution des virus de la dengue au Cambodge.

CHAPITRE 3 : MANIFESTATIONS CLINIQUES DE LA DENGUE

Contexte et objectifs de l'étude

L'expression clinique de l'infection par le virus dengue est très variable. Beaucoup d'études sérologiques ont montré que la forme inapparente de la maladie représente la majorité des infections (Porter et al., 2005; Yew et al., 2009). Cependant, peu d'auteurs ont pu isoler le virus chez les individus apparemment sains et pu décrire de façon exhaustive les caractéristiques biologiques de ces infections inapparentes. Les difficultés rencontrées pour réaliser ce genre d'étude sont l'importance de la taille de la population à inclure, la complexité de la méthodologie et le coût de ce type d'études. L'étude « DENFRAME » a été conçue afin de pouvoir recruter des sujets avec une infection inapparente de dengue à partir de l'identification de cas index (DIC) à l'hôpital. Des visites au domicile des DIC ont été réalisées et les membres de leur famille habitant sous le même toit ont bénéficié de suivis cliniques, biologiques et virologiques pendant 7 jours. Cette approche méthodologique a permis d'étudier de façon exhaustive les différentes formes cliniques de la dengue allant d'une infection inapparente à la forme grave et également des cas non dengue ou « dengue-like » (NDC). Ce projet a été réalisé en Asie du Sud-Est (Cambodge et Vietnam) et en Amérique Latine (Brésil et Guyane Française). Les objectifs étaient : (1) d'estimer la proportion d'infections inapparentes parmi les membres des familles des DIC, (2) de comparer les caractéristiques cliniques et biologiques entre des différentes formes de gravité de la dengue et (3) d'évaluer la performance d'un test de détection de l'antigène NS1.

Résultats et conclusions

Nous avons identifié 215 (48.5%) cas de dengue parmi les 443 patients suspects hospitalisés, 21 (4.8%) cas de dengue convalescente, 187 (42.2%) cas de NDC et 20 (4.5%) cas non classifiables. La fréquence de l'infection par DENV-1, -2 et -3 était similaire en Asie et celle par le sérotype DENV-3 était prédominante en Amérique du Sud. La sensibilité du test NS1 était bonne (83.6%) avec une excellente spécificité (98.9%).

En termes de diagnostic clinique, la dengue se manifeste par des symptômes peu spécifiques et peut être confondue avec de nombreuses maladies tropicales. Ainsi, deux tiers des cas suspects en Amérique Latine et un tiers des cas suspects en Asie du Sud-Est n'étaient finalement pas des cas de dengue. Certains paramètres apparaissent utiles pour distinguer les cas dengue et les autres infections tel qu'une baisse du taux des plaquettes, des lymphocytes et des neutrophiles et une augmentation d'hématocrite et des transaminases chez les patients ayant une dengue. Nous

avons également identifié certains facteurs de risque d'évolution vers une forme sévère tels que le sexe masculin, l'âge supérieur à 7 ans, l'absence de douleurs rétro-orbitaires, un bas taux de monocytes, des transaminases normales et une infection par le DENV-2.

Durant le suivi à domicile, 479 membres de famille de 177 DIC ont été inclus. Parmi les 39 sujets ayant une dengue aiguë confirmée, 29 avaient une infection inapparente. Le virus a été détecté chez ces 29 personnes dans 9 domiciles. Deux analyses comparatives ont été réalisées et des paramètres ont été identifiés permettant de différencier: (1) les cas asymptomatiques (taux bas de neutrophiles et de monocytes) *versus* les membres de la famille non infectés par le virus de la dengue, (2) les cas asymptomatiques (faible taux de détection par le test NS1 et taux de lymphocytes plus élevés) *versus* les cas symptomatiques. En outre, d'un point de vue biologique (en se limitant aux paramètres mesurés dans le cadre de l'étude) et hormis le taux de lymphocytes, les individus avec une infection inapparente n'étaient pas différents des cas symptomatiques. Il serait donc intéressant de mener d'autres études afin d'explorer la susceptibilité génétique de l'homme à l'infection par le virus de la dengue ainsi que les facteurs de virulence du virus.

Dans le chapitre suivant, la performance du test de détection de l'antigène NS1 a été évaluée plus précisément dans le contexte du Cambodge avec des sérums bien caractérisés.

CHAPITRE 4 : DIAGNOSTIC DE LABORATOIRE (CAPTURE D'ANTIGENE NS1)

Contexte et objectifs de l'étude

La dengue est une malade aiguë qui dure en moyenne 7 jours et est caractérisée par une phase "critique" survenant vers le 4ème ou 5ème jour après l'apparition des symptômes. Puisqu'il n'y a pas encore de traitement curatif spécifique ni de vaccin, un diagnostic précoce de la dengue est souhaitable, surtout dans les pays à forte incidence. Des tests sérologiques sont le plus souvent employés car ils sont faciles à utiliser et peu couteux. Cependant, ces tests sont difficiles à interpréter en raison de la réactivité croisée avec les autres flavivirus comme le virus de l'encéphalite japonaise (JEV) et le virus de la fièvre jaune (YFV). Ces analyses requièrent en outre deux prélèvements sanguins réalisés au moins à 5 jours d'intervalle (Gubler, 2002; WHO, 2009). L'isolement du virus et la RT-PCR sont des techniques longues, coûteuses et fastidieuses qui nécessitent des équipements de laboratoire spécialisés et des personnels expérimentés. Une des méthodes les plus populaires à l'heure actuelle est la détection de l'antigène NS1 (Alcon et al., 2002; Kao et al., 2005; Lapphra et al., 2008; Libraty et al., 2002). L'antigène NS1 est sécrété dans le sérum des patients infectés par DENV dès le premier jour de fièvre et le taux de NS1 est bien corrélé avec la virémie (Hang et al., 2009; Vaughn et al., 2000; Young et al., 2000).

Des tests de diagnostic basés sur la détection d'antigène NS1 ont été mis sur le marché et leur performances ont été déjà évaluées à plusieurs reprises mais toujours chez des patients présentant une dengue apparente. Selon les données de la littérature, la sensibilité des tests ELISA de capture d'antigène NS1 varie entre 63 % et 94 % (Blacksell et al., 2008; Chuansumrit et al., 2008; Dussart et al., 2008; Guzman et al., 2010b; Hang et al., 2009; Tricou et al., 2010). Les objectifs du travail décrit dans ce chapitre étaient : (1) d'évaluer la performance du test de détection d'antigène NS1 dans un contexte plus large en tenant compte des facteurs cliniques (incluant des cas asymptomatiques), immunologiques et virologiques et (2) d'évaluer l'association potentielle entre le taux d'antigène NS1, la virémie et la sévérité de la maladie.

Résultats et conclusions

Des échantillons provenant de 243 cas de dengue et 17 cas d'infections asymptomatiques, collectés durant l'épidémie de 2006 et 2007, ont été inclus dans cette étude. La sensibilité s'est avérée plutôt décevante (57.5%) avec toutefois une excellente spécificité (100%). Le test est plus fréquemment positif chez des patients avec une forme DF *versus* DHF/DSS (72,3% *versus* 40,2%, p <0,001), lors d'une infection primaire *versus* une infection secondaire (87,5% *versus* 53,5%, p = 0,001), chez les patients avec une virémie élevée (> 5 log équivalent d'ADNc / mL) *versus* ceux

ayant une virémie faible (91% *versus* 45%, p <0,001) et chez les sujets infectés par le DENV-1. Chez des individus avec une infection inapparente, la sensibilité du test capture d'antigène NS1 était inférieure à celle des patients symptomatiques. La sensibilité de ce test de détection d'antigène NS1 combiné avec un test MAC-ELISA était significativement plus élevée que lors que le test est utilisé tout seul (85,7% *vs* 57,7%, p <0,001) et cette sensibilité était comparable à celle de la RT-PCR. Ces deux techniques (NS1 et MAC-ELISA) sont faciles à réaliser et nécessitent peu d'équipement. De plus, des formats immunochromatographiques de ces deux techniques combinées ont été développés, mis sur le marché et évalués.

L'analyse multivariée a montré qu'une faible virémie ou un faible de taux d'antigène NS1 mesuré à partir du 4ème jour de la maladie était un facteur de risque d'évolution vers une forme sévère de la dengue. Dans un contexte clinique, la technique semiquantitative de la détection d'antigène NS1 permettrait non seulement le diagnostic précoce de la dengue mais pourrait aussi servir de marqueur de pronostic de sévérité.

Ce travail a exploré la relation qui pouvait exister entre la virémie, le taux d'antigène NS1 et le risque de développer une forme sévère mais cela n'a pas permis de comprendre les mécanismes impliqués dans la pathogénèse des formes sévères de dengue. Le chapitre suivant (chapitre 5) étudie de façon plus exhaustive ce problème grâce à une approche d'analyse d'expression génomique réalisée sur des prélèvements provenant des patients ayant développé des formes cliniques différentes de la dengue (DF, DHF et DSS).

CHAPITRE 5 : PROFIL D'EXPRESSION GENOMIQUE DES CAS DE DENGUE AVEC SYNDROME DE CHOC

Contexte et objectifs de l'étude

La forme sévère (DHF/DSS) est une cause importante de mortalité dans la dengue et est caractérisée par des fuites plasmatiques conduisant à une hypovolémie puis à un choc circulatoire accompagné d'une décompensation cardiovasculaire. Les résultats de plusieurs études tentant de décrire les processus physiopathologiques de la DHF avaient déjà été publiés. Divers facteurs ont été incriminés dans la physiopathologie tels que le tropisme tissulaire et la virulence des souches, la prédisposition génétique de l'hôte, des phénomènes immunologiques la présence de facteurs solubles de la réponse anti-inflammatoire (cytokines, complément, et autres médiateurs), etc. Une version plus détaillée de la revue de littérature concernant la pathogénèse de la DHF est décrite dans la partie "revue bibliographique" de ce manuscrit (chapitre I.6.2 ; page 49). Néanmoins, on note encore beaucoup de contradictions entre ces études. On pourrait attribuer cela aux différences méthodologiques (définition des cas sévères, variabilité des caractéristiques de la population étudiée et également des marqueurs et techniques spécifiques utilisées dans chaque étude). Cette disparité empêche de bien identifier les marqueurs altérés et ne permet pas d'avoir une bonne vue d'ensemble des interactions de ces marqueurs au cours de l'infection sévère de la dengue. L'étude de l'expression du génome complet a été souvent utilisée pour expliquer des mécanismes impliqués au cours des maladies inflammatoires systémiques (Cobb et al., 2005; Feezor et al., 2005).

Une étude prospective a été menée durant l'épidémie de 2007 afin de tenter d'étudier certains aspects de la pathogénèse du DSS. Des enfants atteints de différentes formes cliniques de la dengue ont été recrutés et des prélèvements séquentiels de sang ont été collectés. En utilisant l'analyse de l'expression génomique, l'objectif était de d'identifier des marqueurs biologiques et d'étudier le rôle de la réponse inflammatoire dans la fuite plasmatique observée chez les patients DSS.

Résultats et conclusions

Un panel de 48 patients présentant les 3 degrés de sévérité (OMS, 1997) a été soigneusement sélectionné, dont 16 cas de DF, 13 cas de DHF et 19 cas de DSS. Leurs profils d'expression génomique ont été décrits et comparés. Cela a permis d'identifier 2959 signatures génomiques spécifiques aux patients DSS. Quatre résultats principaux sont présentés ici : **(1)** la classification

basée sur le profil d'expression génomique a groupé des patients DF et DHF grade I et II séparément des patients DSS. Ce résultat montre que la dengue n'est pas une maladie avec des entités clairement distinctes et ceci va dans le sens de la nouvelle classification de l'OMS de 2009 qui considère que la dengue est une maladie "dynamique" et que toutes les formes intermédiaires entre forme bénigne et sévère sont possibles; **(2)** cette étude a confirmé le rôle de certains marqueurs biologiques déjà décrits (PTX3, cytokine pro-inflammatoire IL-18 ou IL-10) et a identifié aussi de nouveaux marqueurs jamais encore décrits. Il existe une modification des profils transcriptionnels de la réponse immunitaire et d'une activation importante de la réponse anti-inflammatoire. Ces modifications sont associées à l'immunité innée de l'hôte, aux fonctions des lymphocytes et au métabolisme des lipides. De plus, nous avons pu constater que ces modifications sont transitoires et sans complications, ce qui explique le caractère non-destructeur de la réponse immunitaire dans la dengue classique; **(3)** des mécanismes physiopathologiques survenant dans la DSS sont similaires à ceux observés dans d'autres maladies inflammatoires systémiques. Un certain nombre de voies métaboliques en relation avec la réponse immunitaire, « repair-remodeling » et « metabolic-related » sont altérées pendant la phase de choc. En particulier, la réponse des lymphocytes T et NK est réduite de même que celle des gènes de compensation anti-inflammatoire et **(4)** DAMPs (Danger-Associated Molecular Patterns) et TLRs (Toll-like receptors) pourraient être associés à la réponse inflammatoire primaire inadéquate ou exacerbée conduisant à la réponse inflammatoire secondaire observée chez des patients avec DSS.

Dans cette étude, nous avons identifié des marqueurs biologiques impliqués dans la DSS et démontré leurs interactions conduisant à la fuite plasmatique. Ces marqueurs cellulaires et moléculaires pourraient servir comme base dans la découverte de nouveaux médicaments ou utilisés comme des marqueurs de pronostic dans la progression vers une dengue sévère

CONCLUSION

Les données utilisées dans ce manuscrit de thèse proviennent du PNLD (surtout depuis l'introduction de la surveillance virologique en 2000) et également du projet DENFRAME qui a été mené entre 2006 et 2007. L'objectif principal était donc d'appréhender le problème de la dengue de manière globale et intégrative afin de mieux comprendre la maladie. Le travail a abordé les différents aspects de la dengue grâce à des approches pluridisciplinaires et a fourni pour la première fois des données globales sur la dengue en termes d'épidémiologie, d'évolution du virus et de caractéristiques de la présentation clinique au Cambodge et a permis d'identifier des facteurs contribuant à la gravité de la maladie.

Le profil épidémiologique de la dengue au Cambodge est marqué par des cycles de changement de prévalence du sérotype prédominant circulant entre DENV-2 et DENV-3 tous les 3-4 ans et par de grosses épidémies survenant tous les 8-9 ans qui coïncident avec le changement de sérotype prédominant. L'incidence annuelle varie de 0,7 à 3 pour 1000 habitants et les enfants âgés de moins de 6 ans sont les plus touchés. La lutte anti-vectorielle utilisant le téméphos ainsi que les campagnes d'information via les médias ont peu d'impact sur l'incidence de la dengue. L'incidence supérieure observée durant de l'épidémie en 2007 à la moyenne de celle rapportée entre 2002-2006 au cours des premières semaines de la saison des pluies est une bonne indication d'alerte d'une potentielle large épidémie. Comme prévu, nous avons observé que le DENV-2 était devenu le sérotype dominant en 2009 et ce phénomène indique le probable émergence du DENV-3 dans 3 ou 4 après.

Avec des outils bioinformatiques, nous avons montré que les génotypes de DENV circulant au Cambodge sont similaires à ceux observés dans les pays voisins, en particulier en Thaïlande et au Vietnam, et que les virus circulant au Cambodge trouvent probablement leur origine en Thaïlande. Notre analyse a décrit des phénomènes d'extinction et d'émergence de lignées dans les quatre sérotypes et a également suggéré que le processus évolutif a probablement été influencé par au moins 3 événements : l'introduction de nouveaux génotype ou de nouvelles lignées, des événements stochastiques causés par les inondations et la sécheresse, et enfin en raison de l'existence d'une immunité croisée.

Notre étude des formes cliniques de la dengue en Asie du Sud-Est et en Amérique latine a démontré que les symptômes de la dengue étaient peu spécifiques et souvent difficiles à

distinguer des autres étiologies de fièvre indifférenciée. Parmi les patients suspects de dengue, un tiers des patients en Amérique latine et deux tiers en Asie n'étaient finalement pas infectés par le virus de la dengue. Parmi les cas de dengue (215), 13% étaient des cas de dengue sévère et tous étaient originaires d'Asie. Par ailleurs, parmi les cas de dengue détectés chez des membres de la famille de patients hospitalisés, 74,4% étaient des infections inapparentes. Des paramètres biologiques ou cliniques pouvaient être utilisés pour mieux distinguer les cas de dengue symptomatiques des cas asymptomatiques et des infections liées à d'autres pathogènes. Au Cambodge, nous avons constaté que le kit de capture d'antigène NS1 avait une sensibilité globale modérée (57,7%). Cependant, cette sensibilité variait considérablement selon la forme clinique de dengue avec des performances optimales chez les patients atteints de dengue classique (DF) et moins bonnes lors d'infection asymptomatique. Elle était également plus élevée chez les patients prélevés au cours des 3 premiers jours après l'apparition de la fièvre, lors d'infection primaire, lors d'infection par le sérotype DENV-1, et lorsque la virémie était élevée. De plus, nous avons observé une très bonne corrélation avec les méthodes de référence lorsque le test de détection d'antigène NS1 était utilisé en combinaison avec un test sérologique ELISA IgM anti-DENV. Nous avons également démontré que les formes sévères de la maladie étaient plus volontiers observées chez les patients ayant une plus faible virémie (<5 log10 équivalents d'ADNc/mL) ou un plus faible taux d'antigène NS1 mesurés dans les 4 à 8 premiers jours après l'apparition de la fièvre.

Afin d'étudier les facteurs biologiques associés à la gravité de l'infection par le virus de la dengue, une étude des profils d'expression du génome a été réalisée. Plus de 2950 signatures génétiques différenciant les cas de DSS par rapport aux cas de DF / DHF ont été identifiés. L'analyse a montré que la DF et DHF de grade I et II ne sont pas deux phénotypes distincts de la maladie mais une seule et même entité et ce résultat va dans le sens de la nouvelle classification clinique de l'OMS de 2009. Nous avons constaté que les modifications observées au cours de la réponse immunitaire dans la dengue étaient transitoires, sans complications, et que des mécanismes physiopathologiques survenant dans la DSS étaient similaires à ceux d'autres maladies inflammatoires systémiques. Ces réactions inflammatoires secondaires pourraient être dues à la réponse inflammatoire primaire inadéquate ou augmentée liée à DAMPs et aux TLRs. Nos résultats suggèrent que certains marqueurs cellulaires et biologiques identifiés pourraient constituer d'éventuelles cibles pour le développement de nouveaux traitements ou servir de biomarqueurs pour prévoir un risque d'évolution vers une forme grave.

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