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CHARACTERIZATION OF DENGUE VIRUS ISOLATES
FROM PATIENTS EXPERIENCING DENGUE FEVER, DENGUE HEMORRHAGIC FEVER, AND DENGUE SHOCK SYNDROME

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Stockholm 2011

Karolinska Institutet

Published by Karolinska Institutet.

ISBN 978-91-7457-450-0

Printed by
REPROPRINT AB
Stockholm 2011
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

If you know exactly
what you are going to do,
what is the point of doing it?

Pablo Picasso

To Simon
IN MEMORIAM

and all the other children who left this world too early due to incurable diseases

ABSTRACT

The four serotypes of dengue virus (DENV) belong to the genus flavivirus, and have a positive sense, single-stranded RNA genome of ~11 kb. The DENVs cause the most common arthropod-borne viral disease in man with ~100 million infections per year. The sole measure of control is limiting the mosquito vectors *Aedes aegypti* and *Ae. albopictus*, and there is an urgent need for an effective vaccine and potent anti-viral drugs.

DENV infection can be asymptomatic or a self-limited, acute febrile disease ranging in severity. The classical form, dengue fever (DF), is characterized by high fever, headache, stomach ache, rash, myalgia and arthralgia. Severe dengue, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are accompanied by thrombocytopenia, vascular leakage and hypotension. The fatal condition DSS is characterized by systemic shock.

Dengue research has been hampered by a lack of appropriate animal models of infection and disease. Furthermore, fundamental knowledge such as host cell tropism and virulence markers are still not established. This thesis focuses on the characterization of clinical DENV isolates from all four serotypes and clinical conditions (DF, DHF, and DSS) aimed at identifying viral features involved in pathogenesis.

Attempts to develop a strand-specific qRT-PCR to identify primary target cells for DENV replication, failed due to the self-priming phenomenon of the DENV genome. Self-priming was not restricted to any particular regions of the viral genome, nor to contaminating cellular nucleic acids, nor the lack of a poly(A)-tail at the 3' end. First-strand synthesis *in situ* of the DENV genome is believed to arise due to spontaneous loop-back structures functioning as transient primers for the reverse transcriptase.

In vitro studies in mammalian Vero cells revealed a decreased level of replication for all DENV isolates from DSS patients compared to DENV isolates from DF patients. The replication patterns of the DHF isolates resembled either the DF- or DSS-derived DENV isolates depending on serotype. The DSS isolates were further distinguished from milder case DENV isolates by induction of apoptosis in mosquito C6/36 cells.

Three DENV-1 isolates representing a DF, DHF, and a DSS case, were further characterized *in vivo* in BALB/c mice. Infection with the DF and DHF isolates peaked during the first week with viral RNA found primarily in lungs, liver, and to a certain extent in brain. In contrast, the DSS isolate was primarily neurotropic and persisted longer compared to the DF and DHF isolates.

Genomic sequencing revealed a preference for amino acid substitutions in the viral envelope protein and the non-structural (NS) protein NS1 and NS5. Thus, these viral proteins may influence pathogenesis either by immunomodulation, and/or host cells tropism and replication.

In conclusion, these results based on clinical DENV isolates indicate that DENVs within the same serotype and genotype may have both different phenotypes and genotypes. These intrinsic viral features could influence virus virulence and disease pathogenesis in humans.

SAMMANFATTNING

Denguevirus (DENV) är ett flavivirus, som i sin tur omfattar fyra serotyper, och har ett c:a 11 kb stort enkelsträngat RNA-genom av positiv polaritet. DENV är myggburna, och infekterar årligen 100 millioner människor. För närvarande kan spridningen av DENV endast minimeras genom bekämpning av vektorerna *Aedes aegypti* och *Ae. albopictus*. Behovet av antivirala läkemedel och ett effektivt vaccin är därför akut.

Infektioner orsakade av DENV är i de flesta fall asymptomatiska, men ger i många fall hög feber och andra symtom av olika svårighetsgrad. Klassisk denguefeber (DF) karaktäriseras av hög feber, huvudvärk, utslag, samt muskel- och ledvärk. I allvarligare fall, såsom vid dengue hemorragisk feber (DHF) och dengue chock-syndrom (DSS), ses dessutom blödningar orsakade av trombocytopeni, och ökad vaskulär permeabilitet. DSS är ett livshotande tillstånd.

Bristen på lämpliga djurmodeller har försvårat dengueforskningen och mekanismerna bakom sjukdomsförloppet är fortfarande inte kartlagda. Det är fortfarande oklart i vilka celler DENV primärt replikerar och vilka faktorer som bidrar till virus virulens. Denna avhandling bygger på karaktärisering av kliniska DENV-isolat omfattande samtliga fyra serotyper från patienter med klassisk DF, DHF, eller DSS. Syftet var att få en bättre förståelse för de virusegenskaper som bidrar till de olika sjukdomsförloppen.

Försök att utveckla en sträng-specifik qRT-PCR för identifiering av de celltyper som är nödvändiga för DENV-replikation var ej möjlig pga "självpriming" av virusgenomet. Fenomenet visade sig inte vara begränsat till någon specifik del av genomet, och inte heller bero på DNA/RNA från värdcellen. Avsaknaden av poly-A-svans hos virusgenomet var inte heller orsaken. Troligtvis uppstår självpriming genom att RNA-genomet hybridiserar med sig själv genom en bakåtloop som uppkommer spontant och därmed möjliggör initiering av enzymet omvänt transkriptas.

Studier utförda i Vero-celler påvisade en nedsatt virusreplikation för alla isolat från DSS-patienter, jämfört med isolat från DF-patienter. Virusisolat från DHF-patienter liknade antingen dem från DSS-patienter eller DF-patienter beroende på serotyp. Isolaten från DSS-patienter skiljde sig ytterligare genom att inducera programmerad celldöd i myggceller.

Tre isolat av DENV serotyp 1 från patienter med DF, DHF, eller DSS, studerades i BALB/c möss. Viralt RNA hos mössen som hade infekterats med DF- eller DHF-isolaten påvisades främst under första veckan efter infektion. Viralt RNA påvisades framförallt i lungor, lever, och i några fall i hjärnan. I motsats till dessa fynd påvisades viralt RNA främst i hjärnan hos de möss som infekterats med DSS-isolatet. I dessa möss kunde viral RNA dessutom påvisas under upp till två veckor.

Sekvensering av samtliga virus visade att majoriteten av mutationerna förekom i E-proteinet, samt i NS1- och NS5-proteinerna. Dessa proteiner skulle kunna inverka på sjukdomsförloppet genom att interagera med immunförsvaret, och/eller genom virusets replikation, samt vilka celler som infekteras.

Tillsammans visar dessa resultat att DENV inom samma serotyp och genotyp kan skilja sig både fenotypiskt och genotypiskt. Dessa egenskaper hos virus kan i sin tur inverka på virulensen och sjukdomsförloppet hos människa.

LIST OF PUBLICATIONS

This thesis is based on the following original paper and manuscripts, which in the text will be referred to by their Roman numerals:

I. **Tuiskunen, A.**, Leparc-Goffart, I., Boubis, L., Monteil V., Klingström J., Lundkvist, Å., & Plumet, S. 2009. Self-priming of reverse transcriptase impairs strand-specific detection of dengue virus RNA. *J Gen Virol*, 91, 1019-27.

II. **Tuiskunen, A.**, Monteil V., Plumet, S., Boubis, L., Wahlström, M., Duong, V., Buchy, P., Lundkvist, Å., Tolou, H.J., & Leparc-Goffart, I. 2011. Phenotypic and genotypic characterization of dengue virus isolates differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome. *Arch Virol*.

III. **Tuiskunen, A.**, Wahlström, M., Bergström, J., Buchy, P., Leparc-Goffart, I., & Lundkvist, Å. 2011. Phenotypic characterization of patient dengue virus isolates in BALB/c mice differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome. *Virol J, 8, 398.*

IV. **Tuiskunen, A.**, Duong, V., Buchy, P., Mazurek, J., Leparc-Goffart, I., Douagi, I., & Lundkvist, Å. 2011. Phenotypic and genotypic characterization of patient dengue virus isolates. *Submitted.*

RELATED PUBLICATIONS

I. Leparc-Goffart, I., Baragatti, M., Temmam, S., **Tuiskunen, A**.,Moureau, G., Charrel, R. & De Lamballerie, X. 2009. Development and validation of real-time one-step reverse transcription-PCR for the detection and typing of dengue viruses. *J Clin Virol,* 45, 61-6.

II. Duong, V., Ly, S., Lorn Try, P., **Tuiskunen, A**., Ong S., Chroeung, N., Lundkvist, Å., Leparc-Goffart, I., Deubel V., Vong, S., & Buchy, P. 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, e1244.

III. **Tuiskunen, A**., Hjertqvist, M., Vene, S., & Lundkvist, Å. 2011. Dengue fever in returned Swedish travelers from Thailand. *Infect Ecol Epidemiol*, 1, 7240.

CONTENTS

LIST OF ABBREVIATIONS

ADE	Antibody-dependent enhancement
Ae	*Aedes*
APOIV	Apoi virus
C	Capsid
CCCL	CC‑chemokine ligand
CCHF	Crimean-Congo hemorrhagic fever
CS	Cyclization sequence
CST	Castanospermine
DALY	Disability-adjusted life years
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DDB	Dengue disease burden
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fc(γ)R	Fc-(gamma) receptor
hCF	Human cytotoxic factor
HCV	Hepatitis C virus
HF	Hemorrhagic fever
HFRS	Hemorrhagic fever with renal syndrome
HPS	Hantavirus pulmonary syndrome
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
JEV	Japanese encephalitis virus
KUNV	Kunjin virus
LGTV	Langat virus
M	Membrane
MCP-1	Monocyte chemotactic protein-1
MDA	Melanoma differentiation-associated gene
MHC	Major histocompatibility complex
MMLV	Montana myotis leukoencephalitis virus
MODV	Modoc virus
mRNA	Messenger RNA
MTase	Methyltransferase
MVEV	Murray Valley encephalitis virus
NEU	Neudoerfl
NF-kB	Nucleic factor-kappa B
NHP	Non-human primate

NLS	Nuclear localization sequence
NO	Nitric oxide
NS	Nonstructural
NTPase	Nucleoside triphosphatase
PCR	Polymerase chain reaction
PMO	Phosphorodiamidate morpholino oligomer
POWV	Powassan virus
prM	Precursor membrane
RBV	Rio Bravo virus
RC	Replication complex
RdRp	RNA-dependent RNA polymerase
RIG	Retinoic acid inducible gene
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcriptase
RTP	RNA-triphosphatase
SCID	Severe combined immunodeficiency
siRNA	Small interfering RNA
SL	Stem-loop
SLEV	Saint Louis encephalitis virus
SMS	Smooth membrane structures
sNS1	Secreted NS1 protein
TBEV	Tick-borne encephalitis virus
TGN	Trans-Golgi network
TLR	Toll-like receptor
UTR	Untranslated region
VAS	Vasilchenko
VHF	Viral hemorrhagic fever
VP	Vesicle packets
vRNA	Viral RNA
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

1 INTRODUCTION

1.1 AN ANCIENT VIRUS WITH CURRENT GLOBAL PUBLIC HEALTH REPERCUSSIONS

Dengue is an acute febrile disease caused by the mosquito-borne dengue viruses (DENVs) that are members of the *flaviviridae* family, genus flavivirus (Westaway et al., 1985). The four DENV serotypes (DENV-1 to 4) have emerged from sylvatic strains in the forests of South-East Asia (Wang et al., 2000). It is not known when dengue first appeared in human populations, but the first described epidemic of a dengue-like illness occurred in Philadelphia in the United States in 1780 (Carey, 1971). However, an ancient Chinese medical encyclopedia dating to 992 A.D. is believed to refer to dengue fever (DF) when describing a disease called water poison. The name was thought to be derived from the belief that the disease was connected to flying insects associated with water (Nobuchi, 1979). The name *dengue* itself is believed to be derived from "ki-dinga-pepo", which means "strut, swagger" in Swahili (Johnson, 1939).

Epidemics of dengue are thought to have occurred relatively infrequently from 1780 to 1940, when World War II generated ideal conditions for increased transmission of mosquito-borne diseases (Halstead, 1966). Ecological change and destruction, combined with the migration of human resources created new endemic patterns of disease. In the last decades, both DENVs and the mosquito vectors have escalated in geographic distribution causing an increased frequency and magnitude of epidemic DF as well as the emergence of dengue hemorrhagic fever (DHF) (Gubler, 2002).

1.1.1 Epidemiology

DENV is today the most common cause of arboviral disease in the world and all four serotypes can be found worldwide. More than 100 countries are endemic, primarily affecting tropical and subtropical regions containing over 2.5 billion inhabitants (figure 1) (Guzman and Kouri, 2002). The WHO estimates an annual incidence of 100 million infections, with approximately 500.000 people with DHF requiring hospitalization, a large proportion being children. DHF may develop into dengue shock syndrome (DSS) whereof the mortality rate is approximately 2.5 %, although, without proper treatment, fatality rates may exceed 20 % (WHO, 1997).

The number of reported dengue cases has increased dramatically since the 1980's due to several complex reasons (Thomas et al., 2003, Gubler, 2002). The primary driving forces include rapid, unplanned urbanization combined with substandard living conditions, lack of vector control and surveillance, poor public health programs, international travel, and virus and vector evolution (Rigau-Perez et al., 1994, WHO, 1997, Guzman and Kouri, 2002). The contribution of climatic change is controversial, and it is not known to what extent this enhances the spread of mosquitoes, and indirectly the DENVs (Guzman and Kouri, 2002, Thomas et al., 2003, Focks et al., 1995, Tun-Lin et al., 2000).

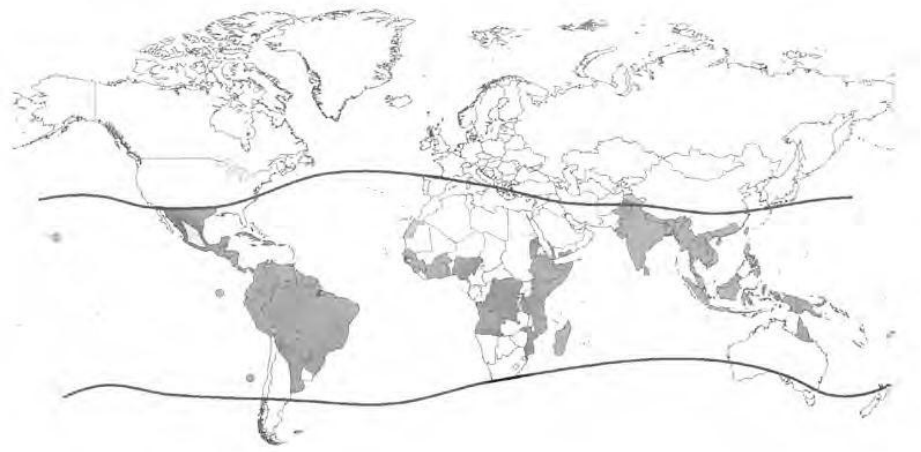


Figure 1. Dark-grey shading indicates countries/areas at risk of DENV transmission, 2008. The contour lines indicate the potential geographical limits of the northern and southern hemispheres for year-round survival of *Ae. aegypti*, the principal mosquito vector of DENVs. *Reprinted by permission from WHO, Dengue: guidelines for diagnosis, treatment, prevention and control - New edition (2009), © 2009.*

1.1.2 Disease burden

With approximately 2.5 billion people living in endemic areas, and with roughly 120 million travelers per year to these regions, the majority of the world's population is at risk of DENV infection. Successful treatment of patients with DHF and DSS is labor intensive and expensive. The dengue disease burden (DDB) in Puerto Rico for the period 1984-1994 was estimated to be an average loss of 658 disability-adjusted life years (DALYs) per million people per year (Meltzer et al., 1998). That is comparable to meningitis, hepatitis, malaria, tuberculosis, intestinal helminthiasis, and the childhood cluster of diseases (polio, measles, pertussis, diphtheria, and tetanus) combined (Torres and Castro, 2007, Hanley, 2010).

In South-East Asia, the estimated loss of 420 DALYs per million people per year corresponds to twice the burden of hepatitis, and one-third of the burden caused by HIV-AIDS in the region (Cho Min, 2000, Halstead et al., 2007, Suaya et al., 2007). Thailand had a calculated DDB at a loss of 427 DALYs per million people per year for 2001 (Clark et al., 2005). Furthermore, the WHO reports that every household of hospitalized dengue patients in Cambodia needs to borrow money in order to compensate for the loss of income to afford hospitalization costs. Out of these household, 88 % reports 'Quite a lot to substantial' economic impact on the household economy due to dengue illness (WHO, 1998).

1.1.3 The vector

Transmission of DENVs is dependent on the vector mosquito *Aedes aegypti,* and to a lesser extent *Ae. albopictus* (figure 2). The spread of DENVs mirrors the vectors'

geographical distribution underlining why mosquito density is an important parameter for predicting DENV epidemics (WHO, 1997).

The *Ae. aegypti* mosquito is well adapted to an urban environment and is a highly competitive vector due to its anthropophilic nature. It thrives in close proximity to humans, and is an intermittent feeder implying a high frequency of multiple host contacts during a single gonotrophic cycle. Thus, the female mosquito can infect multiple persons in order to complete a single blood meal (Harrington et al., 2001). Protective clothing and mosquito-repellent sprays are essential since the *Aedes* mosquitoes are day-active, minimizing the use of bed nets.

The female mosquitoes lay their eggs in artificial water containers such as tires, cans, and jars (Gubler, 1987, Gubler, 2004). Due to water requirements for breeding, mosquito densities peak during wet season, with the direct consequence of rising numbers of dengue cases.

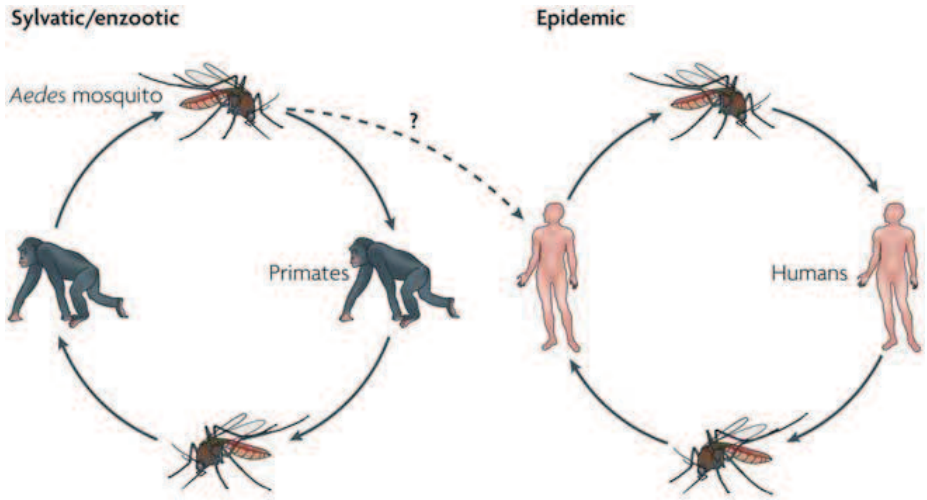


Figure 2. DENV circulates in human and sylvatic cycles. Sylvatic strains are both ecologically and evolutionarily distinct from endemic viruses, although endemic DENVs are hypothesized to have evolved from sylvatic DENV strains. Because of the high level of viraemia resulting from DENV infection of humans, the viruses are efficiently transmitted between mosquitoes and humans without the need for an enzootic amplification host. DENV is spread principally by the *Ae. aegypti* mosquito, which breeds in domestic and peridomestic water containers, increasing the frequency of contact between mosquitoes and humans. In addition, a sylvatic cycle for DENV transmission has been documented in Western Africa (Diallo et al., 2003) and South-East Asia (Wolfe et al., 2001). The contribution of the observed sylvatic cycle of DENV transmission to human infection is unknown, but appears to be minimal. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Stephen, et al.,2007 July; 5:518-528, © 2007.*

1.1.4 Dengue virus – mosquito interactions

In general, *Ae. aegypti* is less susceptible to infection by DENV than *Ae. albopictus,* which could act as a selection mechanism for more virulent strains of DENV; the lower susceptibility would require a higher viral load in the human host in order to infect the mosquito (Gubler, 1987). High viral titers in humans have been seen to be correlated to severe DHF/DSS (Gubler et al., 1978, Gubler et al., 1981, Vaughn et al., 2000, Wang et al., 2003, Tanner et al., 2008). On the contrary, the secondary vector *Ae. albopictus* could

transmit DENV strains that do not replicate to such high titers resulting in less clinically overt or severe disease. This scenario proposes that *Ae. albopictus* could function as a maintenance vector involved in the silent transmission of DENV during inter-epidemic periods (Gubler, 1987, Gubler, 1998a). The susceptibility of the mosquito vector and transmission dynamics, however, are also dependent on DENV strains (Gubler and Rosen, 1977, Anderson and Rico-Hesse, 2006, Hanley et al., 2008), but the mechanisms underlying the inter-specific and inter-strain differences in vector susceptibility to DENV infection remains to be determined.

Once ingested by the mosquito, the DENV establishes a productive infection in the mosquito midgut, wherefrom the virus disseminates and replicates in other tissues. In order to be transmitted to a human (or non-human primate (NHP)) host during the next blood meal, the DENV must ultimately infect the salivary glands and be shed in the saliva. Vector competence is genetically determined and genetic traits influencing both midgut infection and escape barriers have been mapped to various loci on the *Ae. aegypti* chromosomes (Bosio et al., 2000, Gomez-Machorro et al., 2004, Bennett et al., 2005a, Bennett et al., 2005b).

Mosquito cell lines have been generated for studies of mosquito-borne viruses and can be used for field isolation of such viruses. *Ae. albopictus* cells have been used for isolation and identification of DENVs from patient's blood (Singh and Paul, 1969, Pavri and Ghosh, 1969). Arboviral infection of mosquito cell culture yields high concentrations of virus and is characterized by a persistent infection as most mosquito cells are not killed by the infection (Stalder, 1981).

1.1.5 Prevention and control

In the continuing search for an effective vaccine and anti-dengue drugs; two measures to prevent DENV transmission are to reduce the vector population and to educate people in affected areas about basic protection measures (figure 3). Anti-vector control programs include rigorous surveillance, spraying pesticides, minimizing potential breeding sites, and promoting decent housing and infrastructure. Personal protection includes protective clothing and anti-insecticide sprays.

Rapid, unplanned growth of urban centers in South-East Asian and South American countries combined with inadequate water supply and sewerage systems have dramatic consequences on the transmission of DENV (McCall, 2006, McCall and Lenhart, 2008).

Figure 3. Pedagogic material and anti-mosquito campaigns are common and widely used in countries where DENV is endemic. This illustration is from a leaflet for childen in Thailand about dengue; symptoms of the disease, clinical management, and what to do in order to prevent dengue and spread of the disease. Anti-dengue campaigns are often adjusted to a specific target group and illustrative posters are distributed at work places. Households receive information on how to protect their family-members at home. These efforts to limit the spread of the DENV-transmitting mosquitoes are required due to the lack of an effective vaccine. *Courtesy: Krisana Pengsaa, et. al. (2008). Dengue: A Global Health Threat. Thailand Chapter of International Society of Tropical Pediatrics, First Edition, © 2008.*

2 DENGUE VIRUS

2.1 FLAVIVIRUS

The four serotypes (1 to 4) of DENVs are members of the family *Flaviviridae*, genus flaviviruses. The flaviviruses consist of viruses transmitted by mosquitoes or ticks, and viruses without any known arthropod vector. Several of the flaviviruses are known human pathogens, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV), and yellow fever virus (YFV), which is the family prototype (Westaway et al., 1985) (figure 4). The flaviviruses are similar in their morphology, genomic organization, and antigenic determinants. The latter have implied complications by classical serological techniques due to cross-reactivity between antigenic epitopes (Casals, 1954, Henchal et al., 1982).

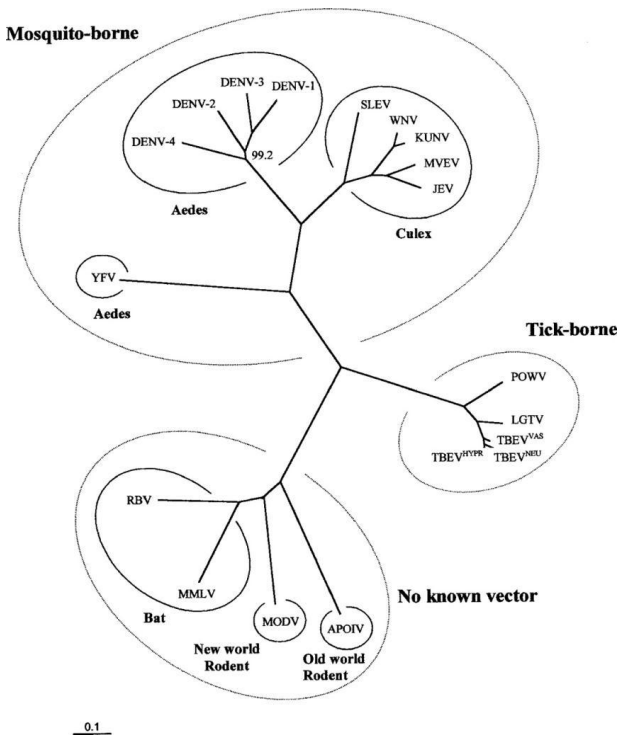


Figure 4. Phylogenetic tree based on the complete coding region of 20 flaviviruses by the neighbour-joining method. Bootstrap statistical analysis was applied with 1000 bootstrap samples (only bootstrap values below 100% are marked). The main vectors or hosts from which viruses were isolated are indicated. Abbreviations not found in the text: Saint Louis encephalitis virus (SLEV), Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), Powassan virus (POWV), Langat virus (LGTV), strain Vasilchenko (VAS), strain Neudoerfl (NEU), strain Hypr (HYPR), Rio Bravo virus (RBV), Montana myotis leukoencephalitis virus (MMLV), Modoc virus (MODV), Apoi virus (APOIV). *Reprinted by permission from Society for General Microbiology, JGV, Charlie et al. 2002; 83: 1875–1885, © 2002.*

2.2 REPLICATION CYCLE

2.2.1 The genome

DENV is an enveloped, single-stranded positive-sense RNA virus. The RNA genome is approximately 10,700 nucleotides and encodes a 3,411 amino acids long precursor polyprotein containing three structural proteins (capsid (C), precursor membrane (prM), and envelope (E)) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (figure 5). The structural proteins are components of the mature virus particle whereas the NS proteins are expressed only in the infected cell, and are not packaged to detectable levels into mature particles. The structural proteins are not involved in replication of the viral genome (Pang et al., 2001a, Pang et al., 2001b, Alvarez et al., 2006a).

The open reading frame is flanked by two untranslated regions (5' and 3' UTR) of approximately 95-135 and 114-650 nucleotides, respectively (Rice et al., 1985, Chambers et al., 1990) (figure 5). The 5'-end contains a type I cap, similar to cellular messenger RNA (mRNA), and the viral RNA (vRNA) is translated by a cap-dependent initiation scanning the 5'-UTR (Cleaves and Dubin, 1979, Wengler and Gross, 1978). The 3'-end lacks a poly(A) tail, but ends in a conserved stem-loop (SL) structure. Both the 5'- and 3'-UTRs are required for efficient translation and replication (Edgil et al., 2006, Lo et al., 2003, Romero et al., 2006, Khromykh et al., 2003, Yu and Markoff, 2005). The UTRs have characteristic secondary structures that confer distinct functions and show high sequence conservation among different DENV serotypes. The 5'-UTR contains a large stem-loop (SLA) that is proposed to act as the promoter for the viral RNA-dependent RNA polymerase (RdRp) NS5 (Filomatori et al., 2006, Yu et al., 2008). Both the 5'- and the 3'-UTRs contain complementary UAR and cyclization sequences (CS) that hybridize in order to mediate genome cyclization and RNA synthesis (Alvarez et al., 2008, Alvarez et al., 2005, Filomatori et al., 2006, Khromykh et al., 2001).

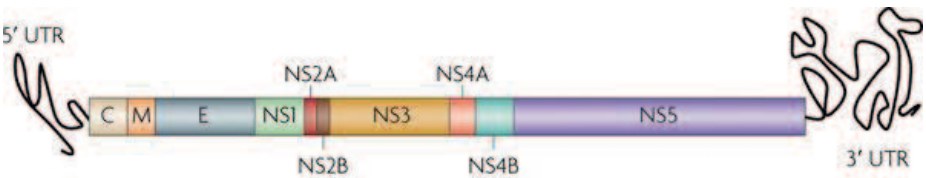


Figure 5. The single open reading frame encodes three structural proteins (C, (pr)M, and E glycoprotein) and seven NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and N55). Each end of the genome is flanked by a UTR that has characteristic secondary structures. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Guzman, et al., 2010 Dec; 8:S7-S16, © WHO, on behalf of TDR (WHO/TDR) 2010.*

2.2.2 Virus entry

Viral entry into the host cell is mediated by receptor-mediated endocytosis through an as yet unidentified cell-surface receptor. Candidate cellular receptors required for viral entry are various glycoproteins (*i.e.,* heparin sulfates), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), or a mannose receptor (Chen et al., 1997, Tassaneetrithep et al., 2003, Miller et al., 2008). The human C-type lectin-like molecule CLEC5A has been suggested to act as a critical macrophage receptor for

DENV, and has been described as a proinflammatory receptor for DENV that contributes to lethal disease in mice (Chen et al., 2008, Watson et al., 2011).

Upon internalization, the acidic pH in the endosome triggers a conformational change in the E protein mediating membrane fusion. The viral nucleocapsid is released into the cytoplasm whereupon the virus uncoats and releases the genome (Heinz et al., 1994).

2.2.3 Polyprotein processing

DENV infection induces intracellular membrane alterations in the cytosol forming vesicle packets (VPs) or smooth membrane structures (SMS) where the viral replication complex (RC) accumulates (Mackenzie et al., 1996, Miller et al., 2006). The induction of membrane structures may serve as a scaffold for anchoring the viral RC.

The input positive-strand vRNA is translated into a single polyprotein, which is cleaved into the individual structural and NS proteins. The input strand translation is followed by a switch from translation to synthesis of a negative-strand intermediate, which serves as a template for new positive-strand vRNA. Multiple rounds of translation produce high levels of viral proteins that together with vRNA are assembled into progeny virions (Clyde et al., 2006) (figure 6).

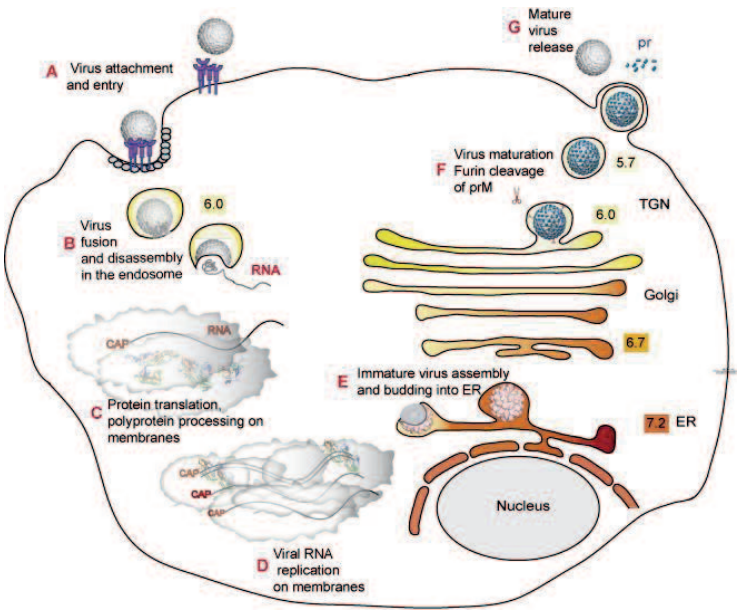


Figure 6. The flavivirus life cycle. (A) Virions bind to cell-surface attachment molecules and receptors, and are internalized through endocytosis. (B) In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of vRNA into the cytoplasm. (C) vRNA is translated into a polyprotein that is processed by viral and cellular proteases. (D) Viral NS proteins replicate the genome RNA. (E) Virus assembly occurs at the endoplasmic reticulum (ER) membrane, where C protein and vRNA are enveloped by the ER membrane and glycoproteins to form immature virus particles. (F) Immature virus particles are transported through the secretory pathway. In the low pH of the trans-Golgi, network (TGN) furin-mediated cleavage of prM drives maturation of the virus. (G) Mature virus is released from the cell. Numbers shown in colored boxes refer to the pH of the respective compartments. *Reprinted by permission from Copyright Clearance Centre Inc, Elsevier, Antiviral Res. Rushika, et al., Antiviral Res. 2008 Oct; 80(1): 11–22, © 2008.*

2.2.4 The structural proteins C, prM, and E

Translation and replication of DENV are dependent on intracellular membrane structures. The C-terminal regions of C, prM, and E contain hydrophobic amino acids that serve as signal sequences for insertion of the remaining protein into the ER membrane (Salonen et al., 2005). An ER signal peptidase together with the viral NS2B-NS3 protease cleaves the structural proteins and NS1 protein into individual membrane-bound proteins (Lobigs, 1993, Yamshchikov and Compans, 1993, Pethel et al., 1992).

Prior to secretion of new viral particles, the prM protein is processed into the mature M protein in the TGN by furin host protease (Stadler et al., 1997). It is believed that prM protects the E proteins from pH-induced reorganization and premature fusion during secretion; hence the maturation event is necessary for infectivity (Guirakhoo et al., 1991, Guirakhoo et al., 1992, Zhang et al., 2003) (figure 6).

There is a general consensus that the viral E glycoprotein affects host cell receptor binding, viral entry, and is a major target for humoral immunity. The E protein is composed of three domains: domain I, domain II harboring the fusion peptide at its distal tip, and domain III responsible for receptor-binding activity (figure 7). In the mature state, E exists as a homodimer with the fusion peptide inaccessible. Low-pH induced trimerization exposes the hydrophobic fusion peptide in a manner consistent with class II fusion protein mediated membrane fusion (Modis et al., 2004). Mutations of residues constituting the ligand pocket at the interface of domain I and II alter the required pH threshold and affect virulence (Rey et al., 1995). There are two potential asparagine (N)-linked glycosylation sites at positions Asn-67 and Asn-153, whereof the former is unique for DENVs and the latter is conserved in most flaviviruses (Heinz and Allison, 2003). The glycosylation pattern differs according to DENV serotype and even among different strains, as well as the cells in which the virus is propagated. The degree and position of N-linked glycans affect the antigenic properties of DENV (Johnson et al., 1994, Lee et al., 1997, Navarro-Sanchez et al., 2003).

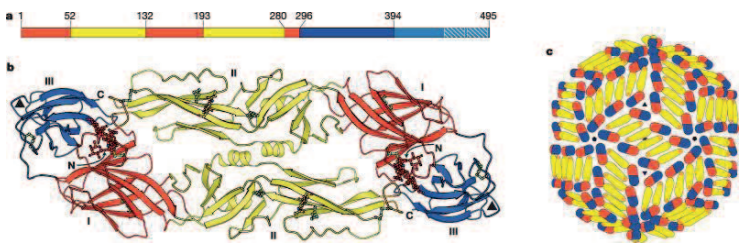


Figure 7. (A). The three domains of dengue E protein. Domain I is red, domain II is yellow, domain III is blue. (B). This is the conformation of an E dimer in the mature virus particle and in solution above the fusion pH. (C). Packing of E on the surface of the virus. *Reprinted by permission from Nature Publishing Group Ltd, Nature, Modis et al. 2004 Jan; 427:313-319, © 2004.*

2.2.5 NS1

The NS1 is a glycoprotein with two glycosylation sites that are conserved among flaviviruses. It is synthesized in the ER as a hydrophilic monomer, but exists as well as a more hydrophobic homodimer. The NS1 dimer is transported to the Golgi apparatus where it undergoes carbohydrate trimming (Winkler et al., 1989, Winkler et al., 1988).

The role of NS1 in virus replication is unknown, but is believed to facilitate viral infection and DENV pathogenesis (Libraty et al., 2002b). NS1 is in addition secreted from infected cells (sNS1), and has been shown to be immunologically important (Schlesinger et al., 1987, Avirutnan et al., 2006). Antibodies raised against sNS1 proteins have been proposed to cause endothelial dysfunction due to cross-reactivity to host proteins and endothelial cells (Lin et al., 2006). Data indicates that sNS1 could be an important modulator of the complement pathway and is proposed to protect DENV from complement-dependent neutralization in solution (Avirutnan et al., 2010).

2.2.6 NS2B-NS3

The NS3 protein acts, together with its cofactor NS2B, as the viral serine protease needed for polyprotein-processing through its N-terminal end (Bazan and Fletterick, 1989, Gorbalenya et al., 1989a, Chambers et al., 1990). This heterodimer protein complex cleaves on the cytoplasmic side of the ER membrane at the junctions between NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5, as well as on the internal sites within C, NS2A, NS3, and NS4A.

The C-terminal end of the NS3 protein has three enzymatic properties: a 5' RNA-triphosphatase (RTP), a nucleoside triphosphatase (NTPase), and a helicase (Gorbalenya et al., 1989a, Gorbalenya et al., 1989b, Li et al., 1999, Wengler, 1993, Bartelma and Padmanabhan, 2002). NS3 forms a complex with NS5 and assists in viral replication through unwinding of RNA and dephosphorylation prior to 5'-end capping (Kapoor et al., 1995, Cui et al., 1998, Johansson et al., 2001).

2.2.7 NS2A, NS4A and NS4B

The remaining NS proteins are cleaved by the viral serine protease NS3 that requires NS2B as a cofactor for catalytic activity (Chambers et al., 1991). A host cell signal peptidase, however, mediates post-translational modifications on the NS4A-4B proteins (Preugschat and Strauss, 1991). The small hydrophobic proteins NS2A, NS4A, and NS4B are less well characterized. Recent findings propose an inhibitory role in interferon (IFN)-mediated signal transduction (Munoz-Jordan et al., 2005, Munoz-Jordan et al., 2003). Their hydrophobic nature potentially implicates them in proper localization of viral proteins and vRNA during replication and virion assembly (Lindenbach and Rice, 2003). Formation of DENV-induced cytoplasmic membrane structures are believed to be an arrangement of the NS4A protein (Miller et al., 2007).

2.2.8 NS5

The largest NS protein encoded in the DENV genome is the NS5 protein, approximately 103 kDa big. The NS5 protein has three major functional domains: the N-terminal S-adenosyl methionine methyltransferase (MTase), the nuclear localization sequences (NLS), and the RdRp activity in its C-terminal domain.

The MTase spans amino acid residues 1 to 239 and is responsible for guanine N-7 and ribose 2′-O-methylations required for the capping of the DENV genome. The cap structure is recognized by the host cell translational machinery (Bartholomeusz and Wright, 1993, Egloff et al., 2002, Ray et al., 2006, Koonin, 1993). The NLS (residues 320-405) interacts with the NS3 viral helicase and is recognized by cellular factors, allowing protein transport to the nucleus (Bartholomeusz and Wright, 1993, Brooks et al., 2002). The NS5 polymerase domain RdRp (residue 273-900) is responsible for

synthesizing new vRNA genomes (Koonin, 1993, Tan et al., 1996, Ackermann and Padmanabhan, 2001).

3 DENGUE – THE DISEASE

DENV pathogenesis remains a challenging jigsaw puzzle with most pieces missing to understand the complex interplay of viral and host factors. Despite intensive research, it is not well understood. The severity of DENV infection is modulated by multiple risk factors such as age (Gubler, 1998a, Guzman et al., 2002a), the genetic background of the host (Guzman et al., 2002b, Halstead et al., 2001), viral serotype (Balmaseda et al., 2006, Gubler, 1998a) and genotype (Messer et al., 2003, Rico-Hesse et al., 1997), and secondary DENV infection by a heterologous serotype (Guzman et al., 2002b, Burke et al., 1988, Halstead et al., 1970, Sangkawibha et al., 1984, Thein et al., 1997).

3.1 CLINICAL FEATURES

The four DENV serotypes can cause a wide range of diseases in humans even though DENV infections may also be asymptomatic. The diseases range in severity from undifferentiated acute febrile illness, classical DF, to the life-threatening conditions DHF/DSS (Gubler, 2002) (figure 8). The WHO has defined criteria for classification of dengue disease, and recognizes four grades according to severity (table 1) (WHO, 1997).

Grade of disease	*Signs and symptoms*
I	Non-complicated undifferentiated febrile illness, or asymptomatic.
II	High fever, rash, headache, arthralgia, myalgia, stomach ache, petichae (DF).
III	Thrombocytopenia, various degrees of vascular leakage, rapid and weak pulse and hypotension (DHF).
IV	Profound shock with undetectable blood pressure and pulse due to severe vascular leakage (severe DHF grade IV, DSS).

Table 1. The WHO criteria for classification of dengue patients (WHO, 1997).

The WHO classification of dengue originated primarily from descriptions of pediatric cases in Thailand during the 1970s (WHO, 1973), and variations from these original description have been reported. Epidemiological changes of dengue have led to problems with the use of the existing WHO classification (Guha-Sapir and Schimmer, 2005, Deen et al., 2006, Rigau-Perez, 2006). In addition, atypical clinical features of dengue are increasingly being reported, but are probably underappreciated (Gulati and Maheshwari, 2007). These alternative symptoms include encephalitis, myocarditits, hepatitis, pancreatis, retinitis, and acute respiratory distress syndrome (Sen et al., 1999, Stephenson, 2005, Thisyakorn and Thisyakorn, 1994, Kabra et al., 1999). These atypical presentations may be due to pathology at different endothelial surfaces (Gulati and Maheshwari, 2007).

The clinical features of DF vary according to patient age (Gubler, 1998a), and some investigations have suggested that only 60 to 70 % of the DHF cases meet the WHO criteria (Maguire et al., 1974, Gubler, 1989). In response to criticism against the rigid WHO criteria, excluding certain DHF/DSS cases with alternative symptoms unless it is accompanied by thrombocytopenia and hypovolemia, the WHO guidelines were revised (Balasubramanian et al., 2006, Bandyopadhyay et al., 2006, Kroeger and Nathan, 2006, Rigau-Perez, 2006). A simplified categorization for dengue case classification has been proposed by WHO's Special Program for Research and Training in Tropical Diseases (TDR) where DHF and DSS cases are grouped together as "severe dengue" (group C) to avoid false-negative DHF/DSS diagnosis (WHO, 2009).

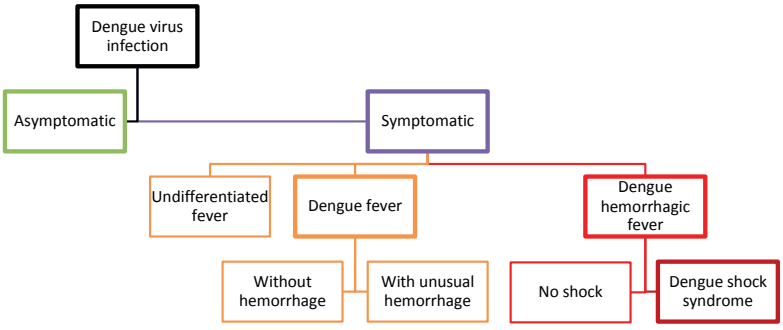


Figure 8. A DENV infection can be cleared by the immune system without any clinical symptoms, or develop into a flu-like disease, classical DF, or the more severe forms of DHF and DSS that may be lethal.

3.1.1 Dengue fever

After an incubation period of 3-15 days (usually 5 to 8), classical DF begins with an abrupt onset of high fever. During the febrile phase, dehydration may cause neurological disturbances and febrile seizures in young children (WHO, 2009). The condition is self-limiting through debilitating illness with headache, retro-orbital pain, myalgia, arthralgia, petechiae rash and leucopenia. A macular-papular recovery rash appears 3-5 days after the onset of fever, and usually starts on the trunk before spreading peripherally (Henchal and Putnak, 1990). DF is sometimes referred to as "break bone fever" due to its incapacitating symptoms with severe muscle and joint pain (Rush, 1789); or "seven-day fever" since the symptoms usually persist for 7 days.

3.1.2 Dengue hemorrhagic fever and dengue shock syndrome

Early symptoms of DF and DHF are indistinguishable, but DHF is associated with hemorrhagic manifestations, plasma leakage resulting from an increased vascular permeability, and thrombocytopenia (<100, 000 platelets/mm^3) (figure 9 and 10). Thrombo-cytopenia is not necessarily restricted to severe dengue, and minor bleeding may occur in mild infections, which can be severe in those with peptic ulcer disease (Tsai et al., 1991). Plasma leakage is characterized by haemo-concentration (haematocrit increase of 20 %), development of ascites, or pleural effusion.

Figure 9. I am holding the hand of a little girl with DHF diagnosis, at the Children's Hospital in the Kampong Sham province of Cambodia, where I collected clinical DENV samples. DENV infections are a major public health concern in Cambodia, and one of the most common causes for hospitalization for children. The plastic bags contain ice cubes that are often placed on the stomach to ease the abdominal pain.

DSS is distinguished from DHF by the presence of cardiovascular compromise, which occurs when plasma leakage into the interstitial spaces results in shock. It is a fatal condition with mortality rates for DSS as high as 20 %, but can also be less than 1 % in places with sufficient resources and clinical experience. Common clinical warning signs for DSS include a rapidly rising haematocrit, intense abdominal pain, persistent vomiting, and narrowed or absent blood pressure (Gubler, 2002).

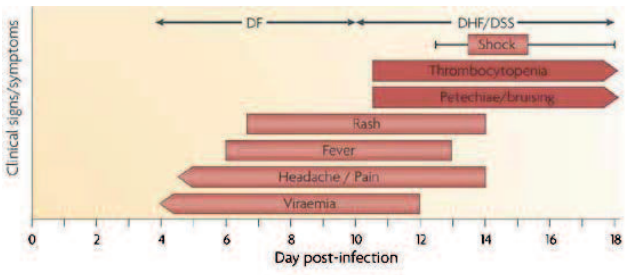


Figure 10. The incubation period before the development of signs of infection generally ranges from 5 to 8 days. Hypovolemic shock can develop during the late stage of disease and usually lasts 1 to 2 days. *Modified and re-printed by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Stephen, et al., 2007 July; 5:518-528, © 2007.*

3.1.3 Laboratory diagnosis

Since laboratory-based dengue diagnosis is often unavailable at the time of care, the preliminary diagnosis relies on a combination of travel history and clinical symptoms. Travel history provides key information that can rule out other potentially life-threatening diseases since the incubation period of DENV is less than two weeks (Rigau-Perez et al., 1998). A confirmed diagnosis for a DENV infection, however, is established by culture of the virus, polymerase-chain reaction (PCR), or serologic assays. There are limitations with each test and detection is based on different virological markers; infectious virus, vRNA, and DENV specific antibodies, respectively.

Culturing the virus requires an acute patient serum with sufficient levels of virus, and the period when DENV can be successfully isolated in patient serum is short. Viremia peaks prior to the onset of symptoms, hence virus levels might have dropped significantly once the patient seeks medical care. Furthermore, rising levels of antibody interfere with virus culture already within a day or two after the subsidence of fever (figure 11). Apart from sample collection limitations, practical considerations limit the use of this method. Culture of the virus is both time- and labor intensive; infectious patient material must be kept cold, and a bio-safety level 3 laboratory is required, demanding professional training of the personnel. These requirements limit this diagnostic tools use, especially in rural areas (WHO, 1997).

The *Ae. albopictus* cell line C6/36 (CRL 1660, ATCC) is commonly used to isolate DENVs from patient material. Specimens that may be suitable for virus isolation include acute phase serum, plasma or washed buffy coat from the patient, autopsy tissues from fatal cases, especially liver, spleen, lymph nodes and thymus, and mosquitoes collected in nature (WHO, 1999). C6/36 cells are most often used to amplify DENVs for research, even though other laboratory cell lines from various vertebrate species are also employed depending on experimental design. Persistent infection in cell culture and multiple rounds of propagation in cells result in laboratory-acquired mutations, leading to cell culture adaptation and altered wild-type features, *e.g.* the plaque size is altered (Igarashi, 1988). The commercial DENV strains available (*e.g.* DENV-2 16681 and P23085) are often

passaged in suckling mice, or may be tissue culture adapted, and can in certain cases be a pool of several patient isolates (www.atcc.org).

Detection of vRNA from serum, plasma, or cells with PCR is based on DENV-specific oligonucleotide primers, and is fast and robust, although sensitive only in the very early stages of disease (Rigau-Perez et al., 1998). PCR is particularly useful in situations when virus culture has not been successful, but nevertheless depends on sample collection during the symptomatic phase (figure 10).

The third laboratory diagnostic option is not based on direct detection, but on the presence of anti-DENV antibodies. Thus, it is not hindered by the limitations of virus culture and PCR, and the timing of sample collection can be more flexible. The acute anti-DENV IgM antibody response lasts for a couple of weeks after infection and the IgG antibodies for several years (figure 11). The immunoglobulins (Ig) are not easily inactivated and do not have the same strict requirements for low temperature as infectious virus specimen. The assay techniques are relatively simple and there are commercial diagnostic kits available (WHO, 1997). The major drawback with serological tests is the considerable risk for false-positive results due to potential cross-reactivity with other flaviviruses, *e.g.* vaccination against YFV (Schwartz et al., 2000).

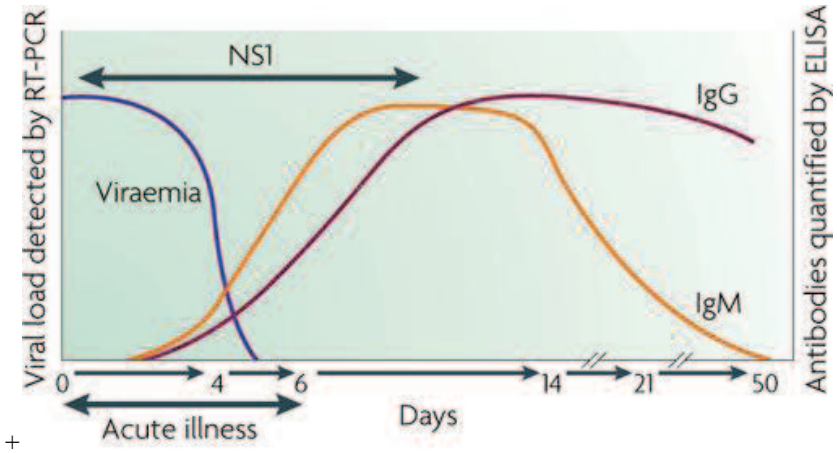


Figure 11. The diagnostic tool that is best suited depends on when the patient seeks medical assistance post-infection since infectious virus and anti-DENV antibodies appear at different time periods. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Guzman, et al., 2010 Dec; 8:S7-S16, © WHO, on behalf of TDR (WHO/TDR) 2010.*

Due to the drawbacks of serological methods to reliably diagnose acute infections, alternative methods based on the detection of the viral NS1 protein have been developed. NS1 can be found both membrane-associated inside the host cell and in a soluble, secreted form.

The amount of secreted NS1 in patient serum correlates with viremia and DENV pathogenesis (Wang et al., 2006, Hang et al., 2009, Libraty et al., 2002b, Vaughn et al., 2000, Young et al., 2000) and the NS1 protein is detectable in serum by enzyme-linked immunosorbent assay (ELISA) from the first day of fever up to nine days post-infection (Libraty et al., 2002b, Alcon et al., 2002, Lapphra et al., 2008, Schilling et al., 2004).

NS1-based ELISAs have become an important diagnostic tool for acute samples in which IgM is not detectable and where PCR is not available. Several commercial NS1 antigen kits are available and are widely used in endemic as well as non-endemic countries. The sensitivity varies from 63 % to 94 % (Hang et al., 2009, Lapphra et al., 2008, Dussart et al., 2008, Phuong et al., 2009), and depends on sample time-point, DENV serotype, and if it is a primary or secondary DENV infection (Duong et al., 2011).

3.1.4 Vaccines

Unlike flaviviruses such as YFV, JEV and TBEV, no licensed vaccine exists for dengue. Vaccination must protect against all four serotypes without predisposing for antibody-mediated enhanced disease (see 6.2 "Antibody-dependent enhancement") and has proven difficult to design. Nearly eighty years of vaccine-related research and development have passed, and over 25 unique DENV vaccine candidates have been tested in clinical trials during the past decade.

To be safe, a dengue vaccine must be functionally tetravalent, eliciting simultaneous protection against all four DENV serotypes (figure 12). Hence, vaccination cannot proceed in an analogous sequential manner, and herein lies the greatest obstacle (Gibbons et al., 2007, Raviprakash et al., 2006, Konishi et al., 2006). Live attenuated vaccines can induce durable humoral and cellular immune responses that mimic natural infection (Wisseman, 1963). The viral replication must, however, be discrete to preclude the development of significant illness. A reasonable range of viremia for a live attenuated vaccine is believed to be approximately 10^1-10^2 infectious units/mL (Guirakhoo et al., 2006) compared to high levels of viremia upon natural infection that can be 10^5-10^7 infectious units/mL (Vaughn et al., 2000).

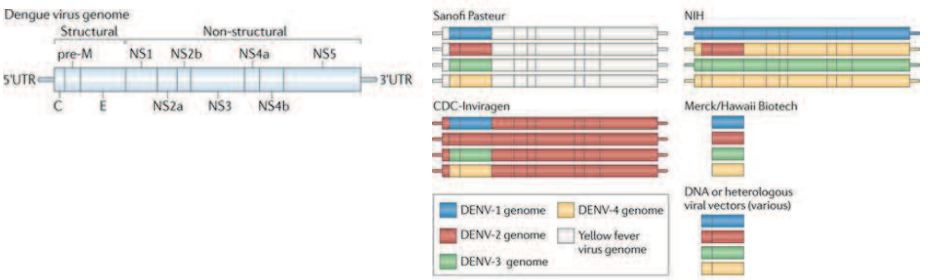


Figure 12. A schematic of the DENV genome is shown on the left. The Sanofi Pasteur vaccine contains four chimeric live flaviviruses, each derived from the YFV genome with the prM and E gene segments replaced by the corresponding gene segments of each of the four DENV serotypes. The US National Institutes of Health (NIH) vaccine contains a mixture of four recombinant DENV genomes; the DENV-2 component is a chimeric DENV derived from a DENV-4 genome with prM and E gene segments replaced by those of DENV-2. This vaccine also contains attenuating mutations in the 3′ NTR that are not shown in the figure. The CDC-Inviragen vaccine contains a mixture of four recombinant DENV-2 genomes; the DENV-1, DENV-3 and DENV-4 components are chimeric DENV genomes. The Merck/Hawaii Biotech vaccine is a recombinant protein vaccine containing the E glycoprotein ectodomains of all four DENV serotypes. Other vaccines in development use plasmids or heterologous viral vectors to express only the prM and E gene segments of all four DENV serotypes. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Immunology, Alan, et al., 2011 Aug; 11: 532-543, © 2011.*

It is expected that a live attenuated vaccine would be successful and require only a single dose since the vaccine against YFV is based on a live attenuated virus. However, it is more likely that booster immunizations will be required based on results from clinical trials using tetravalent formulations of live vaccine candidates aimed at eliciting

neutralizing antibodies (Kanesa-Thasan et al., 2003, Sabchareon et al., 2002, Edelman et al., 1994, Sun et al., 2003). The obvious challenge is when and how to boost; infectivity and immunogenicity in NHP models have not always clearly predicted the outcome of human trials (Men et al., 1996, Guirakhoo et al., 2001). Vaccination compliance may also be lower with a multi-dose vaccination strategy, especially in regions where resources are scarce, and at the same time where the need for a vaccine often is the most acute.

There are currently two live attenuated tetravalent vaccine candidates in phase II clinical evaluation, several live attenuated vaccine candidates in phase I clinical evaluation, and many subunit, -deoxyribonucleic acid (DNA), and vectored vaccines in pre-clinical stages of development. It is hoped that clinical trials evaluating novel recombinant subunit proteins, DNA, and vectored vaccines be initiated in the coming years. These approaches could be part of a prime-boost strategy, or stand-alone. The use of different types of vaccines depends on the purpose of vaccination and target group reflecting the disease setting. In endemic areas, there is an urgent need for routine immunization against dengue for infants and young children 1-3 years of age. A dengue vaccine would be coordinated with current childhood immunization schedules. Due to the socioeconomic status of many endemic countries, this type of vaccine ought to be inexpensive. In contrast, a protective vaccine for international travel, seasonal work personnel, and military staff that visit or work in DENV endemic areas are more tolerant to increased cost. Vaccination in this case will need to be rapid.

Hence, the different requirements for a dengue vaccine vary according to target group and their specific needs (life-long immunity or temporal protection), and efficient antiviral drugs would be a useful complement for protection and/or treatment. In addition, antiviral drugs would be more potent in an outbreak situation than a vaccine when there is no time to complete a multi-dose immunization schedule spanning six months or more.

3.1.5 Treatment and therapeutic approaches

Currently, vector control, regarded as both expensive and ineffective, is the only method for disease prevention (see 1.1.5 Prevention and Control) (Gubler, 1998b, Halstead and Deen, 2002). In the absence of available vaccines and antiviral drugs against DENV infection, specific treatment for dengue patients consist primarily of supportive care including bed rest, antipyretics, and analgesics. Urgent resuscitation with intravenous fluids to replace lost intravascular volume in DSS patients is a pre-requisite; Ringer's lactate has been shown to be effective in moderately severe dengue, and starch or dextran have been suggested for more severe cases (Wills et al., 2005). Aspirin and other salicylates should be avoided due to plasma leakage (WHO, 2009).

The design of novel therapeutic approaches for dengue disease has focused on the various stages of the viral replication cycle. The conformational changes of the E protein and its interaction with prM or M have been a major interest. These transition states present opportunities for antiviral targeting of the entry, assembly or maturation steps of the virus life cycle. Antiviral peptides have been designed and tested for blocking of both DENV and WNV entry with positive results, indicating that antiviral peptides could be a promising form of DENV therapy (Hrobowski et al., 2005, Bai et al., 2007). Targeting of mature virus entry into host cells is an extremely promising candidates since delivery of target compounds into the host cell during stages of fusion and maturation is significantly more challenging.

Another approach to inhibit the structural changes of the E-prM protein interactions has been to synthesize peptides mimicking the pr peptide of the M protein thereby preventing membrane fusion and release of newly synthesized virions. The viral protease is another interesting target for antiviral discovery, since proteases are common to most viruses and generally important for efficient replication. Protease inhibitors for hepatitis C virus (HCV) may eventually be further developed to inhibit the DENV protease NS2B-NS3 (Tomlinson et al., 2009).

Nucleoside analogues are usually prodrugs that need to be converted to their antiviral nucleotide metabolite forms (Parker, 2005). Ribavirin (1-β-d-ribofuranosyl-1*H*-1, 2, 4-triazole-3-carboxamide) possesses broad spectrum antiviral activity (Streeter et al., 1973, Sidwell et al., 1972) and is used in combination with IFN to HCV infection (Benarroch et al., 2004). Ribavirin depletes the nucleotide pool and thereby indirectly affects capping and polymerase activities of both cellular and viral proteins (Benarroch et al., 2004). In addition, ribavirin causes a more error-prone replication of several viral genomes (Contreras et al., 2002, Crotty et al., 2000, Eigen, 2002, Lanford et al., 2001, Zhou et al., 2003). Despite successful *in vivo* results with several RNA viruses, ribavirin has a cytostatic effect in DENV infected cells, and has not been effective in animal models (Koff et al., 1983, Crance et al., 2003, Malinoski et al., 1990).

Nucleic acid-based therapies offer various alternatives. RNA interference (RNAi) is thought to protect the host from viral infections by degrading the extraneous genetic material like vRNA (Ma et al., 2007). It has been used in therapeutic approaches for several infectious diseases, tumors, and metabolic disorders. Small interfering RNA (siRNA) treatment reduces viral load of WNV in mice (Bai et al., 2005), but there are several obstacles yet to overcome; the RNA of flaviviruses are resistant to RNAi since replication occurs in reorganized ER membrane packets (Geiss et al., 2005). In addition, HCV replication was found to be stimulated by the RNAi machinery (Jopling et al., 2005).

Another nucleic acid-based antiviral approach is antisense DNA or RNA decoys, *e.g.* phosphorodiamidate morpholino oligomers (PMOs). These compounds act by forming a stable, sequence-specific duplex with RNA, and thereby blocking access to target RNA by biomolecules required for replication. PMOs targeting the translation initiation site of DENV RNA, the 3' NTR, 5'SL and 3'CS were effective in reducing the viral load in various cell lines (Kinney et al., 2005, Holden et al., 2006). These compounds meet most of the requirements for an anti-DENV therapeutic; non-toxic, cheap, easy to administer, stable for months at variable temperatures, but remain to be tested in animal models (Kipshidze et al., 2001, Iversen et al., 2003).

Sulphated polysaccharides have been investigated for anti-DENV activity, although inconsistency in the activity results indicates that they need to be further tested both *in vitro* and *in vivo (Chen et al., 1997, Marks et al., 2001, Talarico et al., 2005, Lee et al., 2006a)*.

The processing of N-linked oligosaccharides in the ER is important for viral glycoprotein maturation, and inhibition of glucosidase-mediated trimming affects the replication cycle of several enveloped viruses (Mehta et al., 1998). DENV production was inhibited in mouse neuronal cells by two ER α-glucosidase inhibitors, castanospermine (CST) and deoxynojirimycin (Courageot et al., 2000). CST was effective against all four serotypes in

human hepatoma cells, and prevented mortality in DENV-2 infected mice. This effect was restricted to DENV, not being observed against other flaviviruses like WNV and YFV (Whitby et al., 2005). A third ER α-glucosidase inhibitor, *N*-nonyl-deoxynojirimycin, inhibits DENV-2 infection in BHK-cells (Wu et al., 2002). These results with α-glucosidase inhibitors are encouraging and should be investigated further *in vivo*.

Nitric oxide (NO) is generated by macrophages, monocytes, dendritic cells (DCs) and neutrophils; the same cells that are supposed to be the main sites of replication for DENV (Lin et al., 2002b, Wu et al., 2000). *In vitro* assays have revealed that NO specifically affects the viral RdRp activity, suggesting possible viral targets of NO during DENV infection (Takhampunya et al., 2006).

Hence, there are multiple options for designing novel therapeutics for dengue disease. The main concern with most therapeutic approaches, however, is that they are not validated for inhibitory effects on all four DENV serotypes. Additonally, several studies have not been examined in an animal model, and several reported antivirals have been tested at only one time point, pre- or post- infection in tissue culture systems, and therefore need to be subjected to more diverse regimes, and different cell types.

4 DENGUE VIRUS PATHOGENESIS

DENV infection is a systemic and dynamic disease with a wide clinical spectrum. Gross pathological findings in cases of DHF or DSS include hemorrhages in the skin, subcutaneous tissues, gastrointestinal tract, and heart (Bhamarapravati, 1989). Hemorrhage, dilatation and congestion of vessels, and edema of arterial walls are commonly found, and hemorrhagic manifestations in other organs combined with fluid accumulations in body cavities may be substantial (Hotta, 1969, Bhamarapravati, 1967).

The underlying mechanisms, however, of vascular leakage and hemorrhage are not well characterized. Elevated plasma levels of pro-inflammatory and vasoactive cytokines before and at the time of plasma leakage in patients with DHF suggest that excessive cytokine production (a "cytokine storm") induce vascular permeability. Available data propose that the outcome of a DENV infection depends on a balance between favorable and unfavorable immune responses; the former providing control of viral replication, whereas the latter enhances inflammatory and vascular permeability. The lack of reliable immunological markers for either protective or pathological immune responses to DENV and the lack of a suitable animal model for dengue disease hamper the understanding of dengue pathogenesis. Insights into the immune response against DENV infection rely primarily on clinical and epidemiological studies.

4.1.1 Tropism

Identification of the primary target cells of DENV replication has proven to be extremely difficult. Existing data is based on virus detection by immunohistochemical (IHC) analysis with antibodies against viral structural proteins, or by *in situ* hybridization to the positive-strand vRNA. However, it is difficult to prove direct infection of specific target cells by these methods as a positive signal could be due to virus endocytosed or phagocytosed by uninfected cells. Detection of negative-strand vRNA and/or DENV NS proteins would provide much stronger evidence of active DENV replication.

After inoculation by an infected mosquito, the initial round of viral replication is believed to occur in the subdermal Langerhans DCs (Ho et al., 2001, Libraty et al., 2001, Marovich et al., 2001, Wu et al., 2000). These infected cells become activated and migrate to draining lymph nodes (Johnston et al., 2000). The activated DCs elicit a robust IFNα/β and tumor necrosis factor alpha (TNFα) response together with a strong pro-inflammatory response to limit contiguous spread (Libraty et al., 2001). Viral replication continues in still undefined cells in the lymph node. There is a general consensus that candidate cell types belong to the macrophage-monocyte lineage. Autopsies and human biopsies confirm that cells from the mononuclear phagocyte linage probably are the primary targets of DENV infection following initial dissemination from the local skin site. Infiltrating mononuclear cells in affected tissues have been shown to contain DENV antigen (Boonpucknavig et al., 1979, Boonpucknavig et al., 1976, Sahaphong et al., 1980), and DENVs can occasionally be isolated from peripheral blood leukocyte fractions (Scott et al., 1980). Similar observations have been made in rhesus macaques where DENV was recovered from leukocyte-rich tissues such as regional lymph nodes, systemic lymphatic tissues, and disseminated skin sites.

Infection is amplified within the lymph nodes and viremia can be detected when the infectious virus enters the circulation via the efferent lymphatic system and thoracic duct.

Circulating monocytes in the blood are believed to be infected due to the viremia facilitating spread to secondary visceral organs where macrophages within the spleen, liver and bone marrow are infected (Jessie et al., 2004, Diamond et al., 2003, Solomon and Vaughn, 2002, Xiao et al., 2001a, Xiao et al., 2001b, Durbin et al., 2008).

There has been limited and inconsistent dissemination to solid organs (Marchette et al., 1973); DENV antigen has been detected in lymphocytes (Bhoopat et al., 1996, Jessie et al., 2004), hepatocytes (Bhoopat et al., 1996, Couvelard et al., 1999, Huerre et al., 2001, Miagostovich et al., 1997), endothelium (Bhoopat et al., 1996, Hall et al., 1991, Jessie et al., 2004, Ramos et al., 1998), and cerebral neurons and astrocytes (Ramos et al., 1998, Bhoopat et al., 1996). There are in addition other studies with contradicting results where the same tissues have been examined without any detected DENV antigen (Hall et al., 1991, Jessie et al., 2004, Miagostovich et al., 1997).

A further controversy surrounds the role of endothelial cells as the target for DENV infection. Severe dengue disease is characterized by systemic endothelial dysfunction accompanied by vascular leakage, even though destructive vascular lesions are generally absent in fatal cases (Gubler, 1998a). Primary human endothelial cells and human endothelial cell lines are permissive for DENV infection (Andrews et al., 1978, Avirutnan et al., 1998), but endothelial infection, however, does not seem to be required for severe pathologic changes in individual tissues (Balsitis et al., 2009). Their contribution *in vivo* remains to be established.

The presence of DENV antigens in various organs and cell types suggest that the host receptor(s) is broadly distributed. Host receptors for DENV are believed to include mannose binding protein, heparan sulphate, chondroitin sulphate and DC-SIGN (Wang et al., 2011, Miller et al., 2008, Avirutnan et al., 2007). Following DENV infection natural antibodies (IgM), complement, and possibly NK cells control the initial levels of viremia and to certain extent tissue dissemination. Infected cells are targeted by the cellular immune system, upon recognition by cytotoxic T lymphocytes, which will be discussed below.

4.1.2 The humoral immune response

The humoral immune response is hypothesized to be vital for controlling DENV infection and dissemination, and infection with one serotype provides long-lasting protection to that specific serotype (homotypic immunity). Subsequent infection by another serotype results in short-lived protection (heterotypic immunity), and may eventually be harmful and increase the risk of severe dengue disease (see section 6.2) (Sabin, 1950). The transient nature of heterotypic immunity is believed to be due to cross-reactive viral E-protein specific antibodies which are protective above a certain concentration threshold (Whitehorn and Simmons, 2011).

The principal targets of the antibody response to DENV infection in humans are the prM, and E structural proteins, and the NS1 protein. Weak antibody responses to other NS proteins, *e.g.* NS3 and NS5, have also been detected (Valdes et al., 2000, Churdboonchart et al., 1991). Neutralizing antibodies are directed against the viral E protein and inhibit viral attachment, internalization, and replication within cells. There are multiple epitopes residing within each of the three E domains (Roehrig et al., 1998, Sukupolvi-Petty et al., 2010), but not all are equally accessible for antibody binding due to the dimeric

conformation of the E protein on the virion surface, and its tight packing in the mature form (Lok et al., 2008, Kaufmann et al., 2006, Cherrier et al., 2009).

Domain III of the E protein, which contains the putative host receptor-binding site, is the most variable in amino acid sequence between serotypes. As a result, antibodies specific for this domain show the greatest degree of serotype specificity (Lai et al., 2008). Mutations in domain III of the E protein, however, are common for escaping neutralizing antibody (Lin et al., 1994, Lok et al., 2001). Loss of an effective neutralizing antibody response due to sequence variation has also been detected for the C and NS2B proteins (Wang et al., 2002a, Wang et al., 2002b).

Antibodies against DENV may also bind to complement proteins and promote their activation. Anti-prM and/or E protein antibody-mediated complement fixation to virions can inhibit viral infection (Mehlhop et al., 2007). As for other host immune responses to dengue, complement involvement may also be pathological. Complement activation is a feature of severe dengue and is temporally related to plasma leakage. This suggests that complement activation constitutes a major factor in the pathogenesis of dengue hemorrhagic shock (Bokisch et al., 1973, Malasit, 1987). Increased complement activation at endothelial cell surfaces could contribute to the vascular leakage (Avirutnan et al., 2006), and the viral protein NS1 is proposed to be a modulator of the complement pathway. By promoting efficient degradation of C4 to C4b, NS1 may protect DENV from complement-dependent neutralization in solution (Avirutnan et al., 2010).

4.1.3 The cellular immune response

In addition to the humoral immune response, cellular immune responses are also crucial in dengue pathogenesis. The DENV can infect both CD4+ T-cells and CD8+ T-cells (Mentor and Kurane, 1997), and similar to DENV-specific antibodies, the cellular immune responses can be either protective or harmfully reactive. DENV-specific T-cells respond with a diverse set of effector functions, including proliferation, target cell lysis and the production of a range of cytokines. CD4+ T-cells produce IFNγ, TNFα, TNFβ, interleukin (IL)‑2, and CC‑chemokine ligand 4 (CCL4; also known as MIP1β) which may contribute to pathogenesis (Gagnon et al., 1999). The production of T helper type-2 cytokines, such as IL‑4, is less common (Mangada et al., 2004, Bashyam et al., 2006, Imrie et al., 2007, Dong et al., 2007). In uncomplicated DENV infections, relatively more CD8+ T-cells are present resulting in lower levels of IFNγ and TNFα (Duangchinda et al., 2010). CD8+ T-cell clones specific for DENV partially protect mice from lethal DENV challenge (An et al., 2004) (figure 13a).

The role of T-regulatory cells is unclear in dengue, but there is a study suggesting they are functional and expand in acute DENV infection (Luhn et al., 2007).

Following primary infection, both serotype specific and serotype cross-reactive memory T-cells are generated. Upon secondary exposure, both the protective and cross-reactive memory T-lymphocytes are activated and the non-protective memory T-cells will augment infection (Kurane et al., 1990). Activated memory T-cells recognize both conserved and altered peptide ligand epitopes. The antigen sequence differences depend on the specific DENV epitope, but will nevertheless affect the quality of the effector T-lymphocyte response. This in turn modifies the immunological repertoire, and is suggested to be involved in the development of plasma leakage (Rothman and Ennis, 1999). A full agonist peptide will induce a full range of T-cell responses including

production of multiple cytokines (*e.g.* IFNγ, TNF and CCL4) and lysis of the infected cell. A partial agonist peptide, *i.e.* varying at one residue, will cause cross-reactivity in memory T-cells and induce a skewed functional response, involving production of some cytokines but little of other cytokines and inefficient cell lysis (figure 14). Thus, because of sequence diversity between DENV serotypes, the memory T-cells (and B cells) that are re-activated during a secondary DENV infection may not have optimal avidity for the epitopes of the new infecting virus. The "memory" of the primary DENV infection alters the immune response to the secondary infection influencing the clinical outcome.

There is a correlation between the level of T-cell responses and disease severity (Zivna et al., 2002, Mongkolsapaya et al., 2003). The phenomenon of low affinity for the current infecting serotype but a high affinity for a past infection with a different serotype is referred to as *Original Antigenic Sin,* and is the net effect of an altered balance between a protective and pathological outcome (Mongkolsapaya et al., 2003, Mangada and Rothman, 2005).

The pattern of antibody/T-cell responses in secondary DENV infections is also influenced by the sequence and interval between DENV infections (Valdes et al., 1999, Halstead et al., 1983, Alvarez et al., 2006b, Guzman et al., 1999, Gibbons et al., 2007, Pelaez et al., 2004). As for the ADE scenario (see section 6.2), memory T-cell responses exhibiting serotype cross-reactive proliferative activity decades after the primary infection could potentially alter the balance from a protective immune response towards an improper and non-protective immune response. Interestingly, most of the identified CD4+ and CD8+ T-cell epitopes reside in the NS3 protein, which represents only ~20% of the DENV amino acid coding sequence (figure 13b) (Duangchinda et al., 2010).

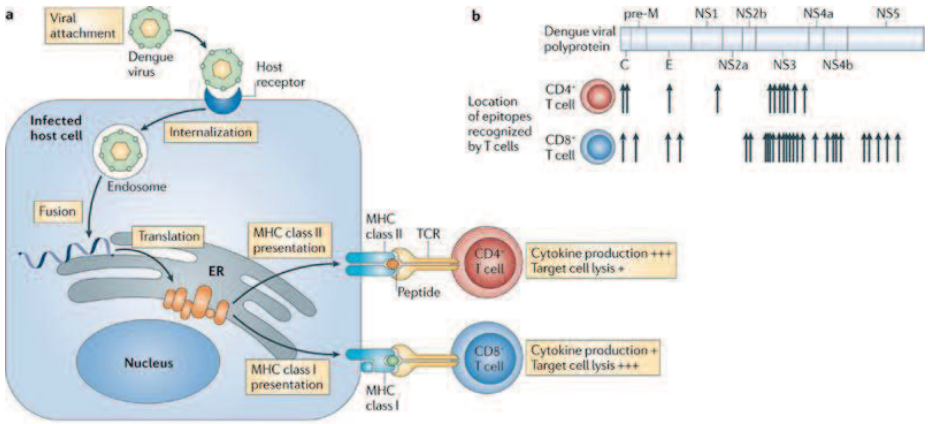


Figure 13. The DENV life cycle and antigen sources are shown. Newly synthesized viral proteins enter the major histocompatibility complex (MHC) class I and II presentation pathways and viral peptide epitopes are presented on the cell surface within the peptide binding groove of MHC molecules. MHC class II molecules present peptides to CD4+ T-cells, which principally produce cytokines but are also capable of lysing infected cells. MHC class I molecules present peptides to CD8+ T-cells, which principally lyse infected cells but also produce cytokines. (b). A schematic of the DENV polyprotein is shown at the top and the locations of well-defined epitopes recognized by human T-cells are marked by arrows. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Immunology, Alan, et al., 2011 Aug; 11: 532-543, © 2011.*

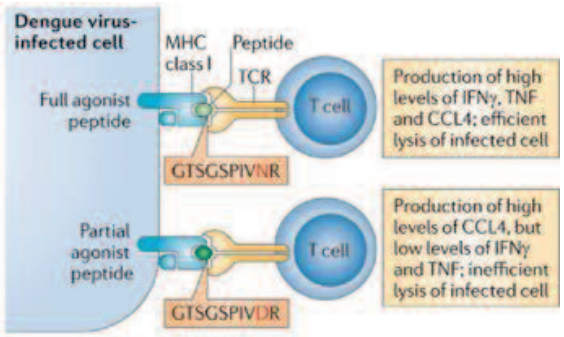


Figure 14. Variant epitopes alter the T-cell functional response. The figure shows two examples. The full agonist peptide (top) induces a full range of T-cell responses; production of multiple cytokines (*e.g.* IFNγ, TNF and CCL4) and lysis of the infected cell. A partial agonist peptide varying at one residue (bottom; altered residue in red) induces a skewed functional response, involving production of some cytokines (CCL4 in this example) but little production of other cytokines (such as IFNγ) and inefficient cell lysis. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Immunology, Alan, et al., 2011 Aug; 11: 532-543, © 2011.*

4.1.4 Cytokines in dengue pathogenesis

Viral recognition by the host cell occurs immediately upon virus entry to raise an appropriate antiviral response. Two main families of pathogen recognition receptors mediate DENV sensing; the extracellular/endosomal toll-like receptors (TLRs) (Akira and Takeda, 2004, Bowie and Haga, 2005), and the cytoplasmic receptor family of DExD/H box RNA helicases (*e.g.* retinoic acid inducible gene 1 (RIG-1) and melanoma differentiation-associated gene-5 (MDA5)) (Meylan and Tschopp, 2006). Binding to a TLR leads to activation of two families of transcriptional factors: the interferon regulatory factors (IRFs), and the nucleic factor-kappa B (NF-kB). These signaling cascades activate production of IFNα/β and proinflammatory cytokines that stimulate maturation of DCs and elicits an antiviral response (Severa and Fitzgerald, 2007, Libraty et al., 2001).

DENV is believed to primarily infect cells of the DC/macrophage/monocyte lineage via receptor-mediated endocytosis and/or enhanced uptake via antibody-virus complexes attached to Fc-gamma receptors (FcγR) (Anderson, 2003). The exact mechanisms behind DHF/DSS are not understood, but the consensus is that infected cells and activated endothelial cells produce TNFα (Carr et al., 2003, Espina et al., 2003), and NO (Charnsilpa et al., 2005, Neves-Souza et al., 2005), increasing vascular wall permeability (Borish and Steinke, 2003). The coincidence of severe disease manifestations with abatement of fever and virus control suggests that the symptoms may be a consequence of the immune response against the virus rather than virus-induced cytopathology. Consistent with this hypothesis is the increased levels of many different cytokines that have been observed in DENV infection (Basu and Chaturvedi, 2008).

Elevated serum levels of cytokines and chemokines include IL-2 (Green et al., 1999, Kurane et al., 1991, Hober et al., 1993), IL-6 (Iyngkaran et al., 1995, Juffrie et al., 2001), IL-8 (Talavera et al., 2004), IL-10 (Libraty et al., 2002a, Green et al., 1999), IL-13, IL-18 (Mustafa et al., 2001), IFNγ (Azeredo et al., 2001, Chakravarti and Kumaria, 2006), TNFα (Iyngkaran et al., 1995, Hober et al., 1996, Bethell et al., 1998, Azeredo et al., 2001), and monocyte chemotactic protein-1 (MCP-1) (Sierra et al., 2010). Furthermore,

these cytokines are associated with secondary infections and severe disease in DHF/DSS patients, implying that severe dengue disease is indeed correlated to immunopathogenesis (figure 15).

It is not fully understood how these cytokines cause malfunction of vascular endothelial cells leading to plasma leakage. A Th1-type response is linked to recovery from acute infection, whereas a Th2-type response is associated with exacerbation of infection and a poor clinical outcome. Patients with DF predominantly have a Th1-type response. Cross-regulation of Th1 and Th2 is primarily mediated by IL-10 and IFNγ, respectively (Mosmann and Sad, 1996). In addition, activated macrophages recruit CD4+ T-cells that produce human cytotoxic factor (hCF), which in turn induces a cytokine cascade that leads to a Th1-type or Th2-type response (Chaturvedi et al., 2000). Levels of hCF can be elevated in severe dengue cases and hCF autoantibodies protect against severe disease (Chaturvedi et al., 2001). As the severity of the illness increases, the response shifts to a Th2-type response, characterized by secretion of IL-4, IL-5, IL-6, IL-10, and IL-13. Infections primarily eliciting a humoral immune response induce a higher expression of Th2-related cytokines (Mosmann and Sad, 1996, Chaturvedi et al., 2000).

Pathological features of DHF are increased capillary permeability in the absence of morphological damage to the capillary endothelium, altered number and functions of leukocytes, increased haematocrit and thrombocytopenia. Thrombocytopenia is accompanied by plasma leaking and deregulated coagulation, and the latter is likely to be mediated by cytokines, *e.g.* TNFα (Chen et al., 2007). Increased levels of IL-6 and IL-8 are associated with deregulated coagulation and fibrinolysis in dengue (Lei et al., 2001, Martina et al., 2009). Thus, it is believed that indirect effects of virus infection render the vascular wall permeable. Secreted TNFα from activated, infected cells promotes increased endothelial permeability and increases the expression of adhesion molecules on endothelial cells (Kallmann et al., 2000, Madan et al., 2002, Dagia and Goetz, 2003, Javaid et al., 2003), whereas increased IL-10 levels correlate to reduced levels of platelets and reduced platelet function (Anderson et al., 1997, Libraty et al., 2002a). This could contribute to the development of bleeding complications. Extensive plasma leakage into various serous cavities of the body, including the pleural, pericardial and peritoneal cavities, may result in profound shock.

The immunopathogenesis of dengue has to a large extent been correlative in nature describing temporal associations between cytokine concentrations and the clinical events of plasma leakage. There is a need to identify causal immunopathogenic mechanisms compared to the abundance of descriptive studies of dengue pathogenesis. It is worth noting that other infectious diseases and inflammatory disorders with elevated cytokine levels are not accompanied by increased vascular permeability as seen in severe dengue. Thus, the challenge is to identify the key elements of the host immune response that are causally linked to papillary permeability from those that constitute the normal host immune response.

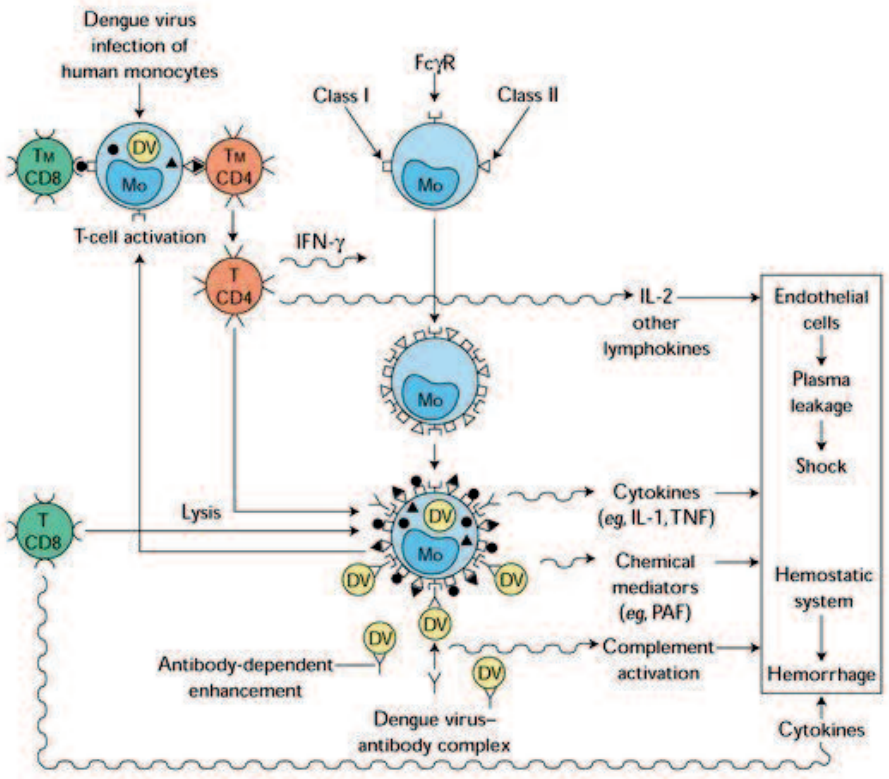


Figure 15. Pathogenesis of DHF and DSS. DENV initially infects a cell of the DC/macrophage/monocyte lineage via receptor-mediated endocytosis and/or enhanced uptake via antibody-virus complexes attached to FcγRs. The heterotypic antibody is not protective, but cross-reacting antibodies attach to the virus and enhance macrophage infection through Fc receptor–mediated endocytosis (as depicted in the bottom part). The secondary antibody response to the new infecting DENV plus the enhanced virus output lead to formation of phlogistic immune complexes. The resulting activated complement components may cause damage directly, and also through interactions with the coagulation system. The cellular immune system is also important. (DV, dengue virus; PAF, platelet activating factor). Memory T-cells (Tm) that recognize cross-reacting epitopes on the new DENV species are activated, proliferate, and secrete IFNγ, TNF, and other cytokines. IFNγ upregulates the FcRs on the macrophages, further increasing the ADE of infection of the dengue target cell. The T-cells also interact with macrophages, with cross-reactive CD8+ T cells able to lyse infected macrophages. The result of this extensive cytokine release and other mediators is the abrupt onset of a shock state due to enhanced vascular permeability. This is referred to as DSS and changes in vascular permeability in DENV infections have classically been measured by monitoring levels of albumin in the plasma. At the time of defervescence, the vascular leak leads to a rising haematocrit as serum proteins leave the vascular system. The resulting falling blood pressure can usually be treated effectively with infusions of crystalloid or, at times, colloid until the condition reverses. *Reprinted by permission from ImagesMD, Viral Hemorrhagic Fevers in Atlas of Infectious Diseases, Peters, et al., 2002 Jan, © 1997, 2004 by Current Medicine LLC.*

4.1.5 Related hemorrhagic viruses

Viral hemorrhagic fever (VHF) is a clinical definition incorporating several diseases caused by viruses from four families: *Arenaviridae, Bunyaviridae, Filoviridae*, and *Flaviviridae* (table 2). Common for these viruses are that they are zoonoses and have a single-stranded RNA genome. Since these viruses are zoonotic agents, their epidemiology is linked to the host-human relation, and the main causes of their outbreaks are associated

to recent ecological perturbations induced by the growing human population. Rodent-borne viruses such as the hantaviruses and the arenaviruses shed infectious particles in urine, faeces and saliva throughout the life of their rodent host (Bowen et al., 1997). Outbreaks are limited to specific geographical regions where these animals live, and humans are infected either by the respiratory route through aerosols contaminated by the urine or faeces of the rodents, or by direct contact of mucous membranes or skin abrasions with excrement or blood of the animal.

In contrast to rodent-borne viruses, mosquito-borne viruses allow large and often unpredictable epidemics. Abundance of water is clearly associated with outbreaks, and freshwater areas created by dams provide breeding sites for mosquitoes and attract humans. Tick-borne viruses, on the other hand, have little opportunity to cause large human epidemics since ticks wait on a vegetative support until an animal/human passes by. The large distribution of ticks, however, is suggested to be due to migratory birds carrying ticks.

The pathogenesis of VHF is not clearly understood, and may vary according to virus. The general symptoms are similar (headache, myalgia, arthralgia and nausea), and the main cause of death is hypovolemic shock due to plasma leakage at the capillary level (Le Guenno, 2001). Macrophages and endothelial cells are assumed to be the most important targets for viral replication (Lewis et al., 1989), except for YFV. These target cells produce TNFα and IL-6 in addition to other proinflammatory cytokines when infected. Thrombocytopenia and/or platelet dysfunction are thought to be due to either infection of endothelial cells causing modifications of the cell membranes, favoring platelet aggregation, or cytokine-mediated actions that inhibit platelet production. Cytokines, coagulation factors and complement factors are involved, but the exact mechanisms underlying VHF are poorly understood (Le Guenno, 2001).

Family	Genus	Virus	Disease	Vector	Geographic location
Arenaviridae	Arenavirus	Junin	Argentinean HF	Rodents	South America
		Machupo	Bolivian HF	Rodents	South America
		Guanarito	Venezuelan HF	Rodents	South America
		Sabia	Brazilian HF	Rodents	South America
		Lassa	Lassa fever	Rodents	South America
Bunyaviridae	Phlebovirus	Rift Valley Fever	Rift Valley Fever	Mosquitoes	Africa (south of Sahara)
	Nairovirus	Crimean-Congo HF	CCHF	Ticks	Asia, Europe, Africa
	Hantavirus	Hantaan	HFRS	Rodents	Asia
		Seoul	HFRS	Rodents	Asia and world-wide
		Belgrade/Dobrava	HFRS	Rodents	Europe
Filoviridae	Filovirus	Marburg	HF	Unknown	Africa
		Ebola	HF	Unknown	Africa
Flaviviridae	Flavivirus	Yellow Fever	Yellow fever	Mosquitoes	South America, Africa
		Dengue 1-4	DHF, DSS	Mosquitoes	South America, Africa, Asia, Oceania
		Kyasanur Forest	Kyasanur Forest disease	Ticks	South Asia

Table 2. HF, hemorrhagic fever, CCHF; Crimean-Congo hemorrhagic fever; HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.

5 ANIMAL MODELS OF DENGUE VIRUS INFECTION AND DISEASE

The pathogenesis of DENV infections is, despite intensive research, not well understood. Many fundamental questions in dengue pathogenesis are difficult to address due to the lack of appropriate animal models of infection and disease. No non-human species naturally exhibits the more severe forms of dengue disease that mimic human DF, DHF, and DSS, and this has hampered the development of a suitable animal model.

5.1 NON-HUMAN PRIMATES

NHPs are the only vertebrates apart from humans known to be naturally infected by DENV. The viral strains isolated from NHPs, however, are genetically distinct from those infecting humans indicating that these transmission cycles diverged a long time ago (Wang et al., 2000). In addition, NHPs do not develop DHF, but only a mild infection.

Animal models of DENV infection were however initially limited to NHP despite the fact that NHPs only develop mild viremia without severe clinical symptoms. Attempts to induce dengue-like disease in other animals failed and the suboptimal NHP models remained the only available model for a long time. Studies in rhesus macaques revealed no abnormalities in haematocrit or prothrombin time, and only a minority of animals displayed a limited decrease in platelet counts (Halstead et al., 1973b, Halstead et al., 1973c, Halstead et al., 1973a). Thus, DENV infection in macaques has been unable to offer any major insights into the molecular mechanisms of DENV pathogenesis.

In contrast, NHPs are widely used in vaccine testing as they are capable of developing neutralizing antibodies in response to DENV infection (Bente and Rico-Hesse, 2006).

5.2 MOUSE MODELS

Rodents are susceptible to DENV infection although they do not exhibit disease similar to that in human DENV infections. To compensate for these limitations, murine models have relied on mouse-adapted DENVs that appear to be attenuated with respect to human infection. New mouse models have been developed to render the mice more susceptible to infection, but none have been able to mimic DENV-specific immune responses.

Mouse models based on intracranial inoculation of DENV have for long been used in parallel to studies in NHPs. Mice usually exhibit a neurovirulent phenotype quite unlike human dengue disease, and succumb to intracranial infection (Bente and Rico-Hesse, 2006). Multiple efforts have generated a diverse set of mouse models for DENV infection, each with distinct advantages and disadvantages (Yauch and Shresta, 2008). The various approaches can be divided into four groups:

* Immunocompetent mouse models
* Severe combined immunodeficient (SCID)-tumor transplant mouse models
* Humanized mouse models
* Interferon-deficient mouse models

Consequently, the various models have contributed in different ways to understanding mechanisms underlying DENV pathogenesis and immunity, and in the development of antiviral drugs and vaccines.

5.2.1 Immunocompetent mouse models

The unimpaired nature of the immune system renders the immunocompetent mice poorly susceptible for DENV infection. Despite the lack of clinical symptoms, these models have been used extensively to study other aspects of DENV pathogenesis (Barreto et al., 2007, Paes et al., 2005, Huang et al., 2000, Chen et al., 2007). In addition, immunocompetent mouse models of DENV infection have been popular for drug and vaccine development studies since the intact immune system is valuable for assessing vaccine immunogenicity.

Clinical and neurological disease can be induced by high-dose infection and/or intracranial injection, as well as mouse-adapted DENV strains that render mice more susceptible to DENV infection (Atrasheuskaya et al., 2003, Zulueta et al., 2006). The relevance of observations made with manipulated DENV strains, however, should be interpreted with caution regarding their relevance to wild-type infections.

5.2.2 Severe combined immunodeficient tumor transplant mouse models

Measurable viremia is a desired trait when studying DENV infection and a common strategy to render the mouse more susceptible to DENV infection is to manipulate the mouse in one or several ways. Transplanted human tumor mass that provides a replication site for infectious DENVs have been applied in SCID mice. Since SCID mice lack an adaptive immune system, DENV infection has been successfully established within the transplanted cells and even some dengue disease features have been reproduced (An et al., 1999, Lin et al., 1998, Blaney et al., 2002). However, it is unclear how any insights into pathogenesis might apply to human pathogenesis since viral replication in restricted primarily to the transplanted cells.

5.2.3 Humanized mouse models

An improved strategy based on the SCID-tumor model is ‘humanized’ SCID mice that are irradiated to destroy the haematopoietic progenitors in the bone marrow, prior to transplantation with human CD34+ haematopoietic stem cells (figure 16). The result is an adaptive immune system consisting exclusively of human cells, with certain parts of the innate system being humanized as well (Bente et al., 2005, Kuruvilla et al., 2007). The advantage with this model is the increased susceptibility to clinical DENV isolates without adaptation to mice, and the mice display some signs of human disease. The exact cell types in which DENV replicates in humanized mice remains to be identified.

The difficulties with the humanized mouse model are multiple, such as the genetic variation in stem cell donors, the hardship to generate sufficient numbers of humanized mice, variability in the degree of human cell engraftment, and radiation sensitivity of SCID-mice (Shultz et al., 1995). A limitation of crucial importance is the lack of lymph nodes that support a human immune system (Cao et al., 1995, DiSanto et al., 1995, Ohbo et al., 1996), as well as the lack of a robust immune response in these humanized mice; both fluctuations in the elicited immune response as well as poor reproducibility (Ifversen and Borrebaeck, 1996, Murphy et al., 1996). Human-specific cytokines required for human cell development and survival are not successfully generated, and there is a low and variable level of T-cell-dependent antibody responses (Shultz et al., 2005, Ishikawa et al., 2005). Thus, humanized mouse models are time-consuming and labor-intensive and,

therefore, not optimal for most drug and vaccine development purposes. These new models may eventually have the potential to answer important and fundamental questions concerning infection of human cells *in vivo*, but there are still major issues to be resolved regarding stem cell development in order to obtain a functional and intact adaptive immune system.

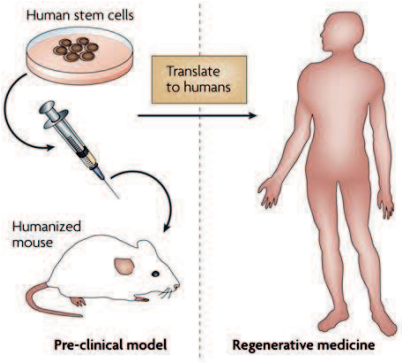


Figure 16. This figure shows schematically the proposed use of humanized mice as a pre-clinical bridge between basic *in vitro* studies and the *in vivo* analysis. On the left (top) are cultures of human stem cells (embryonic stem-cell-derived and adult stem-cell-derived populations) generated *in vitro* and available for analysis of *in vivo* efficacy in humanized mice. Depicted bottom left is a humanized mouse engrafted with human stem cells by various routes of injection or implantation. *Reprinted by permission from Macmillan Publishers Ltd, Nature Reviews Immunology, Shultz et. al., 2007 Feb; 7: 118–130, © 2007.*

5.2.4 Interferon-deficient mouse models

Another way to increase the susceptibility of mice to DENV infection is to generate mice deficient in the IFN pathway (Johnson and Roehrig, 1999, Shresta et al., 2004). The IFN system is potent suppressor of DENV replication, and mice lacking the IFN-α/β and –γ receptors (AG129 mice) can be lethally challenged without intracranial inoculation (Johnson and Roehrig, 1999). The AG129 mouse strain exhibits viral replication in relevant peripheral cell types like macrophages and DCs in spleen and lymph node (Kyle et al., 2007), hepatocytes, and myeloid cells in bone marrow (Balsitis et al., 2009).

A variety of DENV isolates have been used and the clinical symptoms are either neurological disease or a DSS-like vascular permeability syndrome depending on the virus isolate and route of inoculation (Huang et al., 2003, Johnson and Roehrig, 1999, Kyle et al., 2007, Lee et al., 2006b, Schul et al., 2007, Shresta et al., 2006, Stein et al., 2008). Consequently, AG129 mice have been used for numerous studies including tropism, pathogenesis, immune protection and enhancement, as well as for antiviral drug testing and vaccine development (Johnson and Roehrig, 1999, Kyle et al., 2007, Kyle et al., 2008, Schul et al., 2007, Shresta et al., 2004, Shresta et al., 2006, Prestwood et al., 2008). The genetically deficient background does not, however, make the AG129 mouse suitable for genetic studies since it would be time-consuming to introduce a third knock-out gene.

6 RISK FACTORS FOR SEVERE DENGUE

Although the exact mechanisms underlying severe dengue are not known, some risk factors that influence disease outcome have been identified. Among these, host determinants are a key issue in susceptibility to severe dengue disease and include age, chronic disease, ethnicity, and host genotype. An additional contributing risk factor that plays a major role in disease outcome is secondary infection by a heterologous DENV serotype. Finally, the virus serotype and genotype also influence the symptomatic picture of disease and outcome (figure 17). These observations were initially based on epidemiological findings, but accumulating laboratory and experimental data have contributed to the recognition of DENV virulence as an important risk factor.

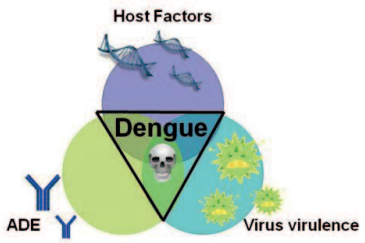


Figure 17. The complex interplay of risk factors for severe dengue disease can be illustrated as a triangular interplay dominated by the three main risk factors: host factors, pre-existing DENV-specific antibodies mediating ADE, and intrinsic virus features influencing strain virulence. The exact contribution of each risk factor may vary from case to case.

6.1 HOST GENETICS

Apart from the influence of viral genetic determinants, the host's genetic background with varying polymorphisms might have important consequences for disease susceptibility (Casanova and Abel, 2007). Improvements in high-throughput genotyping of genetic polymorphisms have permitted a genome-wide approach to the investigation of host genetic susceptibility. Most studies, however, have not attempted functional trials to try to link genetic association with any process in disease pathogenesis.

Indirect evidence of the host's genetic importance has been derived from Cuban dengue epidemics where a reduced risk for DHF/DSS was observed in those with an African ancestry compared to those with European ancestry (Bravo et al., 1987, Gonzalez et al., 2005, Guzman et al., 1990, Guzman et al., 1999, Guzman and Kouri, 2002, Sierra et al., 2007). The Cuban observations coincide with the low susceptibility to DHF reported in African and Black Caribbean populations (Halstead et al., 2001, Saluzzo et al., 1986b). It is interesting that despite the circulation of DENV in 19 African countries, there are only sporadic reports of DHF cases (Gubler et al., 1986, Saluzzo et al., 1986a, Sharp et al., 1995).

In order to better understand these population differences, the polymorphic HLA genes have been among the most studied candidates for genetic associations with DHF/DSS. Several serological studies of HLA class I alleles have been performed in ethnically and geographically distinct populations, and positive correlations of various HLA class I alleles with susceptibility to DHF have been found (Chiewsilp et al., 1981, Loke et al., 2001, Paradoa Perez et al., 1987). A significantly higher frequency of HLA class I alleles A*31 and B*15 have been found in Cuban individuals with symptomatic DENV infection compared to asymptomatic controls, who showed an elevated frequency of HLA II alleles DRB1*07 and DRB1*04 (Paradoa Perez et al., 1987, Sierra et al., 2007). The DRB1*04

was also the most frequent allele associated with resistance to DHF in the Mexican Mestizo populations of the Americas (LaFleur et al., 2002). Since the Mexican Mestizo population and the Cuban population share the same Amerindian genetic background, it is possible that the identification of the same HLA class II allotype could explain the association to dengue disease protection (Centro, 1976, Rivero de la Calle, 1984).

A case-control study in ethnic Thai cases also reported the association of HLA class I alleles (A2, A*0207, B46, B51) with different clinical outcomes (Stephens et al., 2002). A similar study in a Vietnamese population confirmed the association with polymorphism of the HLA class I loci and DHF susceptibility. The same study also found that polymorphisms in the HLA-DRB1 allele are not associated with DHF susceptibility, highlighting the findings in the Amerindian populations (Loke et al., 2001).

The number of studies on polymorphisms within genes other than the HLA loci remains low. Variants of the vitamin D receptor and the FcγRIIA gene are associated with resistance to severe dengue (Loke et al., 2002). Additionally, an allelic variant of the DC-SIGN1 coding gene CD209 is believed to protect against DHF(Despres et al., 2005).

6.1.1 Host health and age

An increased association between severe dengue and bronchial asthma, diabetes mellitus, peptic ulcers, and sickle cell anaemia has been observed (Rigau-Perez, 2006, Rigau-Perez and Laufer, 2006, Bravo et al., 1987, Diaz et al., 1988, Gonzalez et al., 2005, Limonta et al., 2008, Valdes et al., 1999, Guzman et al., 1999). The impact of dengue on chronic diseases and other pathogens, however, needs to be further investigated.

Primary infections are supposed to cause mild disease in children, compared to secondary infections that tend to lead to severe dengue (Egger and Coleman, 2007). In South-East Asia, DHF/DSS is predominantly an illness of children. The greater relative prevalence of DSS in children relative to adults is believed to be due to the intrinsically more permeable vascular endothelium in children (Gamble et al., 2000). There is no clear consensus; studies conducted in South American countries have reported similar (Guzman et al., 2002a) as well as contradictory results indicating that adults are the most affected (Rigau-Perez et al., 2001).

6.1.2 Autoimmune responses in dengue virus infection

Anti-DENV antibodies can cross-react to host proteins and endothelial cells, and this could enhance the endothelial dysfunction observed in DHF/DSS. Antibodies against the viral surface E protein cross-react with plasminogen and have been associated with bleeding in acute DENV infection (Chungue et al., 1994, Markoff et al., 1991), and anti-DENV NS1 antibodies cross-react to host proteins and endothelial cells (Lin et al., 2006, Falconar, 1997). Additionally, immune activation markers (*e.g.* IL-6, IL-8, TNFα, IFNγ, and complement components 3A and 5A) together with altered platelet, DC, monocyte, and T-cell functions suggest that immune responses to various DENV components could contribute to autoimmune processes resulting in DHF/DSS (Falconar, 1997, Lin et al., 2002a, Lin et al., 2003).

6.2 ANTIBODY-DEPENDENT ENHANCEMENT

A secondary infection by a heterologous DENV serotype is an important risk factor for developing DHF/DSS. The explanation lies within the cross-reactive antibodies raised after a primary DENV infection (Endy et al., 2002, Kurane et al., 1998, Kurane, 2007,

Thaung et al., 1975, Burke et al., 1988, Sangkawibha et al., 1984). Serotype-specific antibodies confer life-long immunity to the homologous serotype, whereas cross-protection against heterologous serotypes last for 3-4 months (Sabin, 1952). Beyond this time period of cross-protection, the pre-existing antibodies of sub-neutralizing concentration will instead cross-react with the heterologous virus facilitating viral infection of FcγR bearing cells (figure 18). This phenomenon is known as *Antibody-dependent enhancement* (ADE) (Mady et al., 1991, Green and Rothman, 2006, Kurane, 2007, Littaua et al., 1990). The limited cross-protection between the four DENV serotypes has allowed them to coexist in the same or overlapping geographical areas. Thus, their antigenic uniqueness has implied an evolutionary advantage (Ferguson et al., 1999).

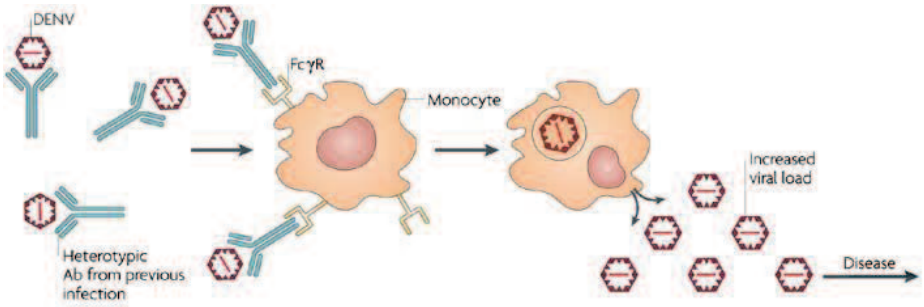
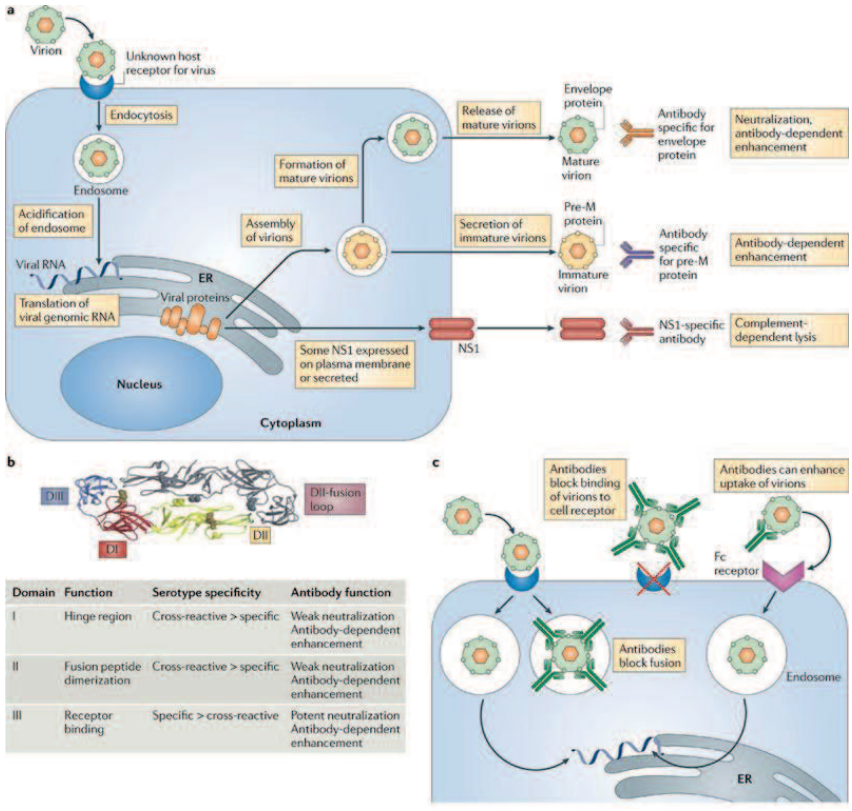


Figure 18. ADE of virus replication occurs when heterotypic, non-neutralizing antibodies present in the host from a primary DENV infection binds to an infecting DENV particle during a subsequent heterotypic infection but cannot neutralize the virus. Instead, the antibody–virus complex attaches to the FcγR on circulating monocytes, thereby facilitating the infection of FcγR cell types in the body not readily infected in the absence of antibodies. The final outcome is an increase in overall viral replication, potentially leading to more severe disease (Vaughn et al., 2000). *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Stephen, et al., 2007 July; 5:518-528, © 2007.*

Low-affinity/sub-neutralizing antibodies and DENV form virus-antibody immune complexes that bind to FcγRs on monocytes. The net result will be a larger number of infected cells compared to the primary infection when there were no cross-reactive antibodies present, or compared to earlier after the primary infection when antibody levels are high enough to achieve neutralization of the heterologous virus. Hence, the viral biomass will be larger during a secondary DENV infection compared to during a primary DENV infection.

In vitro studies indicate that non-neutralizing antibodies against the viral prM protein can potentially mediate ADE. These anti-prM antibodies are in addition non-neutralizing even at high concentrations (Dejnirattisai et al., 2010, Rodenhuis-Zybert et al., 2010). The proposed hypothesis for prM-mediated ADE is based on the fact that the viral prM protein needs to be cleaved to render the virus infectious. Hence, immature virus particles that would otherwise be non- or less-infectious are rendered infectious in combination with anti-prM antibodies that mediate ADE to infect new host cells (Dejnirattisai et al., 2010) (figure 19).



Domain	Function	Serotype specificity	Antibody function
I	Hinge region	Cross-reactive > specific	Weak neutralization Antibody-dependent enhancement
II	Fusion peptide dimerization	Cross-reactive > specific	Weak neutralization Antibody-dependent enhancement
III	Receptor binding	Specific > cross-reactive	Potent neutralization Antibody-dependent enhancement

Figure 19. (a). The DENV life cycle and antigen sources are shown. Dengue virions bind to cell surface receptors (these have not been completely characterized), and the virions are internalized through endocytosis. Acidification of the endocytic vesicle leads to rearrangement of the surface E glycoprotein, fusion of the viral and vesicle membranes and release of vRNA into the cytoplasm. Viral genomic RNA is then translated to produce viral proteins in ER-derived membrane structures, and the viral proteins and newly synthesized vRNA assemble into immature virions within the ER lumen. Cleavage of the viral prM protein by the host cell enzyme furin leads to the formation of mature virions, which are secreted from the cell. In addition, some of the synthesized NS1 protein is expressed on the plasma membrane or secreted, and some virions are secreted in an immature form. Mature and immature virions induce antibody responses to the E protein, and these antibodies can function in neutralization or in ADE of infection. Immature virions also induce antibody responses to the prM protein. Antibodies specific for NS1 can interact with membrane-bound NS1 and cause complement-dependent lysis of virus-infected cells. (b). The structure of the DENV E glycoprotein ectodomain and characteristics of E protein-specific antibodies are shown. The three domains of the E protein are coloured in red (domain I), yellow (domain II) and blue (domain III). (c). The mechanisms of neutralization and enhancement by DENV-specific antibodies are shown. At high levels of epitope occupancy, antibodies can block the binding of virions to the cellular receptor or can block fusion at a post-binding stage. At lower epitope occupancy levels, antibodies can enhance the uptake of virions into cells by interacting with Ig (Fc) receptors. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Immunology, Alan, et al., 2011 Aug; 11: 532-543, © 2011.*

The time interval between heterotypic DENV infections is another parameter influencing the magnitude of ADE; a longer interval between heterologous DENV infections causes higher DHF/DSS ratios (Guzman et al., 2002b). The differences in DENV genotype could influence the pathogenic consequences, but a contributing risk factor is the progressive loss of heterotypic neutralizing antibodies (Guzman et al., 2007). The time effect of

DENV-specific antibodies can be seen in DENV-immune mothers and their infants. Before the age of 3-4 months, the maternally derived DENV-specific antibodies confer protection against a DENV infection. Primary infections in infants aged between 4 and 12 months of age, however, run a higher risk of developing severe dengue due to maternally derived non-neutralizing antibodies. The risk of severe dengue decreases after the age of 1 year as the concentration of cross-reactive antibodies declines (Kliks et al., 1988, Chau et al., 2009, Pengsaa et al., 2006).

A higher viral burden elicits a greater host inflammatory response and increased plasma levels of proinflammatory cytokines. Secondary DENV infections and severe disease in DHF/DSS patients with severe disease have elevated serum levels of IL-2, IL-6, IL-8, IL-10, IL-13, IL-18, IFNγ, TNFα, and MCP-1 (Azeredo et al., 2001, Chakravarti and Kumaria, 2006, Nguyen et al., 2004, Mustafa et al., 2001, Perez et al., 2004, Pinto et al., 1999, Yang et al., 1995b). Thus, an increased infected cell mass would stimulate T-cell and cytokine responses that are proportional to the antigenic stimulus. This hypothesis is consistent with the observations that a high initial viremia or high NS1 concentrations in blood during secondary infections are associated with DHF/DSS (Libraty et al., 2002a, Libraty et al., 2002b, Vaughn et al., 2000, Wang et al., 2003).

Accumulating evidence questions whether ADE of infection alone is sufficient to explain DHF/DSS (Vaughn et al., 2000). Severe dengue with plasma leakage can occur in primary infection without ADE. In addition, by the time plasma leakage occurs, viral titers are several logs below peak levels, and there are patients with high viral titers that do not develop plasma leakage (Vaughn et al., 2000, Vaughn et al., 1997, Libraty et al., 2002a). Thus, increased viremia alone is not the direct cause of plasma leakage and other mechanisms are involved in the cytokine storm. Furthermore, ADE is not a useful correlate of disease risk (Laoprasopwattana et al., 2005, Libraty et al., 2009).

6.3 DENGUE VIRULENCE

ADE has dominated as the explanatory model for severe dengue disease in secondary infections. Evidence for ADE in humans, however, is indirect and controversial results against ADE exist (Endy et al., 2004, Laoprasopwattana et al., 2005). Many parts of the world have become hyperendemic, implying that all four serotypes of DENV co-circulate in the same country (figure 20 and 21), with the consequence that secondary infections are common scenarios. Epidemiological data also indicate that not all secondary infections cause DHF/DSS, and that there are even cases of tertiary and quaternary DENV infections (Halstead, 2007). Studies from Thailand report that 0.08-0.8 % of dengue hospitalizations may be caused by tertiary and quaternary DENV infections (Gibbons et al., 2007). In Cuba, 17.5 % of the total DHF dengue cases were caused by third or fourth infections (Alvarez et al., 2006b).

The four DENV serotypes 1-4 diverge at ~30 % across the polyprotein (Westaway, 1997), but each serotype also consists of phylogenetically distinct 'subtypes' or 'genotypes', which have different geographical distributions (Monath et al., 1986, Trent et al., 1989). The hypothesis that some DENV genotypes have a greater virulence and epidemic potential than others was introduced during the 1970's around the same time that the ADE phenomenon was coined (Barnes and Rosen, 1974, Rosen, 1977, Gubler et al., 1981). In contrast to the ADE hypothesis, however, experimental evidence for increased virulence was for long absent and, therefore, primarily based on epidemiological observations. Recent work has shed light on this question and confirmed

what Rosen, *et al.* proposed almost four decades ago (Barnes and Rosen, 1974, Rosen, 1977).

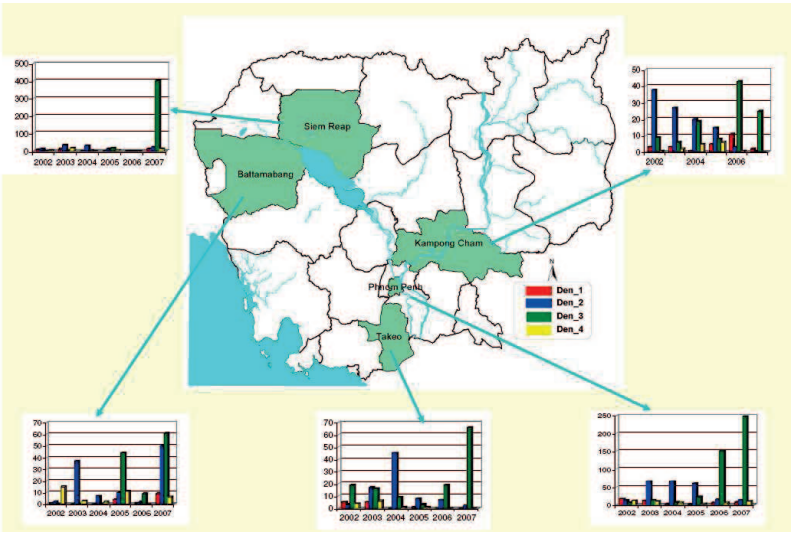


Figure 20. Cambodia in South-East Asia is a hyper-endemic country where all four DENV serotypes are circulating. As a direct result of the presence of multiple DENV serotypes, secondary infections are common. Data come from five sentinel hospitals, coordinated by the Pasteur Institute in Cambodia, from five geographic regions conducting DENV surveillance and serology by ELISA IgM and IgG tests and RT-PCR. *Courtesy Prof. Ngan Chantha, Asia-Pacific Dengue Program Managers Meeting, Singapore 2008, p. 48.*

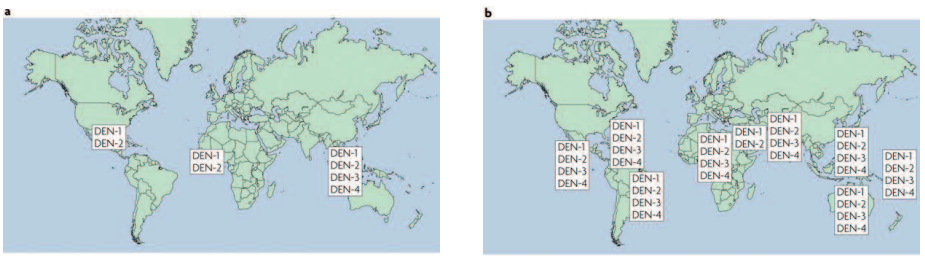


Figure 21. The distribution of DENV has increased dramatically during the last decades. The figure illustrates the distribution in (a). 1970, and (b). 2004. *Reprinted by permission from Nature Publishing Group Ltd, Nature Reviews Microbiology, Guzman, et al.,2010 Dec; S7-S16, copyright 2010.*

There have been specific geographic examples of the appearance of DENV genotypes correlating to DHF/DSS epidemics. The appearance of a South-East Asian DENV-2 strain in the Americas in 1981 resulted in the sudden emergence of DHF/DSS cases. It turned out that DENV-2 could be subdivided into a variety of genotypes, minimally Asian and American. The Asian genotype is more virulent and more likely to result in DHF/DSS than the American genotype even after a secondary infection (Rico-Hesse et al., 1997, Rico-Hesse, 1990). Viruses to the South-East Asian DENV-2 lineage replicate to higher titers in human DCs than American genotype viruses. It was also seen that the South-East Asian genotype infects and disseminates to the head tissue of *Ae. aegypti* mosquitoes more rapidly and in a greater proportion compared to the American genotype

viruses (Anderson and Rico-Hesse, 2006, Armstrong and Rico-Hesse, 2001, Armstrong and Rico-Hesse, 2003, Cologna et al., 2005).

The emergence of group B subtype III DENV-3 strain in Sri Lanka in 1989 is another example of clade replacement correlating with an increase in DHF/DSS (Messer et al., 2002, Messer et al., 2003, Kanakaratne et al., 2009). As for the South-East Asian DENV-2 strain, the invasive DENV-3 strain replicated to higher levels in mosquitoes and disseminated to the head tissue more readily than the displaced, native DENV-3 strain from Sri Lanka (Hanley et al., 2008). Both traits likely enhanced the capacity to spread and displace endemic strains.

Based on the examples given, one hypothetical mechanism for increased virulence suggests that highly pathogenic DENV strains have been selected for enhanced ability to replicate in key human targets, such as macrophages and DCs (Cologna et al., 2005, Cologna and Rico-Hesse, 2003, Vasilakis et al., 2007). Thus, virulent DENVs would produce more viruses per cell, resulting in higher viremia and inflammatory response, than with a low pathogenic strain (Cologna and Rico-Hesse, 2003, Leitmeyer et al., 1999).

The second hypothesis for increased virulence proposes that strains associated with severe DHF/DSS better escape neutralization by serotype cross-reactive antibodies present in the semi-immune host compared to strains associated with DF (Kochel et al., 2005). Enhancement of virus replication following heterologous infection may favor coexistence of multiple serotypes. If such enhancement also results in increased transmission, DENVs from different serotypes would benefit from prior and concurrent circulation of several serotypes in the same location (Ferguson et al., 1999).

It is still not known if the tendency of certain genotypes to cause severe disease results from greater intrinsic virulence, or if greater virulence is a result of enhanced infectivity in the presence of heterologous antibodies, or a combination of the two. Determining whether DENVs differ in virulence, as well as identifying the genetic basis of such differences, is of fundamental importance. This has been the primary focus of this thesis.

7 AIMS OF THE STUDY

Despite the global burden of dengue and intensive research, the mechanisms underlying DENV pathology are not well understood. Dengue research has been hampered by a lack of appropriate animal models of infection and disease. Furthermore, fundamental knowledge such as host cell tropism and virulence markers are still not established. The overall aim of this thesis was to identify viral features involved in dengue virulence by *in vitro* and *in vivo* characterization of clinical DENV. All four serotypes and dengue disease conditions (DF, DHF, or DSS) are represented, and only DENVs passaged once have been used in order to resemble wild-type viruses as much as possible.

The specific aims were:

1. To understand the self-priming phenomenon of the DENV genome and its implications for the development of a strand-specific qRT-PCR for DENV. A strand-specific qRT-PCR would be a valuable tool for identification of target cells for DENV replication.

2. To investigate if clinical DENV isolates from patients experiencing the various forms of dengue (DF, DHF, and DSS) can be differentiated based on their replication profiles and/or apoptosis induction in mammalian Vero cells and mosquito C6/36 cells, and to determine whether any particular virus phenotype correlates with clinical disease severity.

3. To extend the *in vitro* characterization of clinical DENV isolates to *in vivo* conditions using a mouse model and study replication kinetics and organ tropism.

4. To compare the genomes from clinical DENV isolates from all four serotypes and to investigate whether any particular genotype correlates with clinical disease severity and our findings from the *in vitro* and *in vivo* experiments.

8 RESULTS AND DISCUSSION

Below follows a summary of the key findings in each scientific paper included in this thesis. Please see the paper *per se* for details regarding material and methods, complete results including tables and figures, and a comprehensive discussion.

8.1 SELFPRIMING OF THE DENGUE VIRUS GENOME (paper I)

Understanding of the pathophysiological events leading to dengue disease remains limited partly because viral target cells in humans are poorly characterized. One of the most sensitive methods to detect cells supporting active replication of DENV is by identification of the replicative intermediate, the viral anti-genome of negative single-stranded polarity, with RT-PCR.

Attempts to develop a strand-specific qRT-PCR to identify primary target cells for DENV replication, was hampered due to the phenomenon of self-priming of the viral genome. This false priming of the reverse transcriptase (RT) prevented strand-specific detection. The results of the study showed that this event corresponds to cDNA synthesis independent of exogenous primers.

Self-priming applied to all RNAs tested, including negative-sense RNA and cellular mRNA, and was not restricted to any particular regions of the viral genome. Thus, the lack of a poly(A)-tail at the 3' end was not a requirement for self-priming since cellular mRNA (18S and β-actin) were also found to yield cDNA in the absence of exogenous primers. *In vitro* transcribed templates revealed that endogenous priming was not restricted to specific template sequences. Furthermore, self-priming of the template was not associated with small free nucleic acids, such as tRNAs and microRNAs.

Interestingly, self-primed cDNA was slightly shorter than DNA-primed cDNA, suggesting that the 3' end of the RNA looped back on itself to form a short and transient RNA-RNA duplex potentially serving as a primer for the RT. This model is conceivable since the minimal length requirement for AMV RT priming by DNA can be as short as four nucleotides (Falvey et al., 1976). Destabilization of secondary RNA structures by addition of DMSO (10 % of the final concentration), and increased RT temperature to 63˚C did not abolish the intrinsic feature of self-priming.

Hence, self-priming can occur in samples where DNA priming occurs and a significant amount of unexpected cDNA is synthesized, presenting a limitation when designing strand-specific RT-PCR. First-strand synthesis of the DENV genome *in situ* is believed to be due to spontaneous loop-back structures functioning as transient primers for the RT, originating at the 3' end.

The phenomenon of self-priming could explain why many assays proposed for detection of a replicative intermediate are suboptimal and lack specificity. The issue of self-priming needs to be considered when developing molecular methods for virus replication studies, *e.g.* target cell identification.

8.2 *IN VITRO* CHARACTERIZATION (paper II and IV)

DENV strain variations have been acknowledged as a risk factor for severe dengue disease and epidemiological data indicates that virulence differs among DENV genotypes

within the same serotype (Rico-Hesse, 1990, Cologna and Rico-Hesse, 2003, Leitmeyer et al., 1999, Anderson and Rico-Hesse, 2006, Rico-Hesse et al., 1997). Except for attenuated strains generated for vaccine purposes, there is no phenotypic profile of a "virulent" or "avirulent" DENV strain. No specific genetic markers have been identified so far that correlate with increased virulence traits of DENV.

In an attempt to identify viral features involved in virulence, DENV isolates from all four serotypes were characterized phenotypically *in vitro* by analyzing replication kinetics and host cell apoptosis. Human DENV isolates passaged once and originating from Cambodian patients with DF, DHF, or DSS, respectively, were used in order to resemble wild-type viruses as much as possible.

A lower level of replication for all DENV isolates from DSS patients was observed in mammalian Vero cells regardless of serotype compared to DENV isolates from DF patients that all had a higher efficiency of replication. Since Vero cells are commonly used for titration of DENVs, these results have practical implications as wild-type DENVs may have different replication efficiency in these cells.

The difference in viral replication was most notable when measuring viral titers, in contrast to quantifying vRNA copies that almost abolished the observed replication differences. The similar levels of intracellular vRNA despite differences in infectious titer indicate that a significant amount of produced viral particles are not budding competent and/or infectious. DENV virulence is believed to correlate to viral load within the host (Cologna and Rico-Hesse, 2003, Leitmeyer et al., 1999) and defective interfering viral particles have been suggested to influence virulence and interfere in the host immune response (Cave et al., 1985, Huang et al., 1986, Bangham and Kirkwood, 1990). Thus, measuring extracellular infectious viral titer best resembled replication differences between the DENV isolates and the fact similar vRNA levels were observed despite replication differences may have immunological implications in the host during infection.

The replication patterns of isolates from DHF patients were not consistent between the serotypes, resembling either the DF- or DSS-derived DENV isolates. Hence, isolates derived from DHF patients had mixed phenotypes and could not be grouped together with either the DSS or the DF isolates, but instead formed an intermediate group between these two phenotypes.

The DSS isolates were further distinguished from other DENV isolates from milder cases by their ability to induce apoptosis in mosquito C6/36 cells. Apoptosis was not a direct result of viral load since all DENV isolates replicated at equal efficiency in this cell line, and neither the DF, nor the DHF isolates induced apoptosis.

The replication kinetics and apoptosis data gathered groups the four serotypes from DSS cases together, with a common phenotype clearly distinguished from the isolates derived from DF patients. The DHF isolates form an independent group resembling both the DSS and DF isolates. Results showed that phenotypic differences could be associated with different clinical outcome suggesting that DENVs within the same genotype may differ. These intrinsic virus properties could influence pathogenesis in humans.

8.3 *IN VIVO* CHARACTERIZATION IN BALB/C MICE (paper III)

There is no appropriate animal model of DENV infection and disease, and fundamental knowledge such as host cell tropism, and virulence markers are still not established. The aim of this study was to compare wild-type features of DENV-1 isolates passaged only once *in vitro* and never previously inoculated in mouse or cultured in mouse cells prior to direct intravenous inoculation into BALB/c mice. BALB/c mice are susceptible to DENV infection although with discrete pathological features. The mice did not develop clinically apparent disease, and showed only discrete lesions in internal organs. However, vRNA was detected in various organs depending on the inoculated DENV isolate.

Infection with the DF and DHF isolates peaked during the first week and the primary infection sites were lungs, liver, and to a certain extent brain. In contrast, the DSS isolate was primarily neurotropic, infection persisted longer compared to the DF and DHF isolates, and infected the largest number of mice. Neurotropism in mice has been linked with DENV virulence when studying DENV-3 isolated from Brazilian patients (Ferreira et al., 2010). The DSS isolate could be regarded as more virulent than the two other DENV-1 strains since a majority of the DSS-inoculated mice had detectable levels of vRNA. Furthermore, infection by the DSS isolate was less affected by the inoculum dose and persisted longer in a majority of mice.

Cytokines involved in inflammation were quantified in serum collected from inoculated mice and compared in regard to DENV-1 isolate, virus titer inoculated, and time-point post-infection. Overall, the levels of the proinflammatory cytokines (IFNγ, IL-1β, IL-2, IL-6, IL-10, IL-13, MCP-1, RANTES, and TNFα) quantified were low most probably due to poor susceptibility of BALB/c mice to wild-type DENVs. Common for several cytokines, however, was a dose-dependent response to inoculated virus (IFNγ, IL-6, IL-13, and RANTES). In general, the highest levels of measured cytokines were at the beginning of the experimental period (IFNγ, IL-10, and MCP-1 peaked at day 3 post-infection).

A curious finding was that DF-inoculated mice had higher serum levels of IL-10, MCP-1, and RANTES, which are increased in patients with severe DHF and DSS (Chaturvedi et al., 2000, Chaturvedi, 1999, Green et al., 1999, Azeredo et al., 2001, Chakravarti and Kumaria, 2006, Yang et al., 1995a, Yang et al., 1995b). The DHF-and, most notably, the DSS-infected mice had vRNA in predominantly in the brain, which could imply locally high levels of proinflammatory cytokines that do not circulate systemically. This could explain why the IL-10, MCP-1, and RANTES levels dominated in the DF-inoculated mice, since the thoracic and abdominal organs were the primary target for the DF isolate. There was no difference in cytokines like IL-1β, IL-2, and TNFα compared to mock-infected controls and this could be due to the transient nature of many cytokines as well as the limited susceptibility of DENVs in mice.

In summary, these results indicate that DENVs within the same serotype and genotype may differ sufficiently to cause distinct clinical conditions *in vivo*. Furthermore, they confirm a phenotypic distinction observed *in vitro* with the same DENV-1 isolates where the DSS isolate replicated less efficiently in mammalian Vero cells compared to the DF and DHF isolates, but induced apoptosis in mosquito C6/36 cells (paper II). These findings could ultimately provide evidence for differences in virus replication, which in turn could influence the clinical outcomes of the infection, and partially explain some clinical differences observed in humans.

8.4 GENOMIC CHARACTERIZATION (paper II and IV)

Despite accumulating evidence that DENVs differ in their potential to cause disease, no specific genotype or particular genetic signature has been directly associated to increased virulence and severe dengue. The genomic sequences of the open reading frame of the clinical, low passaged, DENVs representing the four serotypes were compared to identify genes potentially involved in the observed *in vitro* phenotypes.

Genomic sequencing revealed in 81 amino acid substitutions in total and a preference for amino acid substitutions in the viral structural E protein, as well as the NS1, NS2A, and NS5 proteins. The twelve clinical DENVs compared differed at eleven positions in the E protein, suggesting that they elicit antibodies that may differ in their epitope specificity. The E protein mediate receptor binding and anti-E antibodies inhibit viral attachment, internalization, and replication within cells which in turn may influence host immune response (Johnson et al., 1994, Lee et al., 1997, Navarro-Sanchez et al., 2003). The Ser→Asn203 in the E protein of the DENV-2 DF isolate could be modified by glycosylation and influence target cell tropism and epitopes for antibodies. Thus, different E protein epitopes could ultimately modulate the course of infection.

Ten amino acid substitutions were localized to both the NS1 and NS2A proteins whose functions are less well characterized. The NS1 protein is believed to be involved in dengue pathogenesis (Schlesinger et al., 1987, Avirutnan et al., 2006, Lin et al., 2002a), and the small hydrophobic protein NS2A is suggested to have an inhibitory role in IFN-mediated signal transduction (Munoz-Jordan et al., 2003). Thus, these two proteins could be involved in the pathogenesis of dengue, but further studies are required to fully understand their significance.

The NS5 protein contained the highest proportion of amino acid substitutions (38 % of the total) and the enzymatic role of NS5 in replication could explain the various replication profiles of the DENV isolates studied. NS5 has three major functional domains and is the largest DENV protein. The N-terminal MTase portion is required for the capping of the DENV genome, which crucial for recognition of the host cell translational machinery (Bartholomeusz and Wright, 1993, Egloff et al., 2002, Ray et al., 2006). The NLS part interacts with NS3 and cellular factors, whereas the C-terminal polymerase domain RdRp is responsible for synthesizing new viral RNA genomes. This suggests that the different phenotypes with distinct replication profiles could be due to genomic variations in the NS5 gene. Enhanced replication capacity in key target cells resulting in higher viral load and a stronger inflammatory response in the host has been proposed to increase virus virulence (Messer et al., 2002, Messer et al., 2003, Kanakaratne et al., 2009, Cologna et al., 2005, Cologna and Rico-Hesse, 2003, Vasilakis et al., 2007). Based on the examples given, one hypothetical mechanism for increased viral virulence suggests that virulent DENVs would produce more viruses per cell, resulting in higher viremia and stronger inflammatory response, than with a low pathogenic strain (Cologna and Rico-Hesse, 2003, Leitmeyer et al., 1999).

Reverse genetics and a larger samples pool are required to identify crucial genes and amino acid sequences involved in dengue pathogenesis. This comparative genomic study highlights certain viral genes known to be involved in antibody response, host immune pathways, and virus replication.

9 GENERAL CONCLUSIONS

Dengue pathogenesis is an interplay between virus and host factors that remain incompletely understood (Guzman and Kouri, 2008). Explaining the heterogeneity between clinical presentations due to a DENV infection is a vital area of research, but has been hampered by the lack of suitable animal models. This thesis work has focused on characterization of clinical DENV-isolates originating in Cambodian children that fell ill to dengue in 2007 (ten patient virus isolates) and 2008 (two patient virus isolates), respectively. Thus, the viral isolates are derived from a homogenous group of individuals with similar age and ethnicity, and the DENV strains within the same serotype belong to the same genotype being >99 % identical. Despite these similarities, the patients developed dengue disease of different severity. The general aim of this thesis was to investigate whether these DENV strains, based on the clinical outcome in their human host, could be discriminated *in vitro* and *in vivo* by phenotypic discrepancies. To complement the phenotypic characterization, whole-genome sequencing was performed to relate any experimental findings with sequence data.

Part of the original characterization was to be based on cell tropism preferences among the twelve viruses analyzed. Cell tropism was to be studied by detection of the replicative intermediate during active virus replication, using a strand-specific RT-PCR. However, it was not possible to develop a strand-specific RT-PCR due to the recognized phenomenon of self-priming, an intrinsic feature of the viral DENV genome. The properties of self-priming required a complete blockage at the 3' end to prevent non-specific, extrinsic, primer independent reverse transcription (paper I).

The phenotype of each clinical DENV isolate was defined by studying the viral replication kinetics and apoptosis induction in mammalian and mosquito cells. Regardless of DENV serotype, all four virus isolates from DSS patients replicated with decreased efficiency, compared to the virus isolates from DF patients. The four virus isolates derived from DHF patients had replication profiles resembling either the ones for the DSS-isolates, or the DF-isolates.

The virus isolates derived from DSS patients were more prone to induce apoptosis in mosquito cells, compared to the virus isolates from milder clinical cases. This feature was independent of replication capacity, since all DENV isolates replicated with equal efficiency in mosquito cells.

Genomic comparison revealed mutations spanning the whole viral genome, affecting both structural genes and NS genes. A majority of the mutations were located in the structural E protein, and in the NS1 and NS5 proteins. The E protein is crucial for virus attachment to the host cell, virus entry, and harbors epitopes recognized by the host humoral immune response. The NS1 protein exists both inside the host cell as well as in a soluble form, being a target for anti-NS1 antibodies. There is a general consensus based on several studies that the NS1 protein is implicated in dengue pathogenesis. The NS5 protein has a dual enzymatic activity, harboring both the MTase and the RdRp playing a vital role in virus replication. NS5 has also been demonstrated to induce transcription and translation of IL-8 (Medin et al., 2005), a neutrophil chemoattractant elevated in severe dengue cases (Talavera et al., 2004). Hence, the amino acid differences identified may explain the

different phenotypes observed *in vitro*, and that they could ultimately influence pathogenesis in humans by being more virulent (paper II and IV).

The three DENV isolates of serotype-1 were characterized *in vivo* in BALB/c mice. Infection was studied during a two week period and the virus isolate from the DSS case was again discriminated from the two milder case isolates. The DSS isolate was unique with its strong preference for brain tissue compared to the two other virus isolates for which vRNA was found primarily in lungs and liver, and only to a limited extent in the brain. Infection of the DF and DHF isolates peaked during the first week, compared to the DSS isolate that peaked during the second week. Hence, the phenotype differences *in vitro* that revealed unique features for the DSS-isolate was confirmed *in vivo*, where the phenotype of the DSS-isolate could be regarded as more virulent (paper III).

The results summarized within this thesis propose that DENVs originating from the same epidemic may differ in phenotype as well as in genotype. All experiments have been performed using minimally passaged viruses (passage 1 from mosquito cells) in order to resemble the wild-type virus as much as possible. The differences could be seen both in various cell systems *in vitro,* and in mice *in vivo,* and were correlated to unique amino acid substitutions in the viral genome. Hence, DENVs within the same serotype and genotype have different intrinsic features that may potentially be involved in virulence, affecting the clinical outcome of DENV infection in humans.

10 ACKNOWLEDGEMENTS

These past few years have involved many meetings with people that have contributed in various ways to the creation and completion of this thesis; some are professional scientists, and some are professional human beings. I wish to express my sincere gratitude to all of you. I would especially like to thank:

My supervisor **Åke Lundkvist** for great companionship and for inviting me into your group. I have learnt plenty by making my own decisions and your belief in me has been encouraging. Thanks for all help with heavy luggage when I moved back to Sweden!

My supervisor **Isabelle Leparc-Goffart** for introducing me to the project from the very beginning, and for sorting out the bureaucracy in order for me to be employed by the French Army Biomedical Research Institute (IMTSSA).

Co-supervisor **Kerstin Falk** for help with revision of text.

My mentor, and source of inspiration, since I was an undergraduate student; **Gunilla ("Nilla") Karlsson-Hedestam**. I am deeply grateful for all professional advice, encouragement, and support during the writing of this thesis.

Philippe Buchy and **Veasna Duong**, Institut Pasteur in Phnom Penh, Cambodia, for invaluable assistance in providing clinical samples and a fantastic collaboration with constructive ideas. **All patients** for their willingness to donate blood, which was absolutely vital for this work.

Stéphanie Devignot, "mon alliée" and best friend at IMTSSA. I miss our revitalizing evenings with romantic comedy movies, pancakes and Nutella!

Paule Verteneuille for her warm personality, concern, and invaluable assistance in writing formal French letters to generals and officers.

Christophe Ritzenthaler, dear friend, professional researcher, and genius: for a never failing support and help with everything throughout the years. Thanks for introducing me to France, and for explaining why French people are so strange but wonderful. I have adopted a second home country and will never stop coming back!

My collaborators and friends at SMI. **Sirkka Vene**, who has an answer to almost everything. **Jonas Klingström** for scientific discussions. **Sandor Bereczky** for advice on mouse dissection, for always being so kind and encouraging. **Marika Hjertqvist** for stimulating discussions and inspirational work together. **Henrik Nordström** for sequencing advice and helpful attitude. **Susanne Johansson** for generous advice regarding splenocyte purification and FACS protocols. **Fredrik Atterfelt** for excellent technical assistance in designing the Luminex experiment. **The staff at**

"Centralförrådet", for being the most cheerful people at SMI. All the former and present **students** for creating a friendly environment.

Maria Wahlström for introducing to the world of SMI with all written and unspoken rules. Thanks for all late hours in the P3 lab, the adventure in Cambodia, and the even more adventurous flight home with twitching stomachs… It has been a pleasure to work with you.

Iyadh Dougi for being absolutely great! Thanks for inspiring scientific discussions, assistance with FACS and PBMC studies, and for being such a warm person.

Karin Sundström, amazing roommate, and for always, soul-mate! So colorful and generous; thanks for your personal encouragement and all the great fun and discussions about life, the life in the lab, and sometime the lab without a life! **Anne-Marie Connolly-Andersen** for good friendship and honest perspective on matters. Your strong mind and big heart is an inspiration. **Nina Lagerqvist** for putting an ironic touch to everything and honest opinion regardless of the subject matter. I will miss the music sessions in the P3 lab! **Elina Tonteri** for great climbing sessions and revitalizing company; two (or actually 1 ½!) stubborn Finns with a strong mind can be challenging, but I have found a long-lasting friend in you. **Jolanta Mazurek** for her unfailing interest and concern, stimulating company in the office, and humoristic attitude. **Sofie Wallerström** for her unique kindness and unfailing assistance in everything.

My beloved brothers **Jan and Per Larsson**. I can finally answer your frequently asked question; now I am done and little sis will finally leave school for good! I am so lucky I have (found) you two.

My most precious little things in life; **Stefan, my Mim-sessa Michaela, Sebastian, Salomon, Ludvig, Felix, and Amanda**. You are the reasons for many smiles and make me strive to be a better person and role-model. Also, my reason for being in science, **Simon**. Forever and ever in my heart and mind; you made me dream and hope that an ounce of information is a step towards a cure.

Vaari for encouragement and persistent interests in my studies during the years. The Tuiskunen sisu is still going strong!

Kerstin and Arne for their personal care and encouragement that I should follow my own heart, mind and path in life. Thanks also to Kerstin for letting me get to know my **grand-mother Alma**, an inspiring woman and symbol for what it means to be human and take care of your next of kin.

All my dear friends whose wonderful personalities have been essential for creating great moments outside the lab and for always being there for me.

My parents and the Bäck family for their infinite warm, generous support. An individual acknowledgement to **my mother** whose care and motto that everything is possible has given me the strength to do what I want in life.

Finally, my most genuine thanks to **Mårten**, my greatest love and future husband. Your care and endless believe in me has given me courage and strength, and your love is the most precious gift in my life. I did not know that people like you existed.

This work was supported by grants from the French Armed Forces Biomedical Research Institute and the Swedish Civil Contingencies Agency (MSB). The French Armed Forces Biomedical Research Institute and the Swedish Institute for Communicable Disease Control provided laboratory workspace and technical laboratory equipment.

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Journal of General Virology (2010), **91**, 1019–1027 **DOI** 10.1099/vir.0.016667-0

Self-priming of reverse transcriptase impairs strand-specific detection of dengue virus RNA

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Dengue virus infection is the most frequent arthropod-borne infection affecting humans in the world. Our understanding of the pathophysiological events leading to mild or severe outcomes of the disease remains limited by the fact that viral target cells in the human body are poorly characterized. One of the most sensitive strategies for detecting cells supporting active replication of this positive-strand RNA virus is the search for the replicative intermediate, an antigenome of negative polarity, by RT-PCR. However, a phenomenon described as 'false priming' of the reverse transcriptase (RT) prevents strand-specific detection. The results of the current study showed that this event corresponds to cDNA synthesis that is independent of any primer addition. This property was general to all RNAs tested and was not associated with small free nucleic acids, such as tRNAs and microRNAs. Rather, it corresponded to initiation of cDNA synthesis from the 3′ end of the RNA template, and a model is proposed in which the template RNA snaps back upon itself and creates a transient RNA primer suitable for the RT. Such a property would explain why many assays proposed for detection of a replicative intermediate are not specific, and may help in the development of a molecular biology protocol that could allow replication studies of RNA viruses of human interest, such as dengue virus, hepatitis C virus and enteroviruses.

Received 13 September 2009
Accepted 23 November 2009

INTRODUCTION

Dengue virus (DENV) is the most frequent mosquito-borne virus infection affecting humans in the world (http://www.cdc.gov/ncidod.dvbid/dengue). It is a member of the genus *Flavivirus*, family *Flaviviridae*, and is represented by four viral serotypes, DENV1–4, that each year affect 50–100 million people in tropical and subtropical areas. Most DENV infections lead to a self-limiting febrile illness (dengue fever), but can also result in more severe diseases, such as dengue haemorrhagic fever and dengue shock syndrome. The mechanisms underlying the more severe outcomes of the infection are poorly understood.

A significant number of host factors have been shown to be required for DENV propagation in insect and human cells (Sessions *et al.*, 2009), but the crucial target cells for DENV remain controversial. DENV infection gives rise to a viraemic phase, and the presence of DENV proteins and nucleic acids has been detected in multiple organs (liver, spleen, kidney, lung and bone marrow; Jessie *et al.*, 2004) and in circulating peripheral blood mononuclear cells, where monocytes, but not B or T cells, have been shown to be permissive to DENV infection (Blackley *et al.*, 2007). However, this detection is not necessarily associated with active replication.

DENV has a positive-sense RNA genome of approximately 11 kb encoding three structural and seven non-structural proteins, and is flanked by 5′- and 3′-untranslated regions (UTRs) that are partially complementary (Alvarez *et al.*, 2005). A hallmark of active DENV replication, as with other members of the family *Flaviviridae*, is the presence of the negative-sense antigenome, which is synthesized directly from the genome and serves as a replicative intermediate (Cleaves *et al.*, 1981; Peyrefitte *et al.*, 2003). Sensitive methods for specific detection of the negative strand of flavivirus RNA have been repeatedly researched.

The most sensitive and specific approach for low-copy-number nucleic acid detection is RT-PCR, and the use of either a reverse or a sense primer during the reverse transcription was first proposed to synthesize cDNA of specific polarity that could be further amplified by PCR.

A supplementary figure showing Mfold RNA structure predictions for the DENV2 *in vitro* transcripts used in this study is available with the online version of this paper.

This solution rapidly appeared to be unreliable due to a phenomenon referred to as 'false priming' of the reverse transcriptase (RT) used (Lanford *et al.*, 1995) from avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (MoMLV). Other methods have been proposed, such as RNase-protection assays (Novak & Kirkegaard, 1991), *in situ* hybridization (Nouri Aria *et al.*, 1993), high-temperature reverse transcription (Lerat *et al.*, 1996) and tagged primers at the RT step (Lerat *et al.*, 1996). New proposals periodically emerge for improved strand-specific RT-PCRs regarding various positive-strand RNA viruses (Bessaud *et al.*, 2008; Billam *et al.*, 2008; Peyrefitte *et al.*, 2003). None, however, has proved to be a convincing solution, and all face the 'false priming' problem of the RT. Some hypotheses have been proposed for this 'false priming': partial inactivation of the RT after the RT step that would still be active in the PCR when forward and reverse primers are present, possible RT activity of the *Taq* DNA polymerase, the existence of a thermostable hairpin at the 3′ end of the RNA template that serves as primer for the RT, and priming by short RNAs such as tRNAs and microRNAs (Lerat *et al.*, 1996). The problem associated with non-specific amplification of the non-targeted RNA strand, however, has never been described precisely.

Considering the importance of establishing the list of target cells during DENV infection, we decided to characterize the 'false priming' event of RNA during reverse transcription, using DENV2 as a model, in order to establish a protocol suitable for strand-specific detection during virus propagation.

RESULTS

As observed previously by others (Peyrefitte *et al.*, 2003), we confirmed that initiation of the RT during RT-PCR with DENV RNA can occur without any exogenous primers at the RT step (Fig. 1a). DENV RNA was added to the RT, either with a reverse primer (exogenous priming) encompassing the very 3′ end of the genome or without primer (endogenous priming). Three regions of the genome were targeted for PCR: nt 2323–2947 (envelope and NS1 genes), 8016–8546 (polymerase NS5 gene) and 10546–10723 (NS5 gene and 3′UTR). A positive signal was detected, whether the RT was exogenously primed or not. Initiation of cDNA synthesis could not be attributed to any background activity of the DNA-dependent DNA polymerase (*Taq* polymerase) as RNA introduced directly into the PCR mix, with forward and reverse primers, could not be amplified (Fig. 1a). Identical results were obtained with another flavivirus, West Nile virus (not shown). Such endogenous priming of the RT in RT-PCR studies has already been described for some single-stranded positive-sense RNA viruses, such as hepatitis C virus (HCV) (Lerat *et al.*, 1996) and enterovirus (Bessaud *et al.*, 2008). We found that endogenous priming was not restricted to this class of viruses, as we observed it with the segmented negative-stranded RNA virus Puumala virus (PUUV; Fig. 1b) and hantaan virus (not shown). Moreover, it was also observed using cellular RNAs as template, such as β-actin mRNA and 18S RNA (Fig. 1b). In each case, contamination by DNA or background RT activity of the *Taq* polymerase could be excluded, as target RNA introduced directly into the PCR mix did not lead to a significant signal (<10 % of the positive tracks). Experiments were conducted with both MoMLV and AMV RT, with identical results. 'False-priming' events in RT-PCR studies therefore involve endogenous priming at the RT step and do not seem to be restricted to genomes of positive-sense RNA viruses. The following experiments focused on DENV 2 Martinique only.

To decipher whether the endogenous priming was due to cellular small nucleic acids (DNA, microRNA, tRNA, etc.) or to an intrinsic property of the RNA template, we

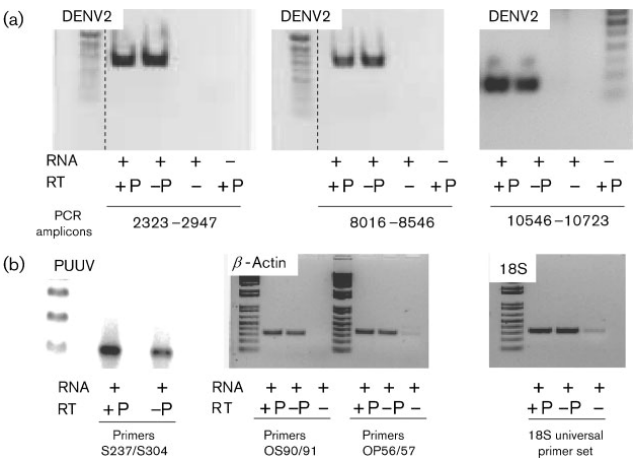

Fig. 1. Endogenous priming of viral and cellular RNAs during reverse transcription. (a) Cell-free DENV RNA was reverse-transcribed (AMV) with or without primer (+/−P), amplified by PCR in three regions (nt 2323–2947, 8016–8546 and 10546–10723) and analysed by agarose gel electrophoresis. As controls, RNA was added directly to the PCR without reverse transcription (RT −) and H_2O was used for whole RT-PCR (RNA −), with primer at the RT step. (b) The same analyses were performed on PUUV extract with primers S237/S304 and on uninfected cell extract for β-actin (primers OS90/OS91 and OP56/OP57) and 18S rRNA (18S universal primer set). Dashed lines indicate that the molecular markers and DNA bands of interest were in the same agarose gel, but not in adjacent wells.

investigated the comparative levels of endogenous and exogenous priming, in correlation with the relative amount of DENV RNA and cellular RNA. We first mixed a set amount of DENV genomic RNA extracted from cell-free virus (2 µl RNA extract from virus particles released into the supernatant of infected cells) with whole RNA purified from uninfected cells (0, 15, 45 and 135 ng). We thereafter performed reverse transcription with or without DNA primer before the quantitative PCR (Leparc-Goffart *et al.*, 2009). The relative level of endogenous priming compared with exogenous priming did not increase when the amount of cellular RNA was increased (Fig. 2a). Thereafter, we decreased the amount of cell-free genomic DENV RNA (from 5×10^7 to 5×10^1 equivalent copies) in a set amount of total cellular RNA (135 ng) and performed the same reverse transcription and quantitative PCR analysis. We did not observe any variation in the relative level of endogenously primed RT compared with exogenously primed RT (Fig. 2b). Thus, the level of endogenous priming was not correlated to the amount of cellular nucleic acids that could have acted as primer: endogenous priming appeared to be a property of the template itself.

Assuming that PCR efficiency is the same whether the RT was primed by DNA primer or not, the PCR quantifications allowed us to estimate the efficiency of the template-mediated priming during cDNA synthesis: on average, it represented 65 ± 15 % of the cDNA production during exogenously primed reverse transcription.

However, we could not exclude the possibility that DENV RNA, extracted from cell-free virus, might contain traces of cellular nucleic acids co-packed in the viral particles that in turn would be used as primers by the RT. This, to our knowledge, has never been described for DENV, but in order to reduce the amount of potential short nucleic acid contaminants relative to the amount of targeted RNA template, we used T7 or SP6 *in vitro* transcripts. These bacteriophage polymerases are known to produce large amounts of full-length RNA transcripts, and are even more efficient with short RNA transcripts (Melton *et al.*, 1984). Therefore, we used dilutions of such *in vitro* transcripts to limit the level of nucleic acid contaminants to a minimum. An artificial mini-genome RNA of 750 nt (miniG) was generated. This contained the first 463 nt of the DENV genome (5′UTR and capsid gene, nt 1–463) and the last 267 nt of the DENV 3′UTR (nt 10456–10723), with 20 irrelevant nucleotides as a spacer (the spacer corresponded to the multicloning site of the plasmid used for construction of the initial DNA template). RT-PCR, with or without primer, respectively, at the RT step was performed, followed by a PCR spanning the entire 3′UTR (nt 10456–10723). As observed previously on DENV full-length genomes, there was a significant amount of RT-PCR product resulting from the RT without added primer. The same experiment was performed on a reverse complementary SP6 *in vitro* transcript, corresponding to a mini-antigenome (miniAG) of negative polarity. Endogenous priming could once again be observed, even in the region corresponding to the entire 3′UTR of the antigenome (nt 1–463 of DENV) (Fig. 3). As it is practically impossible, even with DNase treatments, to completely eliminate the initial DNA template from the *in vitro*-transcribed RNA, we had some weak amplification when using RNA template directly in the PCR (RT −, Fig. 3). This background signal, however, was insignificant compared with the signal observed with RT-PCR.

Due to the position of the reverse primers used at the RT step and in the PCRs (hybridizing to the last 20 nt at the 3′ end of the miniG or miniAG) used in the PCR, positive PCR amplification in the endogenously primed samples indicated that reverse transcription had initiated at the very 3′ end of the template, or at least on the last 10 nt. This

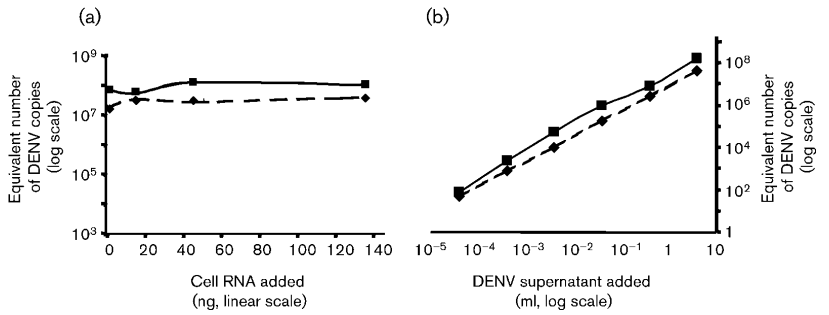

Fig. 2. Lack of correlation between endogenous priming efficiency and the DENV : cellular RNA ratio. (a) A fixed amount of DENV RNA extract (2 µl) was mixed with increasing amounts of total cellular RNA (0–135 ng) and, after reverse transcription with or without exogenous DNA primer (+/−P), an equivalent copy number of DENV genomes was estimated by real-time PCR. (b) A fixed amount of total cellular RNA (50 ng) was mixed with serial dilutions of cell-free DENV RNA (from 5 to 5×10^{-4} µl diluted in water) and analysed as above. ■, +P; ◆, −P.

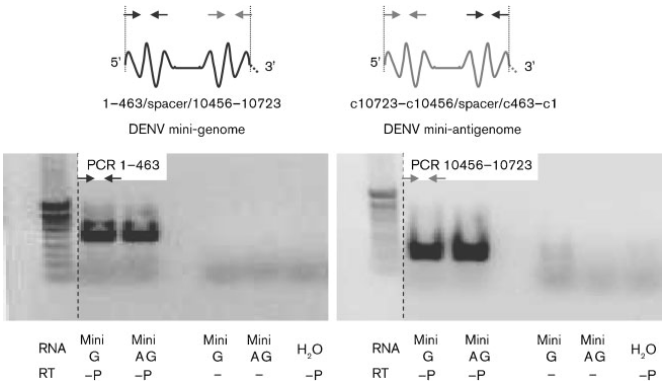

Fig. 3. Self-priming on *in vitro*-RNA-transcribed mini-genome or mini-antigenome. A mini-genome (DENV nt 1–463 + 20 nt spacer sequence + DENV nt 10456–10723) was transcribed *in vitro*, as well as the reverse and complementary mini-antigenome. Both were assayed for endogenous priming by RT (MoMLV) without primer, PCR in regions corresponding to the 3′ end and 5′ end (nt 1–463 and 10456–10723 and corresponding primers) and agarose gel analysis of the products. The absence of significant DNA carry-over from the *in vitro*-transcription template was assessed by submitting the RNA directly to PCR (RT –).

could be concluded, as the synthesized cDNA would otherwise have been too short for reverse primer hybridization at the PCR step. Priming by small contaminating nucleic acids that randomly hybridize on short sequences of the miniG or miniAG template would have led to synthesis of mostly truncated cDNAs that would have been initiated downstream from the 3′ end of the RNA template. This would have implied that no or weak PCR amplification should have occurred. Thus, this result confirms our previous observation that the endogenous priming at the RT step is poorly associated with fragments or small RNA, or even DNA. Rather, our results strongly indicate that the major event leading to priming the RT, in the absence of an exogenous DNA primer, is due to priming close to the 3′ end of the RNA template itself. Thus, the event associated with 'false priming' of the RT when targeting a specific RNA strand appears to be self-priming of the RNA templates.

The 5′ end (capsid gene) and 3′UTR of the DENV genome contain complementary sequences that allow long-range RNA–RNA interactions (Alvarez *et al.*, 2005). To check whether intermolecular or intramolecular interactions between DENV RNAs through their 5′- and 3′-end sequences could play a part in self-priming, we assayed self-priming with RNA *in vitro* transcripts that harboured either the 3′UTR (3′Gen) or a reverse complementary copy of the 5′UTR (3′AGen). Self-priming occurred in both cases, thus excluding the possibility that overlap between the genome 5′ end and the 3′UTR plays a significant role in the event (Fig. 4a). Priming of the RT by RNA secondary structures has also been proposed as an explanation for false priming (Lerat *et al.*, 1996). Flavivirus UTRs are indeed highly structured (Romero *et al.*, 2006), and false priming events are somewhat limited when reverse transcription is performed at high temperatures, for instance using recombinant *Thermus thermophilus* (r*Tth*)

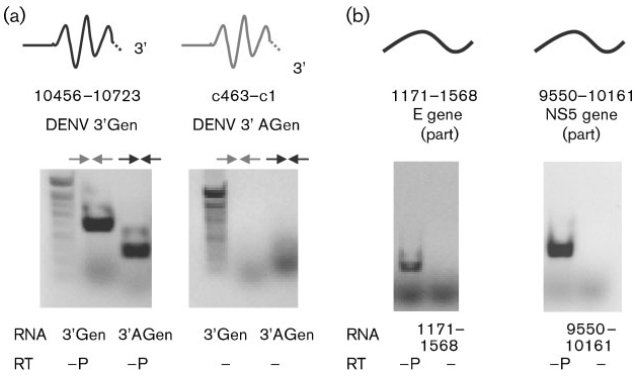

Fig. 4. Self-priming occurs despite the absence of a recognizable 3′ motif. (a) *In vitro* transcripts covering the 3′UTR of DENV genome (3′Gen, black line), or of DENV antigenome (3′AGen, grey line) were submitted to reverse transcription (MoMLV) without primer (–P), PCR (nt 10456–10723 for 3′Gen, black arrows, and nt 1–463 for 3′AGen, grey arrows) and agarose gel analysis of the products. (b) *In vitro* transcripts corresponding to inner regions of the DENV genome (nt 1171–1568 and 9550–10161) were also assayed for self-priming, with the same controls. No particular sequence or motif could be associated with these transcripts, as shown by RNA structure prediction (see Supplementary Fig. S1). The same controls for DNA contamination were used as in Fig. 3.

DNA polymerase at 72 °C, a temperature when RNA structures are destabilized (Lerat *et al.*, 1996). We used two other *in vitro* RNA transcripts corresponding to the inner regions of the DENV genome: nt 1171–1568 and 9550–10161, respectively, which do not include the DENV UTRs. Mfold predictions did not indicate a significant hairpin structure for these transcripts (see Supplementary Fig. S1, available in JGV Online). Self-priming of the two templates could, however, be observed during the RT (MoMLV) step (Fig. 4b). Identical results were obtained using AMV RT (not shown). We also studied the influence of DMSO, a chemical known to diminish the stability of RNA secondary structures. Up to 10 % DMSO in the reverse transcription mix together with DENV RNA was used, but we could not observe any decrease in self-priming (not shown). Thus, our results do not indicate that a particular RNA motif is involved in the self-priming process. This result is perfectly compatible with the variety of RNA templates that have been shown in previous experiments (Fig. 1) to support the intrinsic phenomenon that we refer to as self-priming.

We finally focused on the RT step. The *in vitro* transcript corresponding to sequence nt 10456–10723 of the DENV2 genome (NS5 polymerase gene and 3′UTR) was submitted to reverse transcription by RT (MoMLV) without primer, with a reverse primer, and with both a reverse and a forward primer as additional controls. cDNAs were analysed by electrophoresis on an 8 M urea/acrylamide denaturing gel stained with ethidium bromide. Self-primed cDNA appeared as a single band of ~250 nt, corresponding to a full-length copy of the RNA template (Fig. 5, lane 2). In particular, no other band or a smear was observed, indicating, as described above, that cDNA that is endogenously primed corresponds in a large majority to a single product type, and corresponds to a full-length or quasi-full-length copy of the RNA template. cDNAs synthesized in the presence of a reverse primer appeared to be a mixture of two products (Fig. 5, lane 3): a large product corresponding to the primed cDNA, and a second product, slightly less abundant, with a migration identical to that of the self-primed cDNA of lane 2. As a control, a third sample was analysed, with both forward and reverse primers in the RT. The forward primer did not lead to the synthesis of any additional band compared with RT with only the reverse primer (Fig. 5, lane 4). Identical results were observed with other synthetic RNAs. This confirmed the above results: (i) endogenous priming does not occur randomly along the RNA template, as would be expected if free short nucleic acids were used to prime the enzyme; (ii) endogenous priming, referred to as self-priming, can occur even in samples where DNA priming occurs; and (iii) self-priming of RNAs during reverse transcription leads to the synthesis of significant amounts of unexpected cDNA.

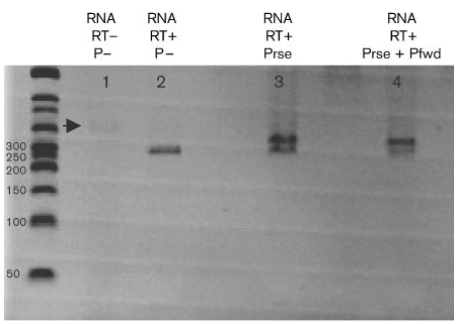

Fig. 5. Denaturing gel analysis of cDNAs produced during self-primed and DNA-primed reverse transcription on *in vitro* transcripts. The 3′Gen *in vitro* transcript (lane 1, faint band, indicated by an arrow) was submitted to reverse transcription (MoMLV) without DNA primer (P−; lane 2), with reverse primer (Prse; lane 3) or with reverse and forward primer (Prse + Pfwd; lane 4). cDNAs were analysed by denaturing urea/acrylamide gels and ethidium bromide staining.

DISCUSSION

AMV and MoMLV RT are currently widely used to synthesize cDNA from RNA templates, using various types of DNA primers such as random oligonucleotides, oligo(dT) and specific primers. These viral enzymes, however, can initiate DNA synthesis from an RNA primer, as described in the manufacturer's instructions. This property is linked to the replication of the virus they originate from: AMV uses $tRNA^{Trp}$ as a primer (Harada *et al.*, 1975), whilst MoMLV uses $tRNA^{Pro}$ (Peters *et al.*, 1977); in addition, the well-studied human immunodeficiency virus RT uses $tRNA_3^{Lys}$. These tRNA primers are even co-encapsidated in the virus particle (Ratner *et al.*, 1985). The self-priming of the RT that we observed *in vitro* on various 'wild-type' or synthetic templates is therefore not surprising, and has already been noted in the literature. Our first results (Fig. 1) confirmed that the phenomenon is not restricted to positive-sense RNA viruses and indicated that it leads to a significant production of unexpected cDNA, corresponding to ~65 % of exogenous DNA priming (Fig. 2), whatever the sequence or structure of the template may be (Figs 3 and 4).

We showed that free nucleic acids are poorly associated with endogenous priming (Figs 3, 4 and 5): this process appears to be only template-mediated. However, we have not characterized the exact molecular events leading to the initiation of cDNA polymerization at the 3′ end of the RNA template. Fig. 5 indicated that self-primed cDNA are slightly shorter than DNA-primed cDNA (see lanes 3 and 4). This could fit a snapped-back RNA priming model in which the 3′ end of an RNA loops back upon itself to form short and transient RNA–RNA duplexes that could serve as primers for the polymerase. Such a mechanism would firstly imply that the RT will prime from an RNA–RNA

hybrid consisting of a few nucleotides, as the 3′ end of an RNA usually does not have any particular complementarity to any sequence elsewhere in the molecule. During the retrovirus cycle, AMV or MoMLV RT priming occurs through hybridization between a primer-binding site, situated at the 3′ end of the retroviral genome, and 15–18 nt of a tRNA (Wakefield *et al.*, 1995). However, the minimal length requirement for AMV RT priming by DNA has been shown to be as few as 4 nt (Falvey *et al.*, 1976). This indicates that, although nucleic acid pairing on 4 nt is thermodynamically unstable, the hybrid between the RNA template and the short priming oligonucleotide can be maintained long enough for the RT to initiate its activity (Isel *et al.*, 1996). The primer requirement by RTs therefore appears to be quite weak. The situation is the same for RNA-dependent RNA polymerases, which can even perform *de novo* initiation (Laurila *et al.*, 2005). As a comparison, *Taq* polymerase can only prime from DNA–DNA hybrids longer than 9 nt (Williams *et al.*, 1990), which may explain why the self-priming phenomenon is not observed at the PCR step, as well as why random hexamers when used in RT do not interfere with the PCR step. Secondly, such snapped-back priming has already been observed by others with RNA-dependent RNA polymerases, such as phage ϕ6 polymerase (Laurila *et al.*, 2005) and with RT: Simpson *et al.* (2004) studied cDNA synthesis from a synthetic 90 nt RNA by retroplasmid RT (pFOXC RT) and used MoMLV RT as a control. Both RTs could perform cDNA synthesis with or without a DNA primer. Self-primed RT products corresponded to an ~170 nt RNA–DNA hybrid (pFOX RT and MoMLV) that the authors associated with elongation of the 3′ end of the RNA template that had snapped back upon itself. Self-primed MoMLV additionally produced a simple DNA of ~80 nt, indeed a cDNA not bound to the 3′ end of the RNA template. Simpson *et al.* (2004) mentioned that snapped-back priming can recover different forms, depending on the enzyme and reaction conditions. This may explain why we observed only a basic form of the cDNA that has the same length as the RNA template (Fig. 5) but not a long hybrid form. The exact mechanism would need further analysis, but this snapped-back priming model is compatible with our observations and other reports. As a comparison, it has been precisely described.

Enzyme suppliers mention that DNA primers are generally more efficient than RNA primers, and one could expect that self-primed cDNA synthesis could be a marginal event when a specific DNA primer is added in a sample. Our results (Fig. 5, lanes 3 and 4) indicate that this is obviously not the case. Additionally, AMV RT has been shown to use both tRNA and DNA primers efficiently to initiate cDNA synthesis, on two loci of a single identical RNA template, and exhibited no drastic preference for one or other primer (Whitcomb *et al.*, 1995). This implies that self-priming will arise in conventional RT reactions where an exogenous DNA primer is present. Any competition that could favour DNA priming compared with RNA priming would be balanced anyway by the fact that RT enzymes are present nowadays in excess in standard RT reactions.

When working on strand-specific detection of viral RNAs, self-priming at the RT step will therefore result in the synthesis of cDNA copies of both positive and negative polarity, in addition to the exogenously primed DNA, whatever strand-specific primer is added. cDNAs of both polarities will then be amplified at the PCR step, without respect to orientation. Due to optimized RT conditions (buffers and RT enzymes), long-range cDNA synthesis can be completed, and any target region of the genome, even far away from the RNA 3′ end, will be affected by intrinsic self-priming, as we confirmed (Fig. 1a).

Various strategies have been reported to achieve strand-specific amplification. In tagged PCR, the RT primer has a unique tag sequence at its 5′ end in addition to the complementary sequence that will be used for specific amplification at the PCR step (Bessaud *et al.*, 2008; Billam *et al.*, 2008; Lerat *et al.*, 1996; Peyrefitte *et al.*, 2003). The tagged primer system, however, cannot overcome the interference of snapped-back priming. During the first cycle of the PCR, the forward primer can hybridize to the self-primed cDNA, whereupon any tagged primer from the RT step will hybridize to this second strand and initiate the synthesis of an amplicon consisting of both the forward and tagged primer sequence. The resulting amplicon will be further amplified through efficient binding of the tagged and forward primer, and the strand specificity will thereby be lost.

Reports with data from purified *in vitro* transcripts or cell-free virus extracts, however, have mentioned that some strand specificity can be achieved, although to a limited extent, using a tagged RT-PCR system. Discrimination between the positive and negative strand can reach a factor of 10^6 (Carriere *et al.*, 2007; Lerat *et al.*, 1996; Mizutani *et al.*, 1998), which should be satisfactory when studying clinical samples of viruses that have a relatively low level of replication, such as hepatitis A or C virus or DENV. Such results were obtained with RNA extracted from quite pure samples. Interestingly, strand specificity of an HCV tagged RT-PCR assay was shown to be better with serum extracts than with liver extracts, although the total quantities of HCV RNA were comparable (Lerat *et al.*, 1996). This indicates firstly that the reliability of a strand-specific assay validated under *in vitro* conditions may not be suitable for clinical studies. Secondly, the discrepancy in the results depending on the sample could find an explanation in a snapped-back model: magnesium ions for instance, which are essentially intracellular, are present at significantly higher concentrations in liver extracts than in sera, and a high Mg^{2+} concentration stabilizes nucleic acid hybridization in RT-PCR. Snapped-back RNA could therefore be favoured in tissue extracts, which would result in a reduced specificity of the assay. Even though various HCV strand-specific systems have been proposed, detection of the negative RNA strand of HCV with tagged RT-PCR is still

controversial (Carriere *et al.*, 2007; Laskus *et al.*, 1997; Lin *et al.*, 2002). Some protocols aimed at reducing the problematic carry-over of tagged primers from the reverse transcriptase to the PCR have also been proposed. These methods involve nuclease digestion and size exclusion (Bessaud *et al.*, 2008; Craggs *et al.*, 2001; Peyrefitte *et al.*, 2003), but none of these methods can completely remove the tagged primers, and will in addition result in loss of material. As detection of the replicative intermediate is of major interest for viruses with a relatively low level of replication (such as hepatitis A, C or E virus or DENV), such methods appear to be of limited interest.

Some authors have used a thermophilic RT approach (Craggs *et al.*, 2001; Lanford *et al.*, 1995; Laskus *et al.*, 1998). The *Tth* RT works efficiently at 65 °C and has been shown *in vitro* to achieve a discrimination factor between strands of up 10^8 transcribed HCV RNAs, whereas the conventional MoMLV RT used at 42 °C could only achieve a factor of 10^4 (Laskus *et al.*, 1998). High-temperature reverse transcription with various combined tagged RT-PCR strategies, however, failed to completely overcome the phenomenon of unwanted detection of the incorrect strand of RNA (Craggs *et al.*, 2001). The snapped-back priming model is again compatible with these observations, as a high temperature will reduce, but not eliminate, the capacity of the template RNA to form short and transient back-loops at its 3′ end.

We investigated several alternative strategies to overcome the snapping back of the non-targeted RNA strand. We first designed tagged primers that had a DENV-specific sequence of only 10 nt. Such primers were expected to hybridize on DENV sequences at low temperature, and thereby to allow the tagged reverse transcription to occur, but would be too short for efficient binding on self-primed cDNA at the PCR step. Endogenous priming of the RT was, however, still efficiently detected at the PCR step (data not shown). It has been reported that oligonucleotides blocked at their 3′ end (to prevent extension) and modified on the last 5′ bases by phosphorothioate (to resist any 5′ exonuclease digestion) (Boiziau *et al.*, 1994) or peptide nucleic acids (Koppelhus *et al.*, 1997) bound to RNA templates can block AMV or MoMLV RT. A derived antisense oligonucleotide strategy could thus stop the RT on a non-targeted RNA strand and prevent full-length cDNA synthesis on the wrong strand. In our hands, however, such a protocol turned out to be unsuccessful (not shown). The strong strand-displacement activity of AMV and MoMLV RT, which is useful for the synthesis of long cDNAs on highly structured RNAs, may have impaired the blocking of the enzyme by the oligonucleotides. RNase H digestion of the non-targeted strand using a strand-specific 3′-end blocked oligonucleotide before the reverse transcription was also unsuccessful (not shown). We then tried to avoid the RT step by employing a ligation-mediated PCR (Hsuih *et al.*, 1996). This approach relies on the hybridization of non-amplifiable hemiprobes on the template, ligation and subsequent assembly of PCR-amplifiable DNA. Such a protocol cannot be applied directly to RNA as there is no enzyme that can ligate DNA oligonucleotides in DNA–RNA hybrids. Strand discrimination could therefore occur only after first-strand synthesis. Standard protocols (Hsuih *et al.*, 1996) or even a SALSA system (MRC Holland), which has been developed for multiplex ligation-dependent probe amplification with a specific ligase that should have a poor activity on non-hybridized hemiprobes, could not achieve satisfying specificity (not shown).

The search for replicative intermediates of flaviviruses therefore remains unsolved, and all protocols published to date remain to some extent unsatisfying. Our data favour a snapped-back priming model of the reverse transcriptase, which clarifies what was reported before as 'false priming'. This model is also compatible with previous observations made on DENV, hepatitis viruses or enteroviruses and can also provide an explanation for some controversial results in *in vitro* or clinical assays. Depending on buffers, the quality of the RT enzymes, reaction temperature and the stability of the RNA back-loops, the rate of RT initiation through snapped-back RNA would vary and thereby affect strand-specific detection. As more efficient enzymes and RT-PCR kits are being developed, snapped-back priming of the RT is a problem of increasing importance in the identification of target cells for flaviviruses and other single-stranded RNA virus infections.

METHODS

Cells. C6/36 cells (*Aedes albopictus* larva cells, ATCC CRL-1660) were grown at 28 °C in Leibowitz's L15 medium (BioWhittaker Europe) supplemented with 2 % tryptose phosphate broth and 5 % fetal calf serum. Vero E6 cells (ATCC C1008) were grown at 37 °C in MEM 199 (Invitrogen) supplemented with 5 % fetal calf serum.

Viruses. DENV2 strain Martinique (H/IMTSSA-MART/DEN2/98-703) was propagated on C6/36 or Vero cells. PUUV (patient isolate) or hantaan virus (strain 76-118) were propagated on Vero E6, as described previously (Hardestam *et al.*, 2008). For experiments with cell-free virus, the supernatant of infected cells was collected before cell death and clarified by centrifugation at 1500 ***g*** for 10 min before RNA extraction.

Viral RNA extraction. Viral RNA was extracted from 140 µl infected cell lysate or supernatant using a QIAmp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. The eluted RNA was stored at –80 °C until use.

RT-PCR. Reverse transcription was performed with AMV (Promega) or MoMLV (Superscript III; Invitrogen) RT according to the manufacturer's instructions. AMV reactions comprised 2 µl RNA extract in buffer containing 50 mM Tris/HCl (pH 8.3) at 25 °C, 50 mM KCl, 10mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 2 U AMV, 1 µl 10 mM dNTP mix (New England Biolabs) and 1 µl specific 10 µM primer (where required; Eurogentec). MoMLV reactions comprised 2 µl RNA extract in buffer containing 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 µl 0.1 M dithiothreitol, 40 U enzyme, 1 µl 10 mM dNTP mix and 1 µl specific 10 µM primer (where required). The reactions were incubated for 40 min at 40 °C, followed by 10 min at 95 °C to inactivate the RT

enzyme. For cDNA analysis on polyacrylamide/8M urea gel, the amounts of RNA were increased to 10 µl. Otherwise, 2 µl of the RT reactions was added to the PCR in a final volume of 20 µl, using standard protocols, using either using *Taq* PCR Mastermix (Qiagen) (35 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 1 min) for standard PCR or Platinum PCR Supermix (Invitrogen) (40 cycles of 95 °C for 15 s and 60 °C for 45 s) for real-time PCR in a LightCycler apparatus (Roche).

The results of end-point PCR were analysed on a 1 % agarose gel stained with ethidium bromide.

Primers. Most primers were synthesized in the laboratory and were used in this study because of availability. They were based on the DENV2 H/IMTSSA-MART/DEN2/98-703 sequence (GenBank accession no. AF208496), nt 2323–2343, 2947–2927, 8016–8036, 8546–8526 and 10723–10703 (Fig. 1); nt 1–20, 463–443, 10456–10476 and 10723–10703 (Fig. 3); and 1171–1191, 1568–1548, 9550–9570 and 10161–10141 (Fig. 4). Other primers were from Eurogentec: OS90 (5′-TCCTGTGGCATCCACGAAACT-3′) and OS91 (5′-GAAGCA-TTTGCGGTGGACGAT-3′), and OP56 (5′-TGGAGTCCTGTGG-CATCC-3′) and OP57 (5′-GTGTAACGCAACTAAGTCA-3′) targeting human β-actin, an 18S PCR primer pair (Quantum RNA Universal 18S; Ambion), S237 and S304 targeting PUUV (Evander *et al.*, 2007) and DenF, DenR and DenP from a pan-dengue Taqman system (Leparc-Goffart *et al.*, 2009).

***In vitro* transcription and PAGE analyses.** *In vitro* transcription was carried out using plasmid constructs or PCR products containing the expected promoter sequences with a T7/SP6 Ribomax large-scale RNA production system (Promega). For denaturing gel analysis, the cDNA was mixed in equal volumes with a buffer containing 95 % formamide, 0.025 % xylene cyanol, 0.025 % bromophenol blue, 0.5 mM EDTA and 0.025 % SDS, boiled for 5 min and put on ice to prevent secondary-structure reformation, and loaded on a denaturing 10 % polyacrylamide gel [7.2 g urea, 3.75 ml 40 % ProSieve 50 (Takara), 1 × TBE, 16 µl TEMED, 120 µl 10 % APS], which was eventually stained in an ethidium bromide bath and visualized under UV.

ACKNOWLEDGEMENTS

The authors thank J. Verner-Carlsson for her contribution to the analyses of hantaviruses.

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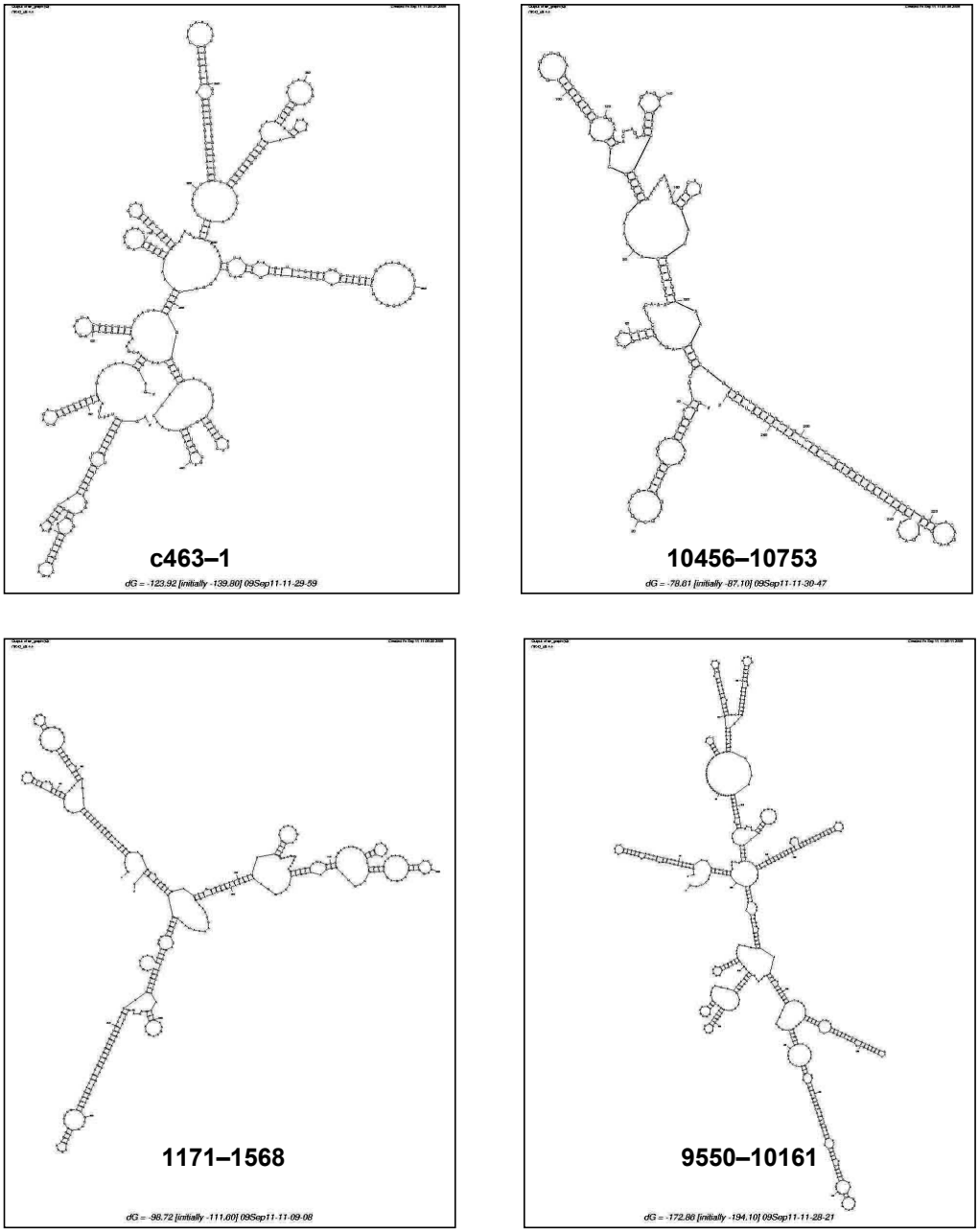

Supplementary Fig. S1. Mfold RNA structure predictions for the DENV2 *in vitro* transcripts used in Fig. 4. DENV2 Martinique sequences (GenBank accession no. AF208496) corresponding to nt 10456–10753 (3′Gen), 463–1 (3′AGen), 1171–1568 and 9550–10161 used as *in vitro* transcripts were submitted to Mfold software analysis (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) for RNA secondary-structure prediction.

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Arch Virol
DOI 10.1007/s00705-011-1100-2

ORIGINAL ARTICLE

Phenotypic and genotypic characterization of dengue virus isolates differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome

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Received: 5 July 2011 / Accepted: 29 August 2011

Abstract Dengue viruses (DENV) cause 50-100 million cases of acute febrile disease every year, including 500,000 reported cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Viral factors have been proposed to influence the severity of the disease, but markers of virulence have never been identified on DENV. Three DENV serotype-1 isolates from the 2007 epidemic in Cambodia that are derived from patients experiencing the various clinical forms of dengue were characterized both phenotypically and genetically. Phenotypic characteristics *in vitro*, based on replication kinetics in different cell lines and apoptosis response, grouped isolates from DF and DHF patients together, whereas the virus isolate from a DSS patient showed unique features: a lower level of replication in mammalian cells and extensive apoptosis in mosquito cells. Genomic comparison of viruses revealed six unique amino acid residues in the membrane, envelope, and in non-structural genes in the virus isolated from the DSS patient.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-011-1100-2) contains supplementary material, which is available to authorized users.

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Introduction

Dengue viruses (DENV) (serotypes 1, 2, 3 and 4) belong to the genus *Flavivirus*, in the family *Flaviviridae.* They are enveloped viruses with a positive-sense single-stranded RNA genome of approximately 10 700 bases. Upon infection, the genome is translated in the cell cytoplasm into a polyprotein, which is secondarily cleaved into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [1].

Dengue is the most frequent arthropod-borne viral infection in the world affecting humans, with one hundred million estimated cases per year. It is endemic in more than one hundred countries and more than 500 000 severe forms of the disease, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), are reported annually, most of them affecting children under 15 years of age [2]. DENV has adapted completely to humans and is maintained in large urban areas in the tropics in human-mosquito-human transmission cycles that no longer depend on animal reservoirs. Such reservoirs, however, can still be found in the jungles of Africa and Southeast Asia in mosquito-monkey-mosquito transmission cycles [3].

Most of dengue infections remain asymptomatic. Apparent disease due to dengue virus varies from a

Published online: 16 September 2011

relatively self-limited though debilitating febrile illness, dengue fever (DF), to the more severe and potentially life-threatening diseases DHF and DSS. The molecular mechanisms underlying dengue illness and the exact factors contributing to disease progress, however, are far from well understood. Host factors, such as age, gender, nutritional status, and genetics are known disease parameters. Furthermore, previous DENV infections and maternal antibodies seem to interfere and affect the outcome of an ongoing DENV infection [4–6].

Epidemiological data indicate, however, that viral factors should also influence the outcome of the disease and that the virulence differs among DENV strains, including strains within the same serotype [7–9].

Although dengue virus strains are easily differentiated on a genotypic basis, no phenotypic profile of "virulent" or "avirulent" strains has been established, except for a few strains that have been attenuated for vaccine purposes.

DENV isolates from children representing the three clinically distinct forms of dengue disease were characterized genotypically by genomic sequencing and phenotypically by analyzing replication kinetics, apoptosis, and adaptation to cell culture. Regarding phenotypic characteristics (replication kinetics and apoptosis patterns), the strain isolated from a patient experiencing DSS, the most severe form of dengue disease, differed significantly from the DENV isolates obtained from patients with DF and DHF. This strain also showed a remarkable ability to rapidly adapt to *in vitro* culture. Interestingly, at the genomic level, this strain exhibited six unique amino acid substitutions.

These observations group DENV obtained from DF and DHF cases together, whereas the DSS virus isolate stands alone when comparing virus isolate characteristics. Together, clinical observations and *in vitro* data suggest that the virus isolated from a DSS case can be distinguished from those obtained from DF and DHF patients, which appear similar.

Materials and methods

DENVs

Three dengue serotype 1 viruses isolated during the 2007 outbreak from patients living in the Kampong Cham province, Eastern Cambodia, were used during this study (Table 1). The isolates were obtained from patients experiencing the three distinct clinical forms of dengue disease: DF, DHF, and DSS, according to the WHO classification [10]. Blood samples were drawn between days 2 and 6 after onset of disease. These samples were collected during the DENFRAME study (a project supported by European Union) and immediately anonymized as stated in the research protocol. The DENFRAME project was approved by the National Cambodian Ethics Committee, and a patient's enrolment was subject to obtaining a written consent signed by the patient or, for those under 16 years old, their legal representatives. The patients specifically agreed that their blood samples may be stored and that further tests on these samples may be undertaken in the future to further understand dengue disease.

Table 1 Epidemiological and clinical characteristics associated with dengue virus serotype 1 isolates used in the study

ID	Epidemic year	Sex	Age	Type of antibody response	Clinical outcome
R0627319	2007	M	7	Secondary	DF
R0627321	2007	F	5	Indeterminable	DHF
R0808258	2007	F	9	Secondary	DSS

Each isolate had been isolated at the Institut Pasteur in Cambodia (IPC) in the standard cell line C6/36, derived from *Aedes albopictus* (CRL 1660, ATCC), and thereafter had undergone only one *in vitro* passage (P1) in order to avoid mutations that might be associated with tissue culture adaptation. Cell culture supernatant was stored at −80°C prior to shipment on dry ice to the French Army Biomedical Research Institute (IRBA, Marseille). The isolates were serotyped at IPC by nested reverse transcriptase polymerase chain reaction according to the Lanciotti procedure modified by Reynes et al. [11].

Cells

Mammalian Vero cells (CCL-81, ATCC) were grown at 37°C, 5% CO_2, in cell culture medium 199 (Gibco) with 5% fetal calf serum (FCS). The *A. albopictus* cell line C6/36 was grown at 28°C, in cell culture medium Leibovitz-15 with 5% FCS and 2% tryptose phosphate (TP).

Immunofluorescence with HB112 and J2 for detection of DENV infection and DENV titration by fluorescent focus assay (FFA)

5×10^5 C6/36 cells per well were seeded in 96-well plates (Corning) and incubated for 24 h prior to infection. Viruses, duplicates of 10-fold dilutions, were prepared with cell culture medium (FCS-free), and 50 µL of diluted virus was inoculated in each well and incubated for 2 h. To immobilize the virus, an overlay medium (0.7% high-viscosity carboxymethyl cellulose (CMC), Sigma-Aldrich, 1X MEM-medium, 2% TP, 5% FCS) was added on top of the cells, followed by a 72-h incubation. The overlay medium was discarded, and the cell monolayer was gently washed once with cell culture medium before fixation with paraformaldehyde (3.7%, pH 7.4) and incubated for 15 min at

room temperature. The cell monolayer was then washed once with PBS (1X) followed by a 5-min incubation with Triton-X 100 (0.5 %) in order to permeabilize the cells. Two washes with PBS (1X) followed before adding a blocking solution (2.5% FCS, 1% BSA in PBS (1X)), and the cells were incubated at 37°C for 30 min. A primary panflavi anti-envelope monoclonal antibody (MAb) (HB112^{TM}, ATCC) or a MAb for staining double-stranded RNA (J2, English and Scientific Consulting, Hungary) was added to the fixed cells and incubated for 30 min at 37°C, followed by two washes with PBS (1X) before applying a goat anti-mouse IgG A488-conjugate (1:250 dilution, Gibco) and incubating at room temperature for 45 min. In order to stain the eukaryotic nuclei, Dapi (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) was added at a 1:5000 dilution, and the cells were incubated for 30 min at 37°C. The monolayer was then washed three times with PBS (1X), and plaque-forming units (PFU) were counted under a microscope.

Viral RNA extraction and real-time one-step quantitative RT-PCR

Viral RNA was extracted from lysed cells and cell supernatant using a QIAmp Viral RNA Mini Kit. RT-PCR reactions were set up according to the manufacturer's instructions (SuperScript III Platinum One-Step Quantitative RT-PCR System, Invitrogen) as already described [12].

Kinetics of DENV replication in various cell lines

Replication kinetics was investigated under three different conditions: on mosquito C6/36 cells at 28°C, on mammalian Vero cells grown at 37°C (5% CO_2) and on Vero cells at 40°C (5% CO_2). The C6/36 cells were seeded to confluence at 170,000 cells per well (3.8 cm^2) in a 12-well plate, and the Vero cells were seeded to confluence at 110,000 cells per well. The cell medium was discarded, and the monolayer of C6/36 cells was washed once with L-15 medium (with 2% tryptose phosphate broth). The monolayer of Vero cells was washed once with PBS (1X). An inoculum of 500 µL per well was used for infection at a multiplicity of infection (MOI) of one. The virus was diluted in cell medium (FCS-free), and virus free-cell culture medium was used for mock infection. The cells were then incubated for 2 h at the appropriate temperature. The inoculum was removed, and the cells were gently washed with PBS (1X). Two mL per well of the appropriate cell medium (supplemented with 5% FCS) was added, followed by incubation at the corresponding temperature. Samples of both supernatant and cells were harvested daily throughout the week and used for viral RNA extraction and virus titration. C6/36 cells were harvested by scraping them off and dissolving them in 2 mL of cell culture medium. Vero cells were harvested by adding 500 µL trypsin 2-3 minutes before adding 1.5 mL of cell culture medium to obtain a homogenous cell suspension.

Evolution of DENV in persistently infected Vero cells

Four T-25 flasks were seeded with 433,000 Vero cells each and infected with one dengue virus isolate each at an MOI of one. The cell medium was discarded and the cell monolayer washed once with PBS (1X) prior to infection and incubated for 2 h at 37°C, 5% CO_2. The inoculum was then discarded and the cells washed with PBS (1X). Ten mL of medium 199 (5% FCS, Invitrogen) was added to each flask, and the cells were incubated for one week at 37°C, 5% CO_2. Seven days post-infection, 2 mL of each supernatant was transferred to a new T-25 flask containing 433,000 cells and infected as described above. This supernatant was then used to infect new cells seven days later, and so on for ten weeks.

The cells from the old flasks were split by first washing with PBS (1X) and then trypsinizing (Gibco), and 1/7 of the cells in a total volume of 10 mL were transferred to a new T-25 flask and incubated for a week. Those cells were subsequently split each week into a new T-25 flask, and the infection was thereby maintained until week 10, when the experiment was terminated.

Samples were harvested each week, and Lab-Tek slides (Nunc) were prepared in parallel to the cell culture flasks to perform immunofluorescence.

Apoptosis TUNEL assay

Vero and C6/36 cells were grown on Lab-Tek chamber slides (0.8 cm^2 per well). Vero cells were seeded with 20,800 and 41,600 cells per well to 50%, and 100% cell confluence, respectively, at the start of infection. C6/36 cells were seeded with 5×10^5 and 16×10^5 cells per well to approximately 50%, and 100% cell confluence, respectively. The cells were infected at an MOI of one and incubated for 24, 48, or 72 h or six days. The cells were then stained for the presence of dengue virus antigen using the HB112 and J2 MAbs, as well as for apoptotic cells using TUNEL staining (terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling). Apoptosis was detected using an In Situ Cell Death Detection Kit Red (Roche) according to the manufacturer's instructions, and cells were stained for 1 h at 37°C. Staurosporine (Sigma) was used as a positive control for apoptosis [13]. One micromolar staurosporine was added to Vero and C6/36 cell cultures, followed by 24 and 48 h of incubation at 37°C and 28°C, respectively.

Sequencing

Viral genomic RNA was isolated from infectious supernatant from the first passage propagated in C6/36 cells, which is believed to confer the lowest possible level of selective pressure, using a QIAamp Viral RNA Mini Kit (QIAGEN). RT was performed using the SuperScript First Strand Synthesis System for RT-PCR (Life Technologies) with gene-specific primers or by self-priming. Phusion Hot-Start High Fidelity DNA Polymerase (Finnzymes) was used to generate overlapping PCR fragments of approximately 1000 nucleotides, which were purified by gel electrophoresis followed by gel extraction using a MinElute Gel Extraction Kit (QIAGEN) and eluted in 10 µL water. DENV1-virus-specific primers were used for two-directional sequencing. Primers were designed for sequencing both strands (Sanger sequencing using ABI3739XL technology and the PhredPhrep program) of the PCR product, from which consensus sequences were assembled.

Results

FFA titration on C6/36 cells

The most common method to titrate DENV for a long time has been based on cytopathic effect (CPE) in monkey Vero cells or rodent BHK-21 cells. This method is widely used for high-passage laboratory DENV strains. Unfortunately, most of primary (or low-passage) DENV isolates from patient sera do not give any CPE on these cells. To overcome this problem, a FFA using C6/36 cells was developed for DENV titration. C6/36 cells are commonly used for DENV isolation and propagation, which explains why these cells were the natural choice for the development of the FFA for virus titration. As seen in Fig. 1, distinct foci (clearly separated from each other) appeared as the virus was more diluted, resulting in a manageable number of foci to be counted for titre estimation.

Depending on the DENV strain, a decrease in the titre was observed after a freezing step at −80°C. For all of our experiments with a specified MOI, it was important to ensure that the initial titre of each strain was stable at each step of freezing and thawing. We have tested different freezing solutions containing sucrose and/or HEPES. The titre was stable for two years at −80°C for viruses frozen in 0.5 M sucrose and 50 mM HEPES and also after few freezing/thawing cycles (data not shown).

Replication kinetics of wild-type DENV isolates obtained from patients experiencing various degrees of dengue illness severity

We investigated the replication kinetics of various clinical DENV isolates in order to characterize them phenotypically in mammalian and mosquito cell lines. Viruses from supernatants and cells were titrated by FFA and by quantification of total viral RNA using a quantitative RT-PCR. Similar kinetics were obtained using these two methods.

All of the isolates replicated with similar efficiency in the mosquito cell line C6/36, with close to 100% of cells infected (Figs. 2a, 4).

In mammalian Vero cells at 37°C, strains isolated from patients experiencing DF and DHF showed identical growth kinetics, reaching viral titres of 10^6 RNA copies/ml (around day 5-6 post-infection). However, the isolate obtained from the patient with the shock syndrome showed restricted replication in Vero cells, with a loss of more than one log in titre (Fig. 2). Immunofluorescence with a pan-flavi anti-envelope monoclonal antibody and with a MAb detecting active virus replication (J2, targeting double-stranded RNA) showed a constantly low level of cells infected with the DSS virus (around 10%) compared to DF and DHF isolates, which infected 100% of the cells (Fig. 3).

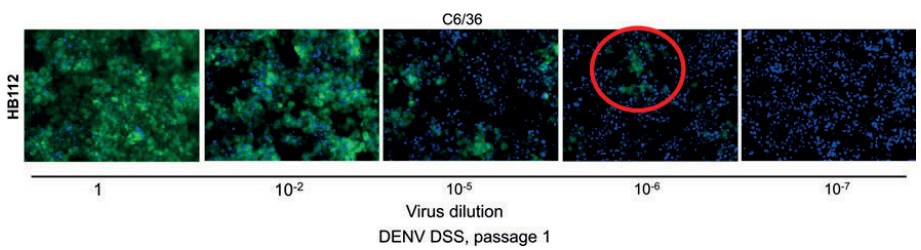

Fig. 1 Titration of a DENV serotype 1 DSS isolate on C6/36 cells, passage 1, stained with HB112 antibody (green) and Dapi (blue). The red circle indicates a fluorescent focus that is well separated from its surroundings

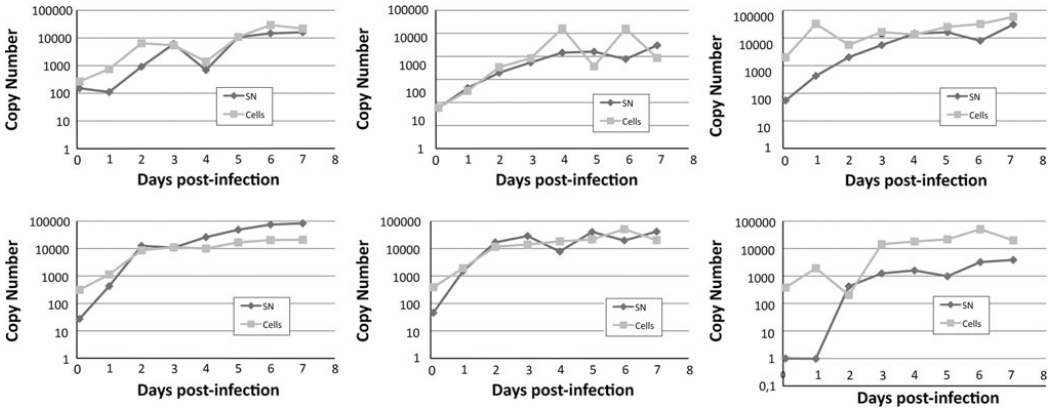

Fig. 2 Replication kinetics of DENV isolates in mosquito C6/36 cells and in mammalian Vero cells at 37°C determined by measuring the copy number by quantitative RT-PCR in supernatants and in cells. These data are the average of three independent experiments. The standard deviation error bars are too small to be seen in this figure on a log scale (see supplementary data)

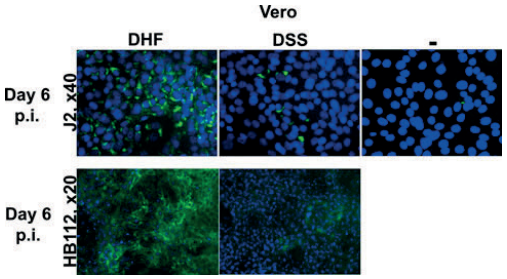

Fig. 3 Replication of DHF and DSS isolates at day 6 p.i. in Vero cells stained with J2 antibody (magnification X40) and DAPI (nucleus) or stained with HB112 antibody and DAPI (magnification X20)

Finally, there was no difference in the kinetic for the three DENVs when infecting Vero cells at 40°C (data not shown).

Apoptosis due to DENV infection

We studied apoptosis in C6/36 and Vero cells to investigate whether the DSS isolate differed from the DF and DHF isolates, as was observed in the replication kinetics. The cells were stained at four time-points (+24, +48, +72 h and +6 days p.i.) in order to follow the development of apoptosis along the course of infection. The experiment was repeated at two different levels of cell confluence, since this parameter influences the degree of apoptosis.

None of the DENV isolates induced a significant degree of apoptosis on Vero cells, regardless of whether the cells were fully confluent or only 50% confluent (data not shown).

The DF and DHF virus isolates did not induce a significant degree of apoptosis on C6/36 cells, regardless of cell confluence (Fig. 4). With the DSS isolate, however, at least 30% of the C6/36 cells were already undergoing apoptosis during the first 24 h of infection, and the number of apoptotic cells continued to increase to 50% during the following days (Fig. 4). This did not depend on the initial cell confluence at the time of infection (apoptosis in 50% and 100% confluent cells was compared). The apoptotic signal one day after infection was found in surrounding, not yet infected cells, rather than in infected cells (Fig. 4). Virus titration and viral RNA quantification using real-time quantitative RT-PCR confirmed that all the three DENV isolates had similar replication kinetics in C6/36 cells (data not shown). Thus, the DSS isolate caused a much higher degree of apoptosis in C6/36 cells compared to the DENV DF and DHF isolates, regardless of replication kinetics.

Adaptation of DENV isolates in persistently infected mammalian Vero cells

After having observed the strong replicative restriction of the DSS strain in Vero cells, we investigated the ability of the virus to establish a persistent infection compare to other isolates. Infected Vero cells were propagated for ten weeks by splitting them weekly, as well as by transferring the infectious supernatant to new uninfected Vero cells. All three DENV isolates had the characteristic of establishing a persistent infection in VERO cells for at least ten weeks (Fig. 5).

For the DSS isolate, the two first weeks of infection showed the same low level of replication with 10% of cells infected, as observed during the kinetics' experiment. However, after 3 weeks, there was an increase in replication,

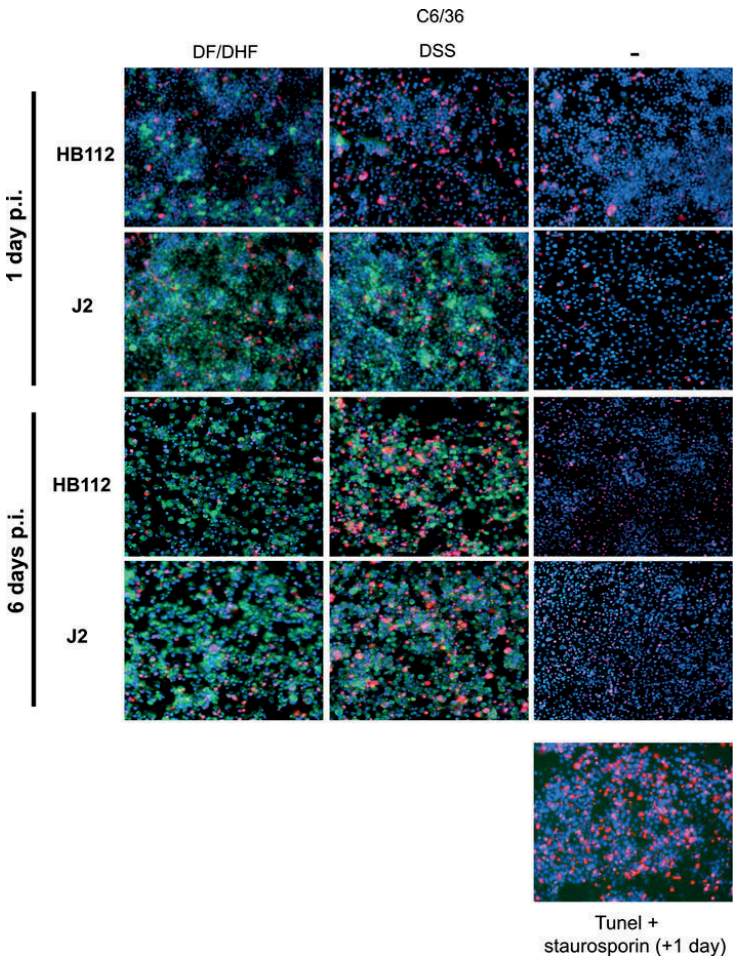

Fig. 4 Apoptosis on confluent C6/36 cells at 24 h postinfection and 6 days postinfection. TUNEL staining (red) and DAPI staining (nucleus, blue) combined with either HB112 or J2 antibodies (green)

with 50% of the cells infected. At week four, there were no longer significant differences in replication activity between the three isolates with 100% of cells infected, leading to viral titres as high as 10^6 to 10^8 FFU/ml (Fig. 5). The passaged viruses showed the same characteristics both for the passaged infected cells and the passaged infectious supernatant. Thus, the DSS isolate could achieve adaptation to Vero cells in only 3-4 *in vitro* passages despite the low level of replication during the first two passages.

Genomic analysis of the three DENV isolates

Complete sequences of viral genomes for the DF (accession number: HQ624983), DHF (accession number: HQ624984) and DSS (accession number: FJ639694) isolates were obtained. These three isolates belong to genotype 1. There are numerous sequence variations at the amino acid level between these three DENV-1 strains, which were isolated during the same outbreak (Table 2). The sequence of the DSS isolate differed from the other two by six amino acids, with mutations located in the membrane gene, the envelope gene, and the non-structural (NS) 1, NS3 and NS5 genes.

The adapted DSS isolate after five passages of Vero cells was also sequenced, and four amino acid changes had appeared in the envelope, 2K, NS4b and NS5 genes (Table 2).

Discussion

Epidemiological, phylogenetic, clinical and experimental data indicate that viral factors could influence the severity of dengue infection [7–9, 14, 15].

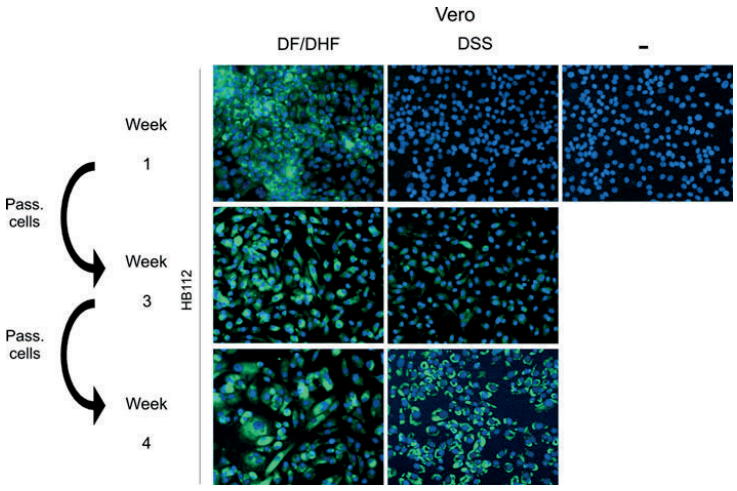

Fig. 5 Adaptation of DENV isolates to VERO cells. Immunofluorescence with HB112 antibody (green) and DAPI staining (blue, nucleus) 7 days postinfection on weeks 1 to 4 of passage of infected VERO cells

This study was aimed at investigating phenotypic as well as genotypic differences between clinical DENV serotype-1 isolates from Cambodian patients experiencing various degrees of dengue disease severity (DF, DHF and DSS). These virus isolates, which were from the same outbreak, represented a good model to investigate if phenotypic properties associated with a specific genetic background could characterize them. It should be noted at this point that this study is preliminary and is limited by the number of isolates used. However, the data obtained showed that both at the genotypic and phenotypic level, differences could be found associated with different clinical outcome.

Previous laboratory experiments have shown that DENV could adapt to cells *in vitro* very quickly, associated with genetic and phenotypic changes [16, 17]. In our study, we also showed that the characteristic of the DSS isolate replicating less efficiently in mammalian cells was lost after three *in vitro* passages and was associated with changes in the viral genome. These results emphasize the importance of using primary viral isolates, or viruses rescued from infectious clones, when studying DENV characteristics, i.e., virulence. Laboratory-adapted strains have passage-acquired mutations that may involve loss of wild-type virus features that are of significant importance for understanding DENV virulence.

Some *in vitro* experiments on human peripheral blood leukocytes have shown differences in DENV replication correlating with the severity of the disease [18]. We observed a similar phenomenon in mammalian Vero cells, with differences in DENV replication depending on the DENV isolate. The clinical DENV isolates derived from patients exhibiting the non-complicated form DF and the more severe form DHF replicated efficiently in Vero cells compared to the DSS virus isolate, which showed a very low level of replication, corresponding to a lower percentage of infected cells. This restricted replication of the DSS isolate in Vero cells could be due to the establishment of a persistent infection. Ten passages of Vero cells infected by DF, DHF or DSS isolates showed that the three isolates are able to establish a persistent infection in mammalian cells. This characteristic has already been described for a laboratory-adapted strain of DENV2 in human mononuclear cell lines [19]. In mosquito cells, none of the three DENV serotype 1 isolates studied showed any differences at the level of viral replication. However, the DSS isolate induce a high level of apoptosis in C6/36 cells as early as 24 h postinfection. Whether apoptosis is the result of infected cells releasing pro-apoptotic mediators rather than being a direct effect of viral infection is difficult to distinguish because most of the C6/36 cells were infected. However, Kanthong et al. recently observed 40% apoptosis in C6/36 cells persistently infected with DENV and treated with 5-kDa filtrate from a supernatant of C6/36 cells acutely infected with DENV [20]. Therefore, it could be hypothesized that the replication of the DSS isolate in C6/36 cells induces the production of apoptosis-inducing cytokines.

Regarding the phenotypic properties of the three different isolates, the DSS isolate behaved differently than the other two isolates. This differentiation was also found in another recent study using a genome-wide microarray to look at the transcriptional profiles of samples from patients presenting with the different clinical forms of DENV infection [21, 22]. The lower level of replication of the DSS isolate in mammalian Vero cells fits the observations

Table 2 Amino acid differences between DF, DHF and DSS isolates in the open reading frame

Amino acid position in polyprotein	Amino acid position in protein	Gene or region	Virus and amino acid			
			DF	DHF	DSS	DSS adapted
75	75	Capsid	Asn	Ser	Ser	Ser
109	109		Val	Met	Met	Met
158	43	Membrane	Phe	Leu	Leu	Leu
171	56		Ser	Thr	Thr	Thr
229	114		Gly	Ser	Gly	Gly
277	162		Pro	Pro	Ser	Pro
335	56	Envelope	Val	Ile	Ile	Ile
450	171		Ser	Thr	Thr	Thr
484	205		Lys	Lys	Lys	Arg
755	476		Leu	Leu	Phe	Phe
891	115	NS1	Lys	Lys	Arg	Arg
1099	323		Lys	Arg	Arg	Arg
1168	40	NS2a	Phe	Leu	Leu	Leu
1199	71		Met	Thr	Thr	Thr
1345	217		Lys	Arg	Arg	Arg
1594	118	NS3	Ser	Ser	Pro	Pro
1807	331		Val	Leu	Val	Val
2225	3	2K	Asp	Asp	Asp	Asn
2265	20	NS4b	Glu	Gly	Gly	Gly
2404	159		Val	Val	Val	Leu
2496	2		Gly	Gly	Gly	Arg
2512	18	NS5	His	Gln	Gln	Gln
2527	33		Met	Thr	Met	Met
2543	49		Thr	Thr	Ile	Ile
2628	134		Val	Ile	Val	Val
3018	524		Ser	Pro	Pro	Pro
3044	550		Lys	Arg	Arg	Arg
3058	564		Asn	Lys	Lys	Lys
3280	786		Val	Ile	Ile	Ile
3298	804		Ala	Thr	Thr	Thr
3324	830		Ser	Ser	Asn	Asn
3326	832		Glu	Gly	Gly	Gly

made by Duong *et al.*, whose data indicate lower levels of virus in patients suffering from DSS compared to patients with DF [23]. This differs from earlier reports that lay the foundation for the current dogma: the higher viremia, the worse the outcome [24].

A significant number of sequence differences were detected by the complete sequencing of the three DENV serotype 1, genotype 1 isolates, showing that different viruses are circulating during an outbreak. By looking at the complete sequences published in GenBank of fifteen DENV-1 genotype 1 isolates from the 2007 epidemic in Cambodia, 69 positions with amino acid variations were found. But even if some variations are unique to one isolate, it can be seen that all of the isolates analyzed are similar to DF sequence or to DHF/DSS sequences. Two sequenced isolates from GenBank are identical to our DSS isolate. The DSS virus had six unique amino acid changes compared to the two other viruses isolated from patients with DF or DHF. Two of these six amino acid substitutions (P277S and L476F) are located in the transmembrane regions of the membrane and envelope proteins. The transmembrane region of the envelope is involved in virion assembly and is required for translocation of the NS1 protein [25, 26]. The role of this domain in virion production could explain a part of the restricted replication observed with the DSS isolate in mammalian cells. The

other mutations were in non-structural genes (NS1-K115R, NS3-S118F, NS5-T49I, NS5-S830N). NS1, and more specifically, the secreted form of NS1, associated with complement has a potential role in the vascular leakage responsible for the shock syndrome [27]. Finally, NS3 and NS5 are proteins with enzymatic activities that are important for viral replication. All of these mutations could be implicated in the *in vitro* and *in vivo* phenotypic characteristics of the DSS isolate. None of the amino acid differences that were found affect the known glycosylation sites or disulfide bonds. It should again be emphasized that our study and our results are preliminary. Further studies are required in order to understand and identify the virulence markers of DENV. More strains of the different DENV serotypes and for each clinical form should be studied for association between phenotypic and genotypic characteristics. Finally, reverse genetic and *in vivo* studies will be required to further identify virulence markers for DENV.

Acknowledgments This work was supported by the French Armed Forces Medical Service, the French Direction Générale de l'Armement (contrat d'objectif 07co405) and from the European Union (DENFRAME project, 6th Framework Programme: 2005-INCO-DEV2-n°517711).

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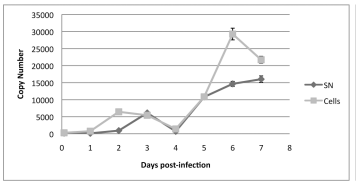
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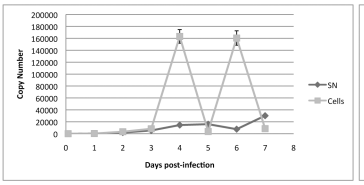
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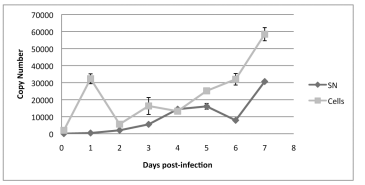
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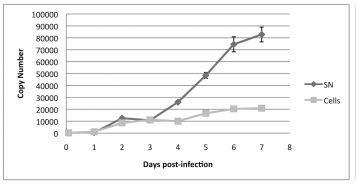
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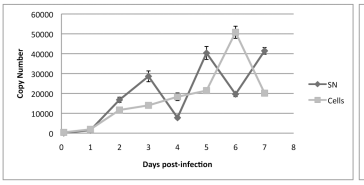
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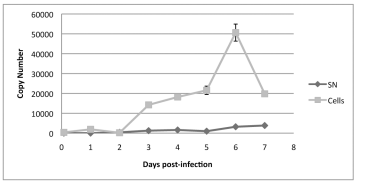
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Phenotypic characterization of patient dengue virus isolates in BALB/c mice differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome

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Abstract

Background: Dengue virus (DENV) infection is the most common arthropod-borne viral disease in man and there are approximately 100 million infections annually. Despite the global burden of DENV infections many important questions regarding DENV pathogenesis remain unaddressed due to the lack of appropriate animal models of infection and disease. A major problem is the fact that no non-human species naturally develop disease similar to human dengue fever (DF) or dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Apart from other risk factors for severe dengue such as host genetics and secondary infection with a heterologous DENV, virus virulence is a risk factor that is not well characterized.

Results: Three clinical DENV-1 isolates from Cambodian patients experiencing the various forms of dengue disease (DF, DHF, and DSS) were inoculated in BALB/c mice at three different concentrations. The DENV-1 isolates had different organ and cell tropism and replication kinetics. The DENV-1 isolate from a DSS patient infected the largest number of mice and was primarily neurotropic. In contrast, the DENV-1 isolates from milder clinical dengue cases infected predominantly lungs and liver, and to a lesser extent brain. In addition, infection with the DENV isolate derived from a DSS patient persisted for more than two weeks in a majority of mice compared to the other DENV-1 isolates that peaked during the first week.

Conclusions: These results confirm the *in vitro* findings of the same DENV-1 isolates, that showed that the isolate derived from a DSS patient can be distinguished based on phenotypic characteristics that differ from the isolates derived from a DF and DHF case [1]. We observed in this study that the DSS virus isolate persist longer *in vivo* with extensive neuroinvasion in contrast to the other DENV-1 isolates originating in milder human cases. 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). The characterization of these clinically distinct DENV-1 isolates highlight that DENVs within the same genotype may have different *in vivo* phenotypes.

Highlights: • Clinical DENV-1 isolates have different organ tropism in BALB/c mice.
• The isolate from a DSS patient is primarily neurotropic compared to the other isolates.
• The DENV-1 isolates have different *in vivo* replication kinetics.
• The isolate from a DSS patient persists longer compared to the other isolates.
• These phenotypic differences confirm our earlier *in vitro* findings with the same DENV-1 isolates. Thus, DENVs within the same serotype and genotype may differ enough to affect clinical conditions *in vivo*.

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Keywords: dengue virus, mouse model, tropism, clinical isolate, cytokines, dengue hemorrhagic fever, flavivirus

Background

The dengue viruses (DENV) belong to the genus flavivirus of the *Flaviviridae* family, and consist of four (1-4) antigenically related, but clearly distinct viruses (serotypes). The DENV particle is enveloped, and has a single-stranded positive-sense RNA genome of approximately 11 kb that resembles a messenger RNA with a cap on the 5' end but no poly(A) tail at the 3' end. The RNA genome encodes a 3411 long precursor polyprotein that contains three structural proteins (C, prM, and E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The open reading frame is flanked by two nontranslated regions (5' and 3' NTR) of approximately 95-135 and 114-650 nucleotides, respectively, that have characteristic secondary structures that are required for efficient translation and replication [2,3].

The DENVs are endemic in tropical and subtropical areas and more than one hundred million people get infected annually. Infection can be either asymptomatic, or cause an acute febrile illness that is characterized by fever, headache, retro-orbital pain, arthralgia, and myalgia. This condition can progress into dengue haemorrhagic fever (DHF), with cardinal signs such as increased vascular permeability, thrombocytopenia, focal or generalized haemorrhages. DF may progress into the life-threatening state of dengue shock syndrome (DSS) [4]. In the recent WHO guidelines, for case management purposes, DHF and DSS cases are now grouped together as "severe dengue" (group C) [5].

Uncontrolled urbanization and globalization have resulted in the geographic spread of the DENV-transmitting mosquitoes *Aedes aegypti* and *A. albopictus*, co-circulation of different DENV serotypes, and increased frequency of dengue epidemics [6]. There has been a severe increase of DHF/DSS in many endemic regions, emphasizing the urgent need of an effective vaccine.

Despite the global burden of DENV infections many important questions regarding DENV pathogenesis remain unaddressed due to the lack of appropriate animal models of infection and disease. A major problem is the fact that no non-human species naturally develop disease similar to human DF or DHF/DSS. Epidemiological, clinical, and laboratory findings indicate that both genetic differences in the virus and the host immune response contribute to the occurrence and severity of disease [7].

In the search for better animal models for dengue, several approaches to investigate infection of DENV in mouse have been proposed. One of two major strategies have been to modulate the mouse to render it more susceptible to DENV infection, e.g. SCID mice transplanted with cultured, DENV-susceptible tumour cells [8-10]. The other major alternative has been to modify the DENV in order to make it more infection-competent in the murine host, e.g. mouse-neuroadapted DENV [11-14], or virus serially passaged in mouse [15,16]. The relevance of these models to human pathogenesis remains unknown.

The immunocompetent BALB/c mouse is susceptible to DENV infection and has been used extensively to study various aspects of DENV infection pathogenesis, despite the lack of clinical symptoms [17-20]. Mouse-adapted DENV strains increase the susceptibility to DENV infection and elicit clinical symptoms in BALB/c mice [11,21]. The relevance of these observations to infection with wild-type DENV strains, however, should be interpreted with caution due to the altered genotype and phenotype of the passaged virus.

We have previously reported that DENV-1 isolates only passaged once in C6/36 cells (passage 1), derived from patients presenting the clinically distinct forms of dengue (DF, DHF, and DSS), can be distinguished *in vitro* based on different replication kinetics in mammalian Vero cells and apoptosis in C6/36. The DSS-derived isolate (accession number: FJ639694) had a unique phenotype compared to the DF- and DHF-derived isolates (accession numbers: HQ624983 and HQ624984, respectively) and genomic comparison revealed six amino acid substitutions unique for the DSS isolate that were located in the structural viral M and E genes that constitute the extracellular mature virus particle, and in the NS1, NS3, and NS5 genes. The E protein mediates host cell receptor binding, viral entry, and is a major target for humoral immunity [22,23]. The role of NS1 in virus replication is not known, but is thought to be involved in facilitation of viral infection and in the pathogenesis of DENV infection [24,25]. The NS3 acts together with its cofactor NS2B as the viral serine protease needed for DENV precursor polyprotein-processing [3,26-28]. The NS5 protein has a dual enzymatic activity with an RNA dependent RNA polymerase activity in its C-terminal domain, and a methyltransferase activity at its N-terminal end. Hence, these six mutations could ultimately affect antibody response, host immune pathways, and virus replication.

The primary aim of the present study was to investigate whether there is a phenotype difference *in vivo*

between the DENV-1 isolates similar to the *in vitro* findings, by studying difference in infectivity in BALB/c mice and to investigate the cell and organ tropism. The second aim was to determine if the inoculated virus concentration and the effects of the infection correlated, primarily by measuring the levels of inflammatory cytokines induced by the infection.

Results

DENV infection and Organ Tropism

All mice had detectable levels of DENV specific IgG antibodies at day 15 post-infection (p.i.), confirming the intravenous route to be appropriate choice in order to successfully infect all mice. In total 54.3% of the DENV-1 infected mice had detectable levels of viral RNA; 48.2% of the mice infected with the DF isolate; 55.6% of the DHF isolate infected mice; and 59.3% of the DSS isolate infected mice (table 1). All mice survived the infection without presenting any clinical signs but pathological lesions such as haemorrhage could be detected, most notably in brain, liver, lung tissue, and to some extent in spleen.

Haemorrhage in lungs was most pronounced in the DF-infected mice, whereas the DHF-infected mice experienced haemorrhage in lungs, liver and to some extent in brain. The DSS isolate seemed to have some neurotropism as the DSS-infected mice had extensive bleedings in the brain, whereas the peripheral organs showed only minor signs of haemorrhage. None of the NC mice exhibited any clinical signs or tissue pathology.

The three DENV-1 isolates showed different tropism; the two DENVs obtained from a DF and DHF case infected primarily lungs, spleen, and liver, whereas the DENV from a DSS patient infected lungs, spleen, and exhibited a strong tropism for brain tissue ($p = 0.001$). The results are summarized in table 1. The effects of infection in lung tissue showed a positive correlation to inoculated virus concentration and time-point, with a peak of infection at day 6 p.i. ($p = 0.003$) combined with the highest titer 1.5×10^6 PFU/mL in the inoclum ($p < 0.001$) (table 2).

Risk of Infection and Kinetics of Infection

The DENV-1 isolates exhibited different risks for infection that depended both on virus concentration in the inoculum ($p = 0.012$) and on time-point p.i. ($p = 0.014$) (Figure 1). The effects of inoculated dose of infectious virus showed a positive correlation to the total number of infected mice for each virus isolate, and infection peaked on day 6 p.i. for all three viruses.

Kinetics of infection differed between the three DENV-1 isolates. Viral RNA in the DF and DHF inoculated mice was predominantly detected during the first week of infection, and declined during the second week. Six inoculated mice tested positive after inoculation with the DF, and DHF isolates, respectively, both on day 3 and 6 p.i. During the second week (day 15 p.i.), however, only 1 and 3 mice tested positive after inoculation with the DF- and DHF-viruses, respectively. The time-course of infection differed in the DSS- infected mice that showed a delayed response to inoculation compared to the DF- and DHF-infected mice. Day 3 p.i. had the lowest number of DSS-infected mice (3 positive mice) whereas at day 6 p.i. 7 mice were infected, and 6 during

Table 1 Viral RNA was found in various organs depending on inoculated DENV-1 isolate (DF, DHF, or DSS), virus concentration in inoculum, and time-point p.i. In total 54.3% of the DENV-1 infected mice had detectable levels of viral RNA

DENV isolate	DF									DHF									DSS								
Titer (PFU/mL)*	1.5×10^4			1.5×10^5			1.5×10^6			1.5×10^4			1.5×10^5			1.5×10^6			1.5×10^4			1.5×10^5			1.5×10^6		
Day (p.i.)**	3	6	15	3	6	15	3	6	15	3	6	15	3	6	15	3	6	15	3	6	15	3	6	15	3	6	15
Heart	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lungs	-	-	-	2	3	-	2	3	1	-	-	-	-	2	-	2	2	1	-	-	-	-	3	1	1	1	1
Spleen	1	-	-	-	-	-	2	-	-	1	-	-	-	-	2	2	1	-	-	1	-	-	1	1	2	-	-
Brain	-	-	-	-	-	-	1	-	-	1	1	-	-	-	-	-	3	-	-	1	2	1	3	2	-	2	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	2	-	-	-	-	-	-	-	-	-	-	-
Kidneys	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Infected mice	1	-	-	2	3	-	3	3	1	2	1	-	1	2	2	3	3	1	-	3	2	1	3	3	2	2	1
Infected mice per titer	1			5			7			3			5			7			4			7			5		
Infected mice per day p.i.	6			6					1 6				6					3 3				7					6
Σ infected mice per virus isolate	13									15									16								
% infected mice per virus isolate	48.2%									55.6%									59.3%								

* Titer of inoculum was significant ($p = 0.012$).

** Time-point post-infection was significant ($p = 0.014$).

Table 2 Risk of infection in lungs

Inoculated virus (PFU/mL)	Risk of infection
1.5×10^4	0
1.5×10^5	0.41
1.5×10^6	0.52
Days p.i.	Risk of infection
3	0.26
6	0.52
15	0.15

Both inoculated DENV-1 concentration and time-point were significant ($p < 0.001$ and $p = 0.003$, respectively). The risk for infection in the lungs with the DF isolate was highest on day 6 p.i. and showed a positive correlation to inoculated virus.

the second week of infection (day 15 p.i.). Thus, the number of mice infected with the DF and DHF isolates peaked during day 3 and 6 p.i. in contrast to the number of DSS-infected mice that peaked during day 6 and 15 p.i (Figure 2).

Overall, the DSS isolate replicated in a majority of infected mice and for a longer period of time compared to the DENVs obtained from milder cases (Figure 2).

Cytokine secretion in serum

IFNγ levels peaked during the first week of infection and most notably on day 3 p.i. that showed the highest levels of IFNγ ($p = 0.007$). There was also a positive correlation to inoculated virus dose where the highest titer (1.5×10^6 PFU/mL) elicited the strongest IFNγ response ($p = 0.002$). A positive titer dependent correlation to IL-6 levels was observed in all three groups of DENV-1 inoculated mice ($p = 0.028$) (data not shown). There was a time and virus dependent difference regarding detected IL-10 levels. The DF-inoculated mice had higher levels of IL-10 compared to the DHF- and DSS-inoculated mice ($p = 0.007$), with a peak at day 3 p.i ($p = 0.009$) (Figure 3). The DF-inoculated mice also dominated in serum levels of secreted MCP-1 and showed the biggest difference compared to the DSS-inoculated mice ($p < 0.0001$), followed by DHF-inoculated mice ($p = 0.011$) (Figure 4). MCP-1 levels peaked at day 3 p.i. whereupon it decreased ($p = < 0.001$). Inoculated virus titer showed a positive correlation to measured serum levels of RANTES in all DENV-1 inoculated mice ($p = 0.0036$), and DF-inoculated mice dominated ($p = 0.048$). IL-13 and RANTES showed a dose-dependent response to inoculated virus. IL-1β, IL-2, and TNFα did not show any statistically significant relationships to any of the analysed parameters (table 3).

Discussion

DENV infections are a major public health problem and constitute a real challenge due to the absence of vaccines and effective antiviral drugs. There are no non-

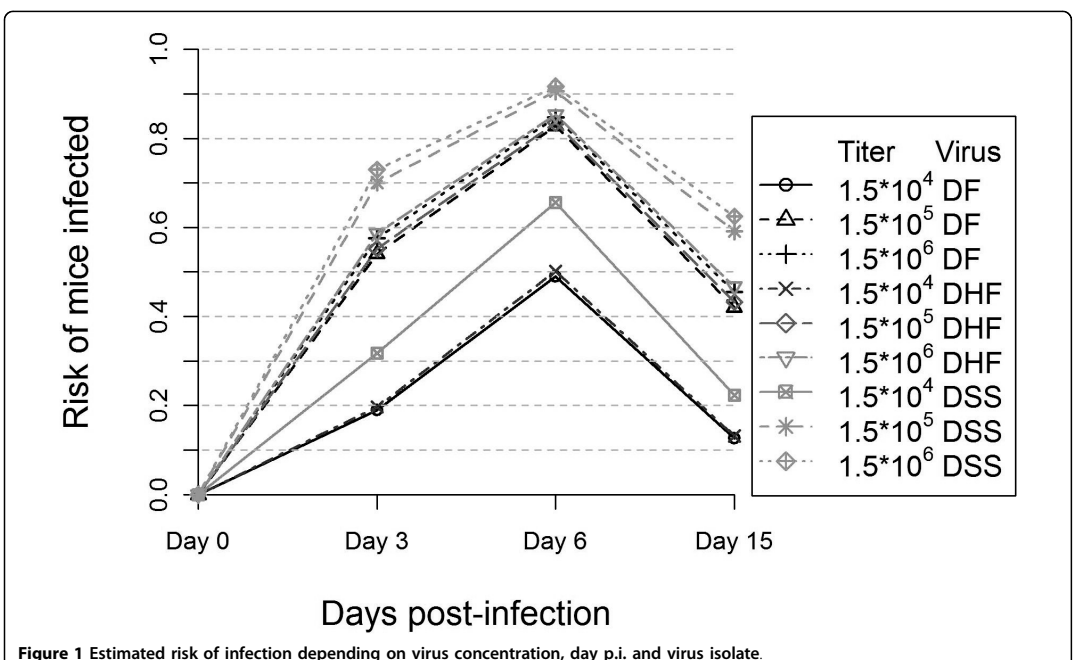

Figure 1 Estimated risk of infection depending on virus concentration, day p.i. and virus isolate.

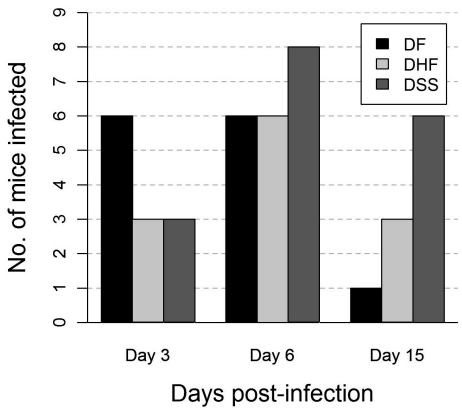

Figure 2 Persistence of infection shown as the number of mice infected out of the total mice inoculated, independent of inoculated virus concentration. Nine mice per DENV isolate and time-point in total were inoculated with the corresponding DENV isolates; DF, DHF, and DSS, respectively.

human species that naturally develops disease following DENV inoculation into peripheral sites and that resemble the clinical symptoms observed in humans [29]. The mechanisms underlying the immune responses to DENV infection are still poorly defined, and the lack of suitable animal models has hampered investigation of host- and virus-specific mechanisms that control primary and sequential DENV infections.

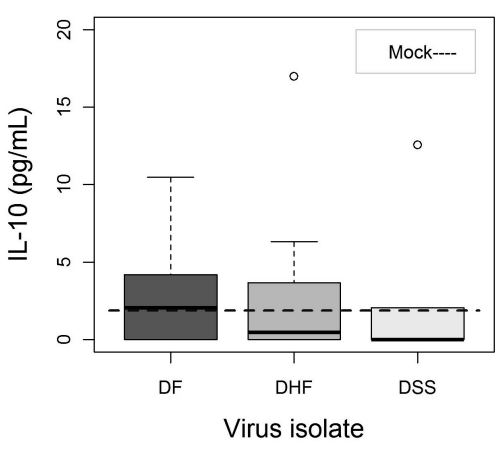

Figure 3 Box plots showing median IL-10 (solid line) by DENV-1 isolates independent of inoculated virus concentration and days p.i. The line in the middle of each box represents the median; the boxes consists of 1st and 3rd quartile, whiskers are 1.5*(3rd quartile-1st quartile). Outliers are marked with an open circle. The DF-inoculated mice had higher levels of IL-10 compared to the DHF- and DSS-inoculated mice ($p = 0.007$), with a peak at day 3 p.i ($p = 0.009$).

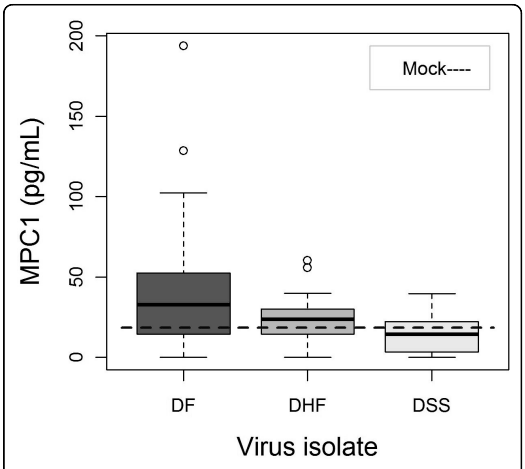

Figure 4 Box plots showing median MCP-1 (solid line) by DENV-1 isolates independent of inoculated virus concentration and days p.i. The line in the middle of each box represents the median; the boxes consists of 1st and 3rd quartile, whiskers are 1.5* (3rd quartile-1st quartile). Outliers are marked with an open circle. The DF-inoculated mice had the highest serum levels of secreted MCP-1 and showed the biggest difference compared to the DSS-inoculated mice ($p < 0.0001$), followed by DHF-inoculated mice ($p = 0.011$). MCP-1 levels peaked at day 3 p.i. and whereupon it decreased ($p = < 0.001$).

In this study, we have compared three minimally passaged DENV-1 isolates from patients experiencing the various clinical forms of dengue disease (DF; DHF; and DSS, respectively) *in vivo* by infection in mouse. We show that the isolate from a DSS patient infected the largest number of BALB/c mice, with a different tropism compared to the DENV-1 isolates obtained from milder clinical cases during the same outbreak. This indicates that DENVs within the same serotype and genotype may differ enough to cause various clinical conditions *in vivo*. These results confirm a phenotypic distinction previously observed *in vitro* with the same DENV-1 isolates where the DSS isolate replicated less efficiently in mammalian Vero cells than the DF and DHF isolates, and elicited apoptosis in mosquito C6/36 cells [1].

The aim of the study was to compare wild-type features of DENV-1 isolates passaged only once *in vitro* and never before inoculated in mouse or cultured in mouse cells prior to direct intravenous inoculation into BALB/c mice. BALB/c mice are known to be susceptible for DENV infection although with discrete pathological features [11,30]. We found that the mice did not develop dengue disease, and did not present any symptoms of the infection. However, viral RNA was detected

Table 3 Serum cytokine levels in mice inoculated with DENV-1 isolates from patients with DF, DHF, and DSS, respectively, and independent of inoculated virus concentration

Day p.i.	Cytokine	DF	DHF	DSS	Mock
3	IFNγ	3.10	3.24	2.08	0.09
	IL-1β	3.15	0.00	0.00	0.00
	IL-2	1.46	2.00	1.05	0.00
	IL-6	1.48	1.65	2.69	3.22
	IL-10	4.20	3.13	0.00	0.00
	IL-13	88.83	73.12	80.23	98.84
	MCP-1	71.58	34.25	21.45	0.00
	RANTES	35.11	13.17	15.93	9.76
	TNFα	6.44	3.97	3.78	0.00
6	IFNγ	2.53	1.65	1.92	0.00
	IL-1β	3.15	0.00	0.00	0.00
	IL-2	1.46	1.44	2.21	2.21
	IL-6	0.36	0.77	3.10	2.69
	IL-10	2.05	0.00	0.00	0.00
	IL-13	70.84	62.33	51.19	121.60
	MCP-1	22.12	14.54	3.37	0.00
	RANTES	20.47	14.16	24.46	10.43
	TNFα	2.45	2.15	0.00	0.00
15	IFNγ	1.24	1.28	1.70	0.87
	IL-1β	0.00	0.00	0.00	0.00
	IL-2	2.20	1.19	5.61	0.00
	IL-6	0.18	1.88	1.65	0.03
	IL-10	1.03	0.00	0.00	0.00
	IL-13	67.20	91.70	84.96	75.57
	MCP-1	21.53	14.54	3.37	0.00
	RANTES	27.68	17.22	20.75	15.02
	TNFα	1.73	0.00	1.48	0.00

The cytokines were measured in pg/mL.

in various organs depending on the DENV isolate injected (table 1). We were unable to detect any systemic viremia and this suggests an absence or a very low level of virus replication in circulating peripheral blood mononuclear cells. Alternatively, the lack of detectable viraemia could indicate that the virus had already disseminated into peripheral sites, since tissue infection was measured from day 3 p.i.

In brief, viral RNA was detected by qRT-PCR in spleen, liver, lungs, kidneys, and brain. Viral RNA was found in the kidneys in only one mouse, which is in line with earlier studies [31]. Organ tropism clearly differed between the three clinical DENV-1 isolates and our findings suggest that the disease severity may correlate with high tissue viral burden, even if plasma viraemia was below the detection limit. The DENV isolate from a DSS case showed a strong preference for brain tissue, compared to the two isolates from milder dengue cases that primarily were found in other organs as lungs and liver. Neurotropism in mice has been previously suggested to be associated with DENV virulence when comparing DENV-3 isolated from Brazilian patients [32].

Breakdown of the blood-brain barrier in DENV infected mice have been described before, and were shown to be dose-dependent [33]. Genomic sequencing performed previously [1] identified a unique amino acid substitution L→F476 in the E protein of the DSS isolate compared to the DF and DHF isolates, that may explain the neurotropic character of the DSS-isolate. The NS1 gene of the DSS isolate harboured another unique amino acid substitution, K→R115 in comparison to the two other DENV-1 isolates. The soluble form of NS1 (sNS1) is a dominant target of humoral immunity and activates complement components in normal human serum, and is proposed to play a significant role in the pathogenesis of disease [25,34]. The NS1 glycoprotein is glycosylated at two sites, N130 and N270, and glycosylation of both residues has been suggested to be required for neurovirulence in mice [35]. It is remains to be elucidated whether the possible breakdown of the blood-brain barrier and neurotropic character of the DSS isolate has any correlation to the disruption of the endothelium observed in humans suffering from vascular leakage. The three remaining amino acid substitutions unique for the DSS isolate were located in the NS protein 3 (S→P118), and 5 (T→I49, and S→N830). These differences could potentially alter the enzymatic activity of these proteins.

The DSS isolate seemed to persist longer *in vivo* since viral RNA was detected in a majority of mice on day 15 p.i. compared to the DF and DHF infected mice, and thereby exhibited infection kinetics differing from the two other DENV-1 isolates (DF and DHF) where viral RNA decreased after day 6 p.i. Thus, the DSS isolate could be regarded as more virulent than the two other DENV-1 strains, since a majority of the DSS-inoculated mice had detectable levels of viral RNA, infection was also less affected by the dose of inoculums and persisted longer in a majority of mice (Figure 1). The wild-type DENVs analyzed here could provide evidence for differences in virus replication, which in turn could influence the clinical outcomes of the infection, and eventually partitally explain some differences in virulence observed in humans.

These results confirm the *in vitro* findings of the same DENV-1 isolates, that showed that the strains derived from a DSS patient can be distinguished based on phenotypic characteristics from the isolates derived from a DF and DHF case [1]. We have previously shown that the DSS virus isolate exhibit much slower replication kinetics in mammalian Vero cells and an apoptotic

response in mosquito cells, and we observed in this study that the DSS virus isolate persist longer *in vivo* with extensive neuroinvasion.

Cytokines involved in inflammation were quantified in serum collected from inoculated mice and compared in regard to DENV-1 isolate, virus titer inoculated, and time-point p.i. Overall, the levels of the proinflammatory cytokines (IFNγ, IL-1β, IL-2, IL-6, IL-10, IL-13, MCP-1, RANTES, and TNFα) quantified were low, which is most probably due to fact that BALB/c mice is poorly susceptible to wild-type DENVs. The mice did not develop clinical apparent disease, and showed only discrete lesions in internal organs. Common for several cytokines, however, was a dose-dependent response to inoculated virus (IFNγ, IL-6, IL-13, and RANTES). The highest levels of measured cytokines were also in general at the beginning of the experimental period (IFNγ, IL-10, and MCP-1 peaked at day 3 p.i.). A curious finding was that DF-inoculated mice had higher detectable serum levels of IL-10, MCP-1, and RANTES, which have been seen to be increased in patients with severe DHF and DSS [36-42]. The preference for the brain of the DHF-, and most notably the DSS-infected mice, could imply locally high levels of proinflammatory cytokines that do not circulate systemically. This could explain why the IL-10, MCP-1, and RANTES levels dominated in the DF-inoculated mice, since the thoracic and abdominal organs were the primary target for DF isolate. Cytokines like IL-1β, IL-2, and TNFα did not show any differences compared to mock-infected controls and this could be due to the transient nature of many cytokines as well as the limited susceptibility of DENVs in mice.

Conclusions

We have previously shown *in vitro* that the isolate from a DSS case could be distinguished based on replication kinetics and apoptosis from isolates originating in milder cases. In this study we have extended the characterization of those clinical DENV-1 isolates derived from patients exhibiting the various forms of dengue illness. Despite their close relatedness, the DENV-1 isolate from a DSS case was phenotypically different from the other two isolates by significant brain invasiveness and a higher infectivity in BALB/c mice. The isolates from a DF and DHF case infected primarily lungs and liver, and to a limited extent brain and infection declined faster after day 6 p.i than in DSS-infected mice.

This is the first time different clinical South-East Asian DENV isolates have been directly compared for the *in vivo* characteristics in BALB/c mice. To define key elements involved in the virulence of these characterized DENV-1 phenotypes reverse genetics systems are needed. Additional low-passage strains of the different DENV serotypes of each clinical forms of dengue are required to fully decipher the complex mechanisms governing DENV pathogenesis.

Materials and methods

Virus

Three dengue serotype-1 (DENV-1) virus strains isolated from patient sera during a DENV outbreak in the Kampong Cham province, Eastern Cambodia, in 2007, were used. The isolates were obtained from patients experiencing the three distinct clinical forms of dengue disease: DF, DHF and DSS, according to the WHO classification [43]. Patient information regarding the analysed clinical DENV isolates is presented in previous work by Tuiskunen, A. et al., 2011. Blood samples were collected between day 2 and 6 after onset of disease and the serotypes were determined at the Institute Pasteur in Cambodia (IPC) by nested reverse transcriptase-polymerase chain reaction, according to Lanciotti procedure modified by Reynes et al. [44]. The virus isolates were obtained during the DENFRAME study, which has been approved by the Cambodian National 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.

Each DENV was isolated in *A. albopictus* mosquito cell line C6/36 (CRL 1660, ATCC), and thereafter propagated by one passage. The C6/36 cells were maintained in Leibovitz-15 medium, supplemented with 2% tryptose phosphate broth, and 5% fetal calf serum (Invitrogen, Stockholm, Sweden) and maintained at 28°C for 6 days. Cell culture supernatant was centrifuged for 10 min at 15000 rpm, titrated as previously described [1] and stored at -80°C in a solution of sucrose [1.5 M] and hepes [1 M]. Virus had not undergone any previous passage in mouse.

Animals

The animal experimental procedures were approved by the Committee for Laboratory Animal Science of the Swedish Board of Agriculture (ethical permit ID no: 339/07).

Female BALB/c mice (Nova, Sollentuna, Sweden) age 6 weeks weighing 20 g were maintained on a standard laboratory diet with water *ad libitum* and housed under specific pathogen-free conditions at the animal facility of the Swedish Institute for Communicable Disease Control (SMI) in Stockholm, Sweden. Mice subjected to the same treatment were kept in groups of 4-5 mice per cage and handled according to the international guidelines for experimentation on animals. DENV-1 infected mice were housed in a biological safety level (BSL) 3 isolator, and negative control mice were housed in a BSL 2 facility.

Table 4 Schematic overview of the experimental design

DENV-1 isolate	DF			DHF			DSS			NC
Titer PFU/mL	1.5×10^4	1.5×10^5	1.5×10^6	1.5×10^4	1.5×10^5	1.5×10^6	1.5×10^4	1.5×10^5	1.5×10^6	N.A
Day 3 p.i	3	3	3	3	3	3	3	3	3	9
Day 6 p.i	3	3	3	3	3	3	3	3	3	9
Day 15 p.i	3	3	3	3	3	3	3	3	3	9
Total no. of mice		27			27			27		18

Mice were divided in groups of three depending on DENV-1 isolate for inoculation; titer of inoculum; and time of euthanasia. There were in total 99 mice included in the study, whereof 81 were inoculated with infectious DENV-1.

N.A. = not applicable

DENV-1 infection in mice

There were 99 mice in total divided into four groups with 27 mice each according to the 3 DENV-1 isolates and 18 mock infected negative controls (NC). Each group of 27 mice were subdivided into three groups of nine mice that were injected intravenously (i.v.) in the tail vein with a single dose (1.5×10^4 PFU/mL; 1.5×10^5 PFU/mL; or 1.5×10^6 PFU/mL, respectively) of infective DENV-1 without adjuvant. Similarly, the NC group of 18 mice received 150 uL of Leibovitz-15 medium supplemented with sucrose [1.5 M] and hepes [1 M] (table 4). The mice were monitored daily for clinical signs.

Blood that was obtained via a cardiac puncture of mice immediately after euthanasia by 4.5 - 4.8% isoflurane gas inhalation on day 3; 6, and 15 p.i. All mice were immediately splenectomised after euthanasia and thereafter stored at -80°C.

Detection of DENV RNA in BALB/c mice

To assess viral burden in tissues of infected mice, organs such as liver, heart, spleen, lungs, brain, and kidneys were harvested, weighed, and homogenized using Stainless Steel Beads (5.0 mm diameter) with a TissueLyser apparatus (Qiagen, Hilden, Germany). Total RNA from tissues was extracted immediately after dissection using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen).

Mouse sera were obtained by centrifugation of whole blood for 3 minutes at 2000 g in microtainer EDTA tubes (Becton, Dickinson and Company, Temse, Belgium). Viral RNA from thawed aliquots of serum (140 uL) was extracted using QIAamp Viral RNA Mini kit (Qiagen). RNA samples, eluted in RNAse-free water, were stored at -80°C. Quantitative measurements of total viral RNA were performed as previously described [45].

IFA for virus-specific antibody detection

An in-house immunofluorescence assay (IFA) was used as previously described [46] essentially to verify that all DENV-1 infected mice had raised a DENV specific antibody response.

Cytokine dosage

Cytokine levels in mouse sera collected from all inoculated mice, including mock infected, were measured as duplicates with Milliplex Mouse Cytokine/Chemokine Panel (Millipore) in a Luminex 100 (Luminex, Bio-Rad, Sweden). The following cytokines and chemokines were analysed according to the manufacturer's instructions: IFNγ, IL-1β, IL-2, IL-6, IL-10, IL-13, MCP-1, RANTES, and TNFα.

Statistical Analysis

To test the effect of virus, titer and days p.i. on the risk of DENV infection in mouse organs binomial regression with a log link was used. Parametric bootstrap with 10.000 replicates was drawn from an assumed binomial probability model. The effect of virus, titer and day between smaller and larger models were then evaluated by comparing the deviance, extracted from the replicates. The largest model, within each analysis of the infection risk in an organ, included the main effects virus, titer and day plus the interactions virus by titer, virus by day and titer by day.

Median regression was used to evaluate the relationship between virus isolate, day, and titer on the different cytokines. As most cytokines displayed highly right skewed distributions in some virus isolates the use of median estimates yielded more robust results than with the use of mean based methods. Day and titer were analyzed separately with two main models per cytokine: Cytokine = group + day + group by day interaction; and Cytokine = group + titer + group by titer interaction. P-values < 0.05 were used as statistically significant results. All analyses and graphs were performed using the statistical software R (version 2.13.1, 2011).

Acknowledgements

We thank the animal technicians Christel Werner and Olov Rosendahl at the animal facility of the Swedish Institute for Communicable Disease Control, and Dr Mikael Andersson for invaluable help with the statistical analysis.

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Authors' contributions

Conceived and designed the experiments: AT, ILG. Performed the experiments: AT, MW. Analyzed the data: AT, ÅL, ILG. Wrote the paper: AT. Reviewed the paper: PB, ILG, ÅL. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 28 July 2011 Accepted: 11 August 2011
Published: 11 August 2011

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doi:10.1186/1743-422X-8-398
Cite this article as: Tuiskunen *et al.*: Phenotypic characterization of patient dengue virus isolates in BALB/c mice differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome. *Virology Journal* 2011 **8**:398.

IV

Phenotypic and genotypic characterization of patient dengue virus isolates

Short Title: Characterization of dengue virus isolates

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Key words: dengue virus, dengue hemorrhagic fever, dengue shock syndrome, clinical isolate, replication, genome, flavivirus, Vero cells, C6/36 cells, PBMC, monocyte, dendritic cells

Number of words, summary: 249

Number of words, main text: 5254

Number of figures: 4

Number of tables: 4 (+4 tables and +1 figure in supplementary tables and figures)

Summary

The dengue viruses (DENVs) cause the most common arthropod-borne viral disease in man, but despite the global burden, the pathogenesis of dengue remains poorly understood. In addition to host genetics and previous DENV infection history, variations of DENV strain virulence are acknowledged to be a risk factor. The genetic traits that increase virulence, however, are yet to be discovered.

In this study, clinical DENV isolates of passage one derived from patients experiencing the various symptomatic forms of DENV infection (dengue fever, DF; dengue hemorrhagic fever, DHF; and dengue shock syndrome, DSS) were characterized in human primary PBMCs, monocytes, monocyte-derived dendritic cells (moDCs), Vero cells and C6/36 with regard to replication kinetics and induction of apoptosis. The clinical isolates from all four DENV serotypes, derived from DSS patients, were clearly distinguished from the isolates derived from milder cases, based on replication kinetics in moDCs and Vero cells. The DSS isolates also induced apoptosis in C6/36 cells, in contrast to the DENV isolates from milder cases. Replication differences were most notable when measuring infectious titer in comparison to viral RNA levels. This could suggest the presence of defecting interfering particles.

Whole genome sequencing of the clinical DENV isolates revealed multiple amino acid substitutions, whereof a majority was located in the envelope glycoprotein, and in the non-structural protein 1 and 5.

These findings highlight genotype and phenotype differences between clinical DENV isolates that closely resemble wild-type viruses, and suggest that intrinsic viral traits may potentially influence virus virulence in dengue pathogenesis.

Introduction

Dengue is an acute febrile disease caused by the mosquito-borne dengue viruses (DENVs) that are members of the *Flaviviridae* family and consists of four serotypes (1-4) (Westaway et al., 1985). The single-stranded positive-sense RNA genome comprises approximately 10,700 nucleotides and encodes a 3,411 amino acid long precursor polyprotein that contains three structural proteins (C, prM/M, and E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

DENV is transmitted by the mosquito *Aedes aegypti,* and to a lesser extent *Ae. albopictus*, and is the most common cause of arboviral disease in man today (WHO, 1997). All four serotypes can be found worldwide in tropical and subtropical regions where more than 2.5 billion of the world's population live (Guzman and Kouri, 2002). DENV infection can cause a wide range of diseases in humans, even though DENV infections may also be asymptomatic. The diseases range in severity from undifferentiated acute febrile illness, classical dengue fever (DF), to the life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gubler, 2002). The WHO estimates an annual occurrence of 100 million infections whereof approximately 500,000 people with DHF require hospitalization, a very large proportion of who are children. Classical DF begins with an abrupt onset of high fever and is a self-limited though debilitating illness with headache, retro-orbital pain, myalgia, arthralgia, rashes and leucopenia (Henchal and Putnak, 1990). Early symptoms of DF and DHF are indistinguishable, but DHF is associated with hemorrhagic manifestations, plasma leakage resulting from an increased vascular permeability, thrombocytopenia (<100, 000 platelets/mm^3). Cardiovascular compromise accompanies DSS and occurs when plasma leakage into the interstitial spaces results in shock (Gubler, 2002). In the continuous seek for an effective vaccine and anti-dengue drugs the sole measure to prevent DENV transmission is still to reduce the vector population.

The pathogenesis of dengue is complex, and despite intensive research in the area, not well characterized. Several risk factors influencing the severity of DENV infection has been identified such as age (Gubler, 1998, Guzman et al., 2002a), the genetic background of the host (Guzman et al., 2002b, Halstead et al., 2001, Sakuntabhai et al., 2005), viral serotype (Balmaseda et al., 2006, Gubler, 1998) and genotype (Messer et al., 2003, Rico-Hesse et al., 1997), and secondary infection by a heterologous serotype mediating antibody-dependent enhancement (ADE) (Guzman et al., 2002b, Burke et al., 1988, Halstead et al., 1970, Sangkawibha et al., 1984, Thein et al., 1997). The impact and contribution of each individual risk factor, however, is far from fully understood.

There have been specific geographic examples of the appearance of DENV genotypes correlating to DHF/DSS epidemics. The introduction of a South-East Asian DENV-2 strain in the Americas in 1981 resulted in sudden emergence of DHF/DSS cases. The Asian genotype was later proved to be more virulent and more likely to result in DHF/DSS than the American genotype even after a secondary infection (Rico-Hesse et al., 1997, Rico-Hesse, 1990). There are no specific genetic markers, however, that have been identified so far to confer the increased virulence traits of DENV. It is not known if the tendency of certain genotypes to cause severe disease results from greater intrinsic virulence, or if greater virulence is a result of enhanced cross-reactivity in the presence of heterologous antibodies, or a combination of the two. Determining whether DENVs differ in virulence, as well as identifying the genetic basis of such differences, is thereby of fundamental importance. We have previously shown that clinical DENV-1 isolates passaged only once in mosquito cells, derived from patients experiencing DF, DHF, and DSS, respectively, differed both genotypically and phenotypically *in vitro* and *in vivo (Tuiskunen et al., 2011a, Tuiskunen et al., 2011b)*. In addition to unique amino acid substitutions in both structural and NS proteins, the isolate obtained from a patient experiencing DSS replicated less efficiently in mammalian Vero cells and elicited apoptosis in mosquito C6/36 cells, compared to the strains isolated from a DF and DHF patient (Tuiskunen et al., 2011a). The phenotypic difference was also observed in BALB/c mice

where the DSS isolate persisted longer and was primarily neurotropic, in contrast to the two other DENV-1 isolates that infected predominantly internal organs (Tuiskunen et al., 2011b).

The focus of the current study has been to extend the phenotypic and genotypic characterization of the DENV-1 isolates to include isolates of serotype 2, 3 and 4 (DENV-2, -3 and -4). Thus, we have characterized nine additional clinical DENV isolates from patients experiencing the various symptomatic forms of DENV infection; DF, DHF, and DSS. A genomic comparison of the clinical DENV isolates revealed several amino acid substitutions, and replication kinetic differences in mammalian Vero cells, human peripheral blood mononuclear cells (PBMCs), monocytes and monocyte-derived dendritic cells (moDCs). In addition, variations in the apoptotic response in C6/36 cells distinguished the DENV isolates from DSS patients from the remaining DENV isolates from milder dengue cases. These observations complement and agree with our previous findings on DENV-1, and suggest that DENVs derived from the same epidemic may differ both phenotypically as well as genotypically.

Results

Replication kinetics of wild-type DENV isolates obtained from patients experiencing the various degrees of dengue illness severity

We investigated the replication kinetics of the various clinical DENV isolates in order to characterize them phenotypically both in mammalian and mosquito cell lines. Viruses from supernatants and cells were titrated by a focus forming assay (FFA) and the amount of viral RNA (vRNA) determined by quantification of total vRNA using a quantitative RT-PCR (qRT-PCR).

All isolates replicated with similar efficiencies in the mosquito cell line C6/36 with nearly 100% of cells infected 5 days post-infection (pi) (data not shown). In mammalian Vero cells at 37°C,

however, the strains isolated from patients experiencing DF replicated better than strains isolated from patients experiencing severe dengue (DHF or DSS) (Figures 1 and 2). Immunofluorescence was performed in parallel using a panflavi anti-envelope monoclonal antibody (HB112) and a mAb detecting active virus replication (J2, targeting double-stranded RNA). Cells infected with DSS viruses showed a constantly low level of replication, compared to DF-derived DENVs that replicated well, and the DHF isolates that resembled either the DSS isolates or the DF isolates, depending on serotype. The replication differences were most notable when measuring infectious titers, as compared to quantification of the vRNA levels (Figures 1 and 2). Both the DHF and DSS isolates of DENV-2 replicated less efficiently than the DF isolates, which was clearly distinguishable in infectious titre, but not when quantifying intracellular vRNA levels. The same phenomenon was observed with the DENV-3 clinical isolates. The isolates from DHF and DSS patients replicated at a similar low level as compared to the isolate from a DF patient that replicated better in Vero cells. The difference of replication efficiency was best illustrated when measuring infectious titer, whereas vRNA levels were similar for all three DENV-3 isolates. The DSS isolate of DENV-4 replicated less efficiently as compared to the isolates of DF and DHF that replicated with similar efficiency. The difference between the DF and DHF isolates, however, was smaller in intracellular virus levels and vRNA levels, than in supernatant. Together these results suggest that the viruses may differ in the amount of defective viral particles that are synthesized during the replication cycle. Despite similar levels of vRNA, the various strains analyzed differed in the amount of infectious particles produced that distinguished the DSS isolates from the isolates derived from milder cases.

Infection in human primary cells

The primary cells targeted by DENV are suggested to be cells from the mononuclear phagocyte linage (Boonpucknavig et al., 1979, Boonpucknavig et al., 1976, Sahaphong et al., 1980, King et al., 1999, Alen et al., 2011). Therefore, we aimed to directly compare the susceptibility of total PBMC

to infection with the twelve clinical DENV isolates (serotype 1-4) derived from patients experiencing DF, DHF, and DSS. C6/36 cells, PBMC, monocytes, and moDCs were infected with DENV at an MOI of 1 and 10, and RNA extracted 72hpi to quantify DENV RNA levels in the infected versus the uninfected cells. In contrast to the mosquito cells, a very low level of replication or none were observed in both PBMC and monocytes. Yet, significant levels of DENV RNA were found in moDCs at 72 hpi. The DSS isolates from all four serotypes had higher levels of vRNA compared to the DENV isolates derived from DF cases (Figure 3).

Apoptosis due to DENV infection

We studied apoptosis in C6/36 and Vero cells to investigate whether any difference between the clinical DENV isolates of serotype 2-4 could be found. The cells were stained at four time-points (24, 48, 72h and 6 days pi). None of the DENV isolates induced apoptosis of Vero cells (data not shown). Minimal amount of apoptosis was observed in the C6/36 cells infected by the patient isolates from DF and DHF cases compared to the DSS-derived DENV isolates (Supplemental figure 1). The DSS-isolate belonging serotype-3 elicited the highest degree of apoptosis (23%), followed by the DSS-isolate of serotype-2 (21%), and serotype-4 (15%). Since virus titration and vRNA using real-time qRT-PCR confirmed that all nine DENV isolates had a similar replication kinetic in C6/36 cells, the DSS isolates induced a higher degree of apoptosis in C6/36 cells compared to the other viruses obtained from DF and DHF patients regardless of replication kinetics. A unique feature of the DSS-isolate of DENV-3 was the formation of syncytia. This cytopathic effect was clearly visible already on day 3 pi, and remained clearly visible throughout the week of infection (Figure 4).

Genomic characterization

There is no specific genetic marker that today is recognized to increase virulence of DENVs or that

is linked to severe dengue disease in humans. In order to investigate whether certain DENV genes or particular mutations could be involved in virus virulence, the complete open reading frame of each DENV isolate characterized in this study was sequenced. In total, 58 amino acid substitutions were found with a preference for the structural E protein, and the NS proteins 1 and 5, followed by NS2A protein (supplementary table 1). Considerable sequence variation was found both in structural and NS genes (Table 1-3). The DENV-2 isolates obtained from a DF and DHF patient, respectively, were nearly identical, only differing at three positions in the NS5 gene (E→Q262, V→A613, and A→V617), where DHF was identical to the DSS isolate. Regarding the three DENV-3 isolates, there were multiple amino acid differences scattered throughout the genome, including both structural and NS genes. The DSS isolate had a unique amino acid substitution (V→A59) in the prM gene, two in the E protein (Y→H132 and N→S346), one in the NS1 gene (N→S17 and A→T288), and one in the NS3 gene (S→N86). Additional amino acid substitutions were found in the NS2A and NS5 genes, and two in the E gene, that differed between the three isolates. The DF and DHF isolates from serotype-4 differed at four positions. The DF isolate had two unique amino acid substitutions in the E gene (E→D177), and in the NS1 gene (S→P166), whereas the DHF isolate had two unique amino acid substitutions, both located in the NS5 gene (S→L393 and V→S772). The DSS-isolate had six unique amino acid substitutions in the methyltranserase (MTase) region of NS5, and seven in the RNA-dependent RNA polymerase (RdRp) domain. Thus, the clinical DENV isolates were genotypically different with single amino acid substitutions predominantly in the E, NS1 and NS5 proteins (Table 1-3).

Discussion

Despite the public health importance of dengue disease, its pathogenesis remains unclear. There is a complex interplay of viral and host factors. Secondary infection by a heterologous serotype has been reported to be the greatest single risk factor for DHF/DSS, although the ability to cause severe

dengue disease in primary infection varies by DENV strain (Balmaseda et al., 2006). There is no clear correlation, however, between a particular DENV serotype or genotype(s) and disease outcome, but there are indications that certain DENV-2 and DENV-3 genotypes are more often associated with severe disease (Messer et al., 2003, Rico-Hesse et al., 1997). DENV-2 strains of Asian genotype replicated to higher titers in human monocyte-derived macrophages and moDCs than American genotype DENV strains (Cologna and Rico-Hesse, 2003). The emergence of Group B subtype III DENV-3 strain in Sri Lanka in 1989 is another example of clade replacement correlating with an increase in DHF/DSS (Messer et al., 2002, Messer et al., 2003, Kanakaratne et al., 2009). The magnitude of intra-genotype virulence differences and its influence in pathogenesis during an epidemic, however, are not known.

In this study, nine clinical DENVs belonging to serotype-2, -3 and -4 isolated in the Kampong Cham region in Cambodia were phenotypically and genotypically characterized and compared. This current study extends our previous analysis characterizing three DENV-1 isolates *in vitro* and *in vivo* (Tuiskunen et al., 2011a, Tuiskunen et al., 2011b). The DENV-1 to 3 strains originated from the same epidemic in 2007. The clinical isolates of serotype-4 were collected during the 2007 and 2008 epidemics but still in the same region as the other strains. All clinical samples were isolated from young children in order to minimize the inter-host variability. Of note, children have an inherent higher vascular permeability relative to adults (Gamble et al., 2000).

Common phenotype differences for all low-passage DENV isolates characterized in this study and previously, were poorer replication of the DSS isolates in Vero cells. In contrast, the clinical isolates derived from DF patients replicated most efficiently in Vero cells regardless of serotype. The DENV-2 and -3 isolates derived from DHF patients replicated at similar levels than the DSS isolates of the same serotype. In contrast, the DENV-4 strain isolated from a patient experiencing DHF

resembled more the DF isolate than the strain obtained from a DSS patient. Hence, these results from DENV serotype-2, -3 and -4 viruses, after only one passage on mosquito cell culture, confirm the earlier observations made with DENV-1 viruses (passage one) that had highest replication rates when obtained from DF patient than from a DSS case (Tuiskunen et al., 2011a).

Measuring extracellular infectious viral titre reflected best the true replication differences between the DENV isolates. The difference of intracellular vRNA levels between the DENV isolates within the same serotype was smaller than the difference of viral titre, indicating that a significant amount of viral particles produced are not budding competent and/or infectious. Disease severity is believed to be correlated to viral load measured in the blood (Cologna and Rico-Hesse, 2003, Leitmeyer et al., 1999) and defective interfering viral particles have been suggested to influence virulence and interfere with the host immune response (Cave et al., 1985, Huang et al., 1986, Bangham and Kirkwood, 1990). Thus, the observations that the DENV isolates have similar vRNA levels despite their replication differences are interesting findings that may have immunological implications in the host during infection.

Infection with all twelve clinical isolates only passage once on mosquito cell lines was assessed in human primary cells. Immature DCs in the skin are believed to be the first target cells during natural DENV infection in humans (Wu et al., 2000), and other cells of the myeloid lineage such as macrophages are also crucial target cells for DENV replication (Palucka, 2000, Kyle et al., 2007). In this study, the moDCs seemed to be the most susceptible for DENV infection compared to the PBMCs and monocytes. Common for all three cell-types, however, was that the DSS-derived DENV isolates from all four serotypes had higher vRNA levels as compared to the DF isolates. This interesting finding supports the hypothesis that a higher viral biomass causes a worse clinical outcome (Vaughn et al., 2000). Additional low-passage, clinical DENV-isolates, however, are needed to extend this observation.

In addition to replication differences in mammalian cell lines and in human primary cells, the DSS isolates from all three serotypes elicited a higher degree of apoptosis in mosquito cells. Efficiency of replication in C6/36 cells measured both in viral titre and in quantified vRNA were similar for all nine DENVs compared indicating that apoptosis was not a consequence of different levels of viral load. Previous results of a DENV-1 strain isolated from a DSS patient showed increased level of apoptosis in C6/36 cells compared to isolates obtained from milder clinical cases (Tuiskunen et al., 2011a). The significance of these observations requires further investigation in order to identify the factors that modulate apoptosis in this arthropod cell line. An additional feature observed in mosquito cells infected with the DENV-3 strain isolated from a DSS case was the phenomenon of syncytia, fusion from within, or cell-cell fusion. This cytopathic effect was clearly distinguishable from the two other DENV-3 isolates characterized, and is proposed to be mediated by the viral E protein (Koblet, 1987, Omar, 1989, Omar and Koblet, 1988). The DENV-3 DSS-isolate had two unique amino acid mutations in the E protein (Tyr→His132 in domain I, and Asn→Ser346 in domain III of the E protein), suggesting that these may be at the origin of the syncytia observed during cell infection.

The genomic sequences of the open reading frame of all nine DENVs (serotype 2-4) were compared to identify potential markers associated with the different phenotypic expressions observed during *in vitro* experiments. In total, 58 amino acid substitutions were found with a preference for the structural E protein, and the NS proteins 1 and 5, followed by NS2A protein. The nine clinical DENVs compared in this study differed by eight amino acids in the E protein, which could elicit the production of antibodies that may differ in their epitope specificity. The E protein binds to receptors, e.g. DC-SIGN on immature DCs, and anti-E antibodies inhibit viral attachment, internalization, and replication within cells (Alen et al., 2011, Johnson et al., 1994, Lee et al., 1997, Navarro-Sanchez et al., 2003). Both receptor binding efficiency and initial translation may impact the cellular tropism of

the virus. The Ser→Asn203 mutation in the E protein of DENV-2 DF strain could potentially be modified by glycosylation and influence target cell tropism and epitopes for antibodies since DENV entry in human cells is carbohydrate-dependent (Alen et al, 2011). Thus, different E protein epitopes may modulate the infection evolution. As for the E protein, eight amino acid substitutions were found in the NS1 protein. The exact function of the NS1 proteins remains unknown, but is believed to play a role in dengue pathogenesis (Schlesinger et al., 1987, Avirutnan et al., 2006). The NS1 protein is immunogenic and antibodies raised against soluble NS1 protein has been proposed to cause endothelial dysfunction due to cross-reactivity between anti-NS1 antibodies to host proteins and endothelial cells (Lin et al., 2006). The largest number of amino acid substitutions for all three serotypes (2-4) was located in the NS5 protein. The NS5 protein has three major functional domains and is the largest DENV protein. Nine of the mutations were in the N-terminal S-adenosyl methionine MTase part of the protein, which spans amino acid residues 1 to 239 and is responsible for guanine N-7 and ribose 2_-O methylations required for the capping of the DENV genome. The cap structure is recognized by the host cell translational machinery (Bartholomeusz and Wright, 1993, Egloff et al., 2002, Ray et al., 2006). The nuclear localization sequences (NLS) (residue 320-405) interact with the NS3 viral helicase and is recognized by cellular factors, allowing protein transport. Only one amino acid substitution was found in the NLS domain (DHF-isolate of DENV-4 Ser→Leu393). The NS5 polymerase domain RdRp (residue 273-900) is responsible for synthesizing new vRNA genomes and 15 of the NS5 mutations were located in this domain, suggesting an influence in the different replication phenotypes observed.

Hence, clinical DENVs of the same serotype, isolated during the same epidemic, and passaged only once to resemble wild-type as much as possible, may differ both in phenotypes and in genotypes. We have compared replication abilities in mammalian Vero cells, commonly used for titration of DENVs, human PBMCs, monocytes, moDCs, and C6/36 cells, and found replication differences distinguishing isolates obtained from severe dengue cases from those isolated from patients experi-

encing milder symptoms in all cell types except for C6/36. A similar diversification of the DENV isolates was seen in C6/36 cells, however, where the DSS isolates stimulated apoptosis. These findings highlight genotype and phenotype differences between passage 1 clinical DENV isolates, and confirm what we previously found with three DENV-1 isolates originating from the same epidemic in Cambodia 2007 (Tuiskunen et al., 2011a). The viral genomes differed at multiple sites also within the same serotypes, and the observed phenotypic differences may depend on external features of the viral E protein, NS1 and the multi-enzymatic activities of NS5, since 36 of 58 amino acid substitutions were found within these three proteins. Further studies are now required to understand the contribution of each mutation and its potential impact on dengue pathogenesis. These results strongly suggest that DENVs from the same genotype and epidemic may differ both in their genomic set-up as well as in their expressed viral proteins, and that these intra-genotype differences are not restricted to one serotype. The contribution of these discrepancies in dengue pathogenesis in humans remains to be further characterized.

Materials and methods

DENVs

Nine dengue viruses of serotypes-2, -3 and 4 isolated during outbreaks in 2007 and 2008 from patients living in the Kampong Cham province, Eastern Cambodia, were used during this study (Table 4). The isolates were obtained from patients experiencing either classical DF, DHF, or DSS, according to the WHO classification (WHO, 1997). Blood samples were drawn between day 2 and 6 after onset of disease. These samples were collected during the DENFRAME study (a project supported by European Union) and immediately anonymized as stated in the research protocol. The DENFRAME project was approved by the Cambodian National 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.

Each strain had been isolated at the Institut Pasteur in Cambodia (IPC) in the standard cell line

C6/36, derived from *Ae. albopictus* (CRL 1660, ATCC), and thereafter undergone only one *in vitro* passage (P1), in order to avoid mutations that might be associated with tissue culture adaptation. The isolates were serotyped at IPC by nested Reverse transcription (RT) PCR according Lanciotti procedure modified by Reynes et al. (Reynes et al., 2003).

Cells

Mammalian Vero cells (CCL-81, ATCC) were grown at 37°C, 5 % CO_2, in cell culture medium 199 (Gibco, Paisley, UK) with 5 % fetal calf serum (FCS). The *Ae. albopictus* cell line C6/36 was grown at 28˚C, in cell culture medium Leibovitz-15, with 5 % FCS and 2 % tryptose phosphate (TP) (all from Invitrogen Life Technologies, Gibco, Paisley, UK). PBMCs were obtained from healthy donors by collection of buffy-coats provided by the blood bank at the Karolinska Hospital, Stockholm, Sweden, and following Ficoll-Paque (GE Healthcare) density gradient centrifugation. PBMCs were plated in 96-well culture plates at a density of 300, 000 cells per well with RPMI-1640 medium supplemented with 10 % FCS, 2 mM L-glutamin, 100 U/mL penicillin, 100 M streptomycin, 2 % HEPES (all from Invitrogen Life Technologies, Gibco, Paisley, UK). PBMCs were enriched for monocytes following plastic adhesion on to cell culture flasks for 2 h at 37°C, 5 % CO_2. Adherent monocytic cells were detached from the flask and resuspended in the same RPMI-1640 medium used for PBMCs. Isolated cells were plated in 96-well culture plates at a density of 300, 000 cells per well.

The moDCs were generated from monocytes separated from PBMC using CD14-positive magnetic beads (Miltenyi, Bergisch Gladbach, Germany) and were more than 98% pure, as assessed by flow cytometry. Monocytes were cultured in RPMI-1640 medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen Life Technologies, Gibco, Paisley, UK), and the following cytokines: 100 ng/ml rhIL-4 and 75 ng/ml rhGM-CSF (both from Peprotech, Rocky Hill, NJ, USA). On day 2 in culture, half of the cell culture medium was replaced, and new

cytokines were added. On day five, moDCs were harvested and used in further experiments.

DENV infection in cells

The procedures for infection of Vero and C6/36 cells have been described elsewhere (Tuiskunen et al., 2011a). In short, an inoculum of 500 µL virus per well on a 12-well plate was used for infection at a multiplicity of infection (MOI) of 1. The virus was diluted in cell medium (FCS-free), and virus free cell culture medium was used for the mock infected cells. The cells were thereafter incubated for 2 h at the corresponding temperature. The inoculum was removed and the cells were gently washed with PBS (1X). Two mL per well of corresponding cell medium (culture medium 199 for Vero cells, and Leibovitz-15 for C6/36 cells) supplemented with 5 % FCS was added, followed by incubation at corresponding temperature. Samples, both supernatant and cells, were harvested daily throughout the week and used for viral RNA extraction and virus titration.

PBMCs, monocytes and moDCs were infected with DENV at an MOI of 1 or 10, or mock infected with FCS-free cell culture medium or heat-inactivated DENV (60°C, 30 min), and incubated at 37°C in a 5 % CO_2 humidified atmosphere for 2 h. Following incubation cells were centrifuged and inoculums discarded, and the cells were gently washed with DPBS (1X). Two hundred uL per well of cell culture medium (supplemented with 10 % FCS) was added and the cells were incubated at 37°C, 5 % CO_2 for 72 h. Following infection cells were centrifuged and supernatants were saved for further analysis. The cells were kept for immunofluorescence staining.

Immunofluorescence with HB112 and J2 for detection of DENV infection and DENV titration by fluorescent focus assay (FFA)

The infectious titer of DENVs in C6/36 cells was determined by FFA as described previously (Tuiskunen et al., 2011a). Briefly, 50 µL of diluted virus was inoculated in each well on a 96-well plate and incubated for 2h at 28˚C. Plaque-forming units (PFU) were counted in a light microscope 72h post-infection (hpi). A primary panflavi anti-envelope monoclonal antibody (MAb) (HB112^{TM},

ATCC) or a mAb staining for double-stranded RNA (J2, English and Scientific Consulting, Hungary) were used together with a goat anti-mouse IgG A488-conjugate (1:250 dilution, Gibco, Paisley, UK). In order to stain the eukaryotic nuclei, Dapi (4', 6-diamidino-2-phenylindole) binding (Sigma-Aldrich) was used in a 1:5000 dilution.

Viral RNA extraction and real-time one-step quantitative RT-PCR

Viral RNA was extracted from lysed cells and cell supernatant using the QIAmp Viral RNA Mini Kit. The RT-PCR reactions were set-up according to the manufacturer's instruction (SuperScript III Platinium One-Step Quantitative RT-PCR system, Invitrogen Life Technologies) as described previously (Leparc-Goffart et al., 2009).

Apoptosis TUNEL Assay

Vero and C6/36 cells infected with DENV were grown on Lab-Tek Chamber slides (0.8 cm^2 per well) in order to study apoptosis. The method has been described previously (Tuiskunen et al., 2011a). Briefly explained, the cells were infected with a MOI of 1 and incubated for 24, 48, 72h, and six days, respectively. The cells were then stained for the presence of DENV antigen, using the HB112 and J2 MAbs, as well as for apoptotic cells using TUNEL-staining (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling). Apoptosis was detected with the In Situ Cell Death Detection Kit Red (Roche), according to the manufacturers' instructions and cells were stained for 1h at 37˚C. Staurosporin (Sigma-Aldrich) was used as positive control for apoptosis.

Sequencing

Genomic vRNAs were isolated from infectious supernatant from the first passage propagated in C6/36 cells, which is believed to confer the lowest possible level of selective pressure, using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). RT was performed using the SuperScript

First Strand Synthesis System for RT-PCR (Life Technologies) and random hexamer primers. Phusion Hot-Start High Fidelity DNA Polymerase (Finnzymes) was used to generate overlapping PCR fragments of approximately 1000 nucleotides, which were purified by gel electrophoresis followed by gel extraction using the MinElute Gel Extraction Kit (Qiagen, Hilden Germany), and eluted in 10µL water. DENV serotype specific primers were used for two-directional sequencing (supplementary tables 2a.-c.). Primers were designed to sequence both strands (by Sanger sequencing using ABI3739XL technology and the PhredPhrep program) of the PCR product, from which consensus sequences were assembled.

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Tables and Figures

Table 1. Amino acid differences between DF, DHF and DSS isolates in the open reading frame of DENV-2 strains. (*) denotes that the amino acid is located in the MTase region of the NS5 protein, (±) is the NLS domain, and (#) the RdRp domain. Roman numbers indicate domain I/II/III in the viral E protein. Bold text indicates unique amino acid substitution among the three clinical isolates of the same serotype.

Amino acid position in polyprotein	Amino acid position in protein	Gene or region	Virus and amino acid		
			DF	DHF	DSS
266	61	M	**Val**	Ala	Ala
440	160[I]	E	**Met**	Lys	Lys
483	203[II]		**Asn**	Ser	Ser
508	228[II]		**Glu**	Gly	Gly
904	129	NS1	**Tyr**	His	His
1039	264		**Ile**	Thr	Thr
1121	346		**Ile**	Val	Val
1189	62	NS2A	**Ala**	Thr	Thr
1192	65		**Ala**	Thr	Thr
1247	120		**Val**	Ile	Ile
1266	139		**Asn**	Asp	Asp
1457	112	NS2B	**Ile**	Val	Val
1821	346	NS3	**Ser**	Thr	Thr
1827	352		**Met**	Thr	Thr
2186	93	NS4A	**Ile**	Val	Val
2509	27*	NS5	**Ile**	Thr	Thr
2777	262*		Glu	**Gln**	Glu
3128	613[#]		Val	**Ala**	Val
3132	617[#]		Ala	**Val**	Ala

Table 2. Amino acid differences between DF, DHF and DSS isolates in the open reading frame of DENV-3 strains. (*) denotes that the amino acid is located in the MTase region of the NS5 protein, (±) is the NLS domain, and (#) the RdRp domain. Roman numbers indicate domain I/II/III in the viral E protein. Bold text indicates unique amino acid substitution among the three clinical isolates of the same serotype.

Amino acid position in polyprotein	Amino acid position in protein	Gene or region	Virus and amino acid		
			DF	DHF	DSS
173	59	prM	Val	Val	**Ala**
412	132^{I}	E	Tyr	Tyr	**His**
626	346^{III}		Asn	Asn	**Ser**
643	363^{III}		Val	**Ile**	Val
763	483		Ile	**Val**	Ile
790	17	NS1	Asn	Asn	**Ser**
948	175		Tyr	**His**	Tyr
1061	288		Ala	Ala	**Thr**
1199	74	NS2A	**His**	Tyr	Tyr
1342	217		Arg	**Lys**	Arg
1559	86	NS3	Ser	Ser	**Asn**
2678	188*	NS5	Thr	**Asn**	Thr
2926	436$^{\#}$		Lys	Lys	**Arg**
2972	482$^{\#}$		Tyr	**Phe**	Tyr
3049	559$^{\#}$		**Gly**	Glu	Glu
3120	630$^{\#}$		Lys	**Glu**	Lys
3315	825$^{\#}$		Glu	**Gly**	Glu
3385	895$^{\#}$		**Thr**	Ser	Ser

Table 3. Amino acid differences between DF, DHF and DSS isolates in the open reading frame of DENV-4 strains. (*) denotes that the amino acid is located in the MTase region of the NS5 protein, (±) is the NLS domain, and (#) the RdRp domain. Roman numbers indicate domain I/II/III in the viral E protein. Bold text indicates unique amino acid substitution among the three clinical isolates of the same serotype.

Amino acid position in polyprotein	Amino acid position in protein	Gene or region	Virus and amino acid		
			DF	DHF	DSS
456	177[I]	E	**Asp**	Glu	Glu
793	18	NS1	Ile	Ile	**Val**
941	166		**Pro**	Ser	Ser
1159	23	NS2A	Met	Met	**Ile**
2417	175	NS4B	Ala	Ala	**Val**
2429	187		Phe	Phe	**Leu**
2441	199		Leu	Leu	**Met**
2533	34*	NS5	Lys	Lys	**Arg**
2538	39*		Ile	Ile	**Thr**
2540	41*		Tyr	Tyr	**His**
2546	47*		Thr	Thr	**Ser**
2558	59*		Val	Val	**Ile**
2582	83*		Lys	Lys	**Gln**
2892	393[±]		Ser	**Leu**	Ser
3050	551[#]		Ile	Ile	**Val**
3313	814[#]		Ile	Ile	**Thr**
3368	869[#]		Val	Val	**Met**
3373	874[#]		Ala	Ala	**Val**
3375	876[#]		Arg	Arg	**Lys**
3380	881[#]		His	His	**Pro**
3381	882[#]		Phe	Phe	**Ser**

Table 4. Epidemiological and clinical characteristics associated with dengue virus serotype-2, -3 and -4 isolates used in the study. I.D. indeterminable.

ID	Serotype	Epidemic year	Sex	Age	Type of antibody response	Clinical outcome	GenBank accession number
R0712259	2	2007	M	7	Secondary	DF	GU131897
R0802298	2	2007	F	4	Primary	DHF	FJ639717
R0919234	2	2007	M	8	Secondary	DSS	JN368476
R0627315	3	2007	M	3	I.D.	DF	JN368477
R0711230	3	2007	M	5	Secondary	DHF	GU131913
R0808265	3	2007	F	4	I.D.	DSS	GU131915
S0702299	4	2008	F	3	I.D.	DF	JN638570
R0818104	4	2007	F	12	Secondary	DHF	JN638571
S0813047	4	2008	M	8	Secondary	DSS	JN638572

Figure 1. Intracellular replication differences illustrated as ratio based on copy number of vRNA (top), or infectious titer (bottom) in Vero cells. Data are representative of three independent experiments.

Figure 2. Replication differences illustrated as ratio based on copy number of vRNA (top), or infectious titer (bottom) in supernatant of Vero cell culture. Data are representative of three independent experiments.

Figure 3. Clinical DENV isolates of serotype-1, -2, -3 and -4 were used to infect (a.) C6/36 cells, (b.) moDCs, (c.) PBMCs, and (d.) monocytes, with an MOI of 1 and 10. Infection was quantified by measuring vRNA day 3 pi. Data are representative of three replicates per sample, from five independent donors. HI = heat-inactivated.

Figure 4. The DENV-3 isolate from a DSS patient induced syncytia, in C6/36 cells (MOI = 1). (a.) Mock infected cells (20X magnification), (b.) The multinuclear giant cells were clearly visible already at day 3 pi (40X magnification). (c.) day 6 pi (40X magnification). These data are the average of three individual experiments.

Figure legend for supplemental figure:

Figure 5. Clinical DENV isolates of serotype-2, -3 and -4 were used to infect C6/36 cells with an MOI of 1. Apoptosis on day 3 pi is expressed in percent of the total number of cells (in total, 250 cell nuclei were counted per sample, corresponding to 100 %). Mock infected cells had naturally ~5 % apoptosis and the positive control cells treated with staurosporin had approximately 50 % of apoptosis. Data are representative of three independent experiments.

Figure 1

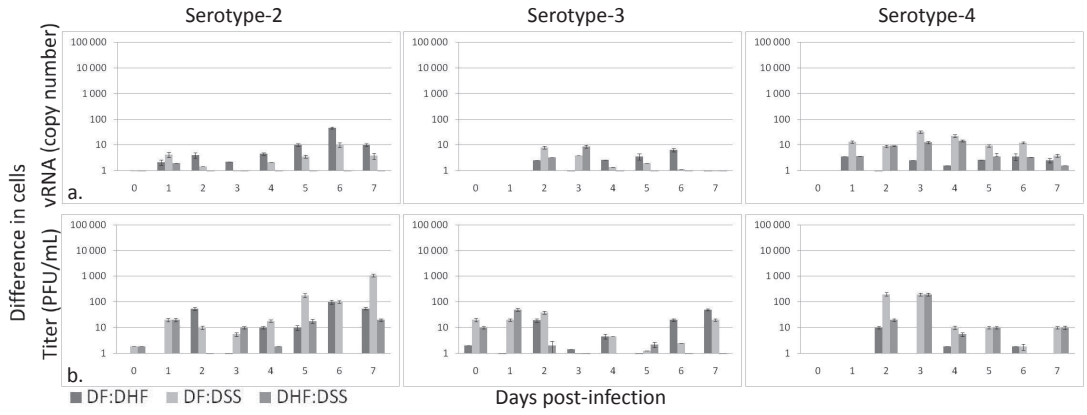


Figure 2

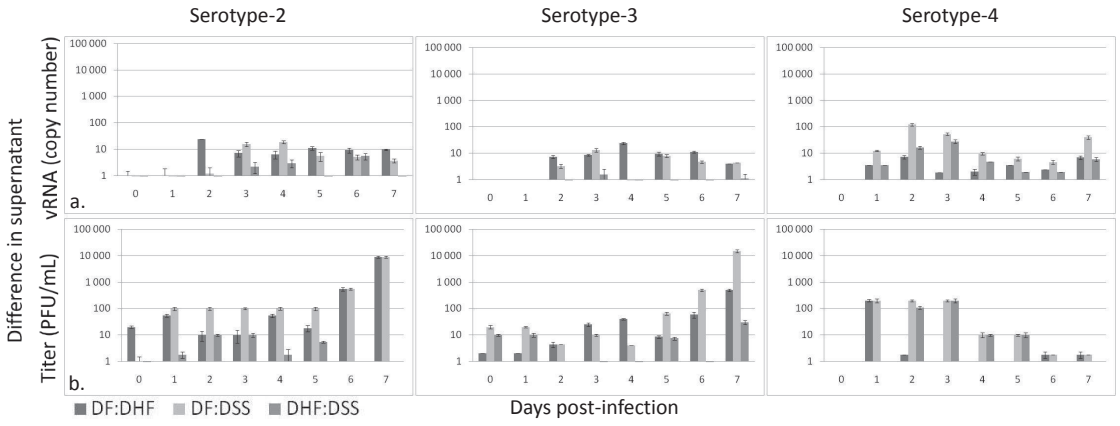


Figure 3

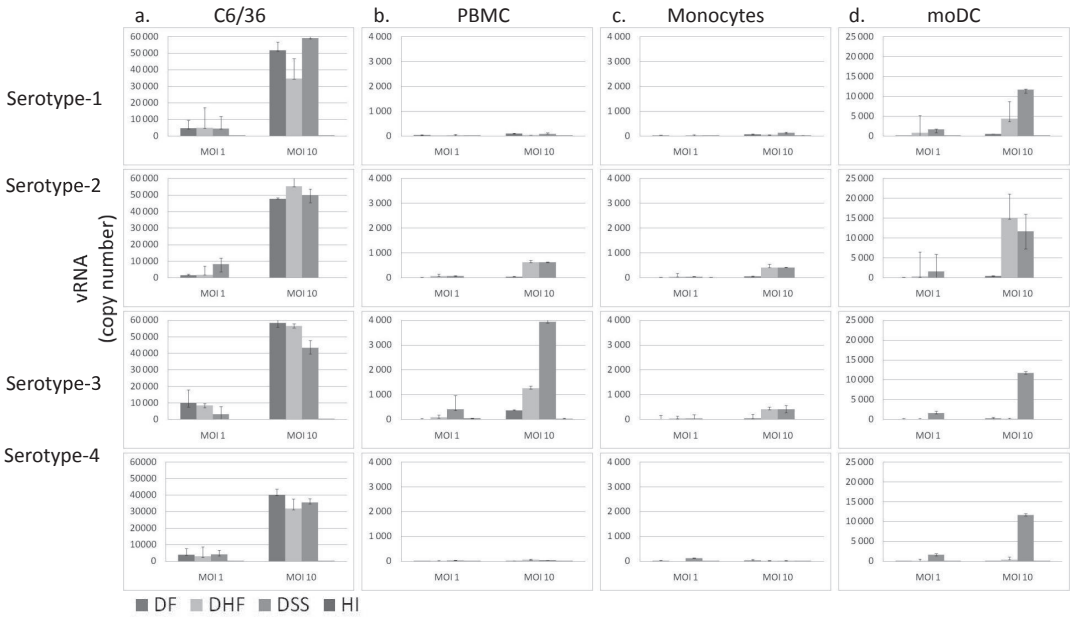
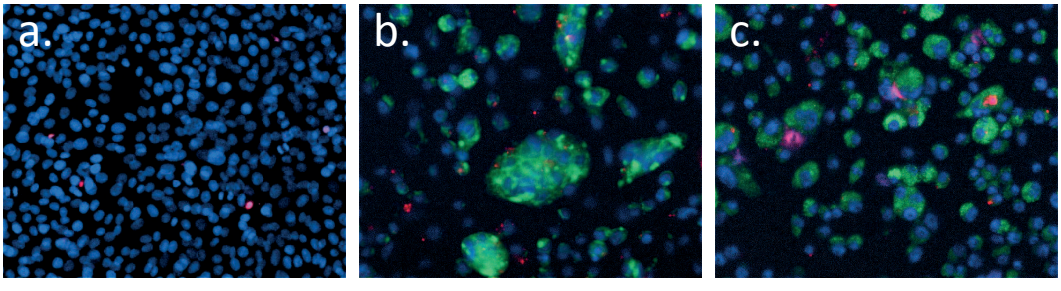
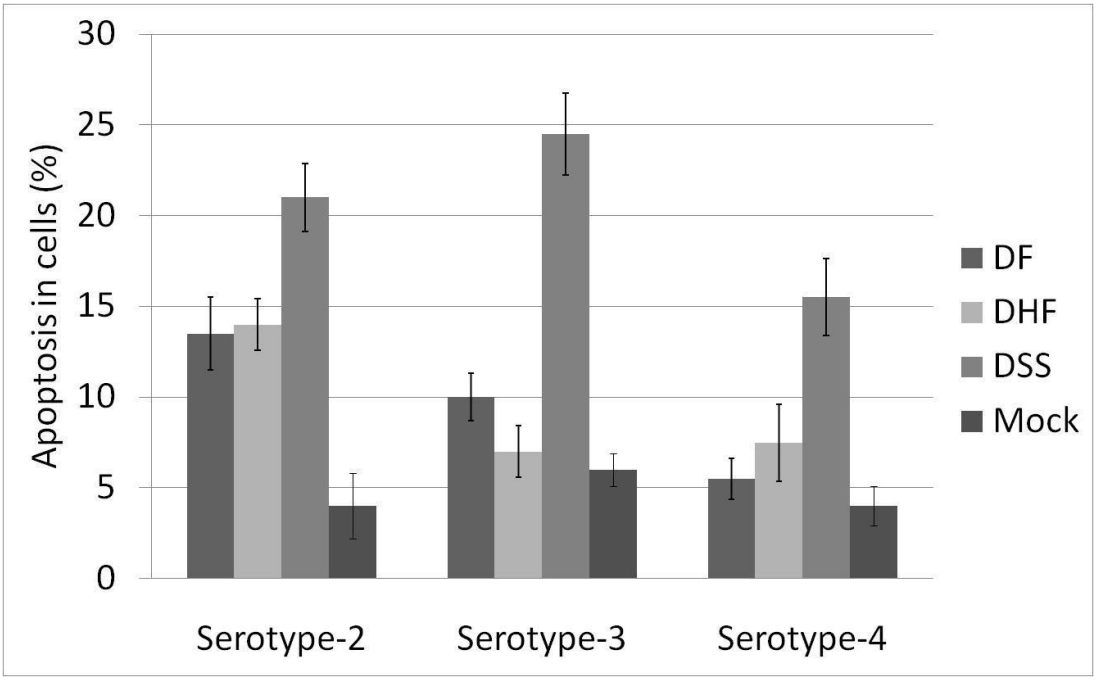


Figure 4



Supplemental figure: Figure 5



Supplemental tables

Table 1. The clinical DENV isolates of serotype-2, -3, and -4 differed at multiple sites throughout the genome. Certain genes particularly prone to amino acid substitutions were E, NS1, and NS5, and this was consistent between the three serotypes.

Gene	DENV-2	DENV-3	DENV-4	**Total**
C	0	0	0	**0**
prM/M	1	1	0	**2**
E	3	4	1	**8**
NS1	3	3	2	**8**
NS2A	4	2	1	**7**
NS2B	1	0	0	**1**
NS3	2	1	0	**3**
NS4A	1	0	0	**1**
NS4B	0	0	3	**3**
NS5	4	7	14	**25**
Totalt	**19**	**18**	**21**	**58**

Table 2 (a). Oligonucleotide primers for genomic sequencing of DENV serotype-2.

Name*	Sequence (5' → 3')	Position	Length
DV2F_16-39	GTG GAC CGA CAA AGA CAG ATT CTT	1	24
DV2R_983-1005	ATG TCA ACC CAR CTY CCT CCT GA		23
DV2F_765-786	CAT GGA TGT CRT CAG AAG GGG C	2	22
DV2R_1868-1891	TCC ATG TTG YGT TTC TGC TAT TTC		24
DV2F_1601-1620	CCC GGA GCR GAY ACA CAA GG	3	20
DV2R_2591-2613	TCC ART CTT GTT ACT GAG CGG AT		23
DV2F_2514-2538	ATA YAA RTT CCA ACC AGA ATC CCC	4	24
DV2R_3468-3490	CTG YCC ATG TCC GGC TGT GAC CA		23
DV2F_3380-3402	CRC TAA GRT ACA GAG GTG AGG A	5	22
DV2R_4490-4512	TTY ACT TCC CAC AGG TAC CAT GC		23
DV2F_4403-4425	GAA GAR CAA ACA CTG ACC ATA CT	6	23
DV2R_5510-5532	CTT TCY TCR TCC ATG ATT GGT GC		23
DV2F_5494-5418	CAG CAA GYA TAG CRG CTA RGG ATA	7	24
DV2R_6789-6812	CCT GGG AAT GTG YTG CAT AAT CAC		24
DV2F_6622-6645	CCA CCA CTG TRA GGA TGG CTA TGA	8	24
DV2R_7880-7902	TCR TGT CCT GGT CCT CCT TTT GT		23
DV2F_7678-7701	GGA YAG AAC CYT RGC AAA AGA AGG	9	24
DV2R_8645-8717	GGT TCT TGR GTT CTC GTG TCC AC		23
DV2F_8502-8526	CAA ACA RAC TGG ATC AGC ATC ATC	10	24
DV2R_9536-9559	ATC TCC ACT GAT GGC CAT TCT TGA		24
DV2F_9298-9318	GGT GGT GCG TGT GTG CAA AGA CC	11	23
DV2R_10198-10221	GGC ATG TAR TCT GTG TAT TCC TCA		24
DV2F_10095-10117	GAG AAG ACC AAT GGT GCG GCT CA	12	23
DV2R_10697-10720	CTG TTG ATT CAA CAG CAC CAT TCC		24

* Primer names with F indicate forward direction and R indicate reverse direction.

Table 2 (b). Oligonucleotide primers for genomic sequencing of DENV serotype-3.

Name*	Sequence (5' → 3')	Position	Length
DV3F_0-24	GTG GAC CGA CAA AGA CAG ATT CTT	1	24
DV3R_989-1011	TCR AGC ACC ACR TCA ACC CAC GT		23
DV3F_927-950	CCA TGA CAA TGA GAT GYG TGG GAG	2	24
DV3R_1892-1915	TTC CCC TTT GTA CTC RGA CCT TAA T		25
DV3F_1811-1833	AAG GGG ATG AGC TAT GCA ATG TG	3	23
DV3R_2854-2877	ACC TCC CAC ACR TTC CAT GCT CTT		24
DV3F_2758-2781	GGC AAA AAT AGT GAC AGC TGA AAC	4	24
DV3R_3921-3945	GCA ACA GTC AAC GTG AAA ATT GTG		24
DV3F_3836-3857	GCT CTG GGG CTC ATG ACT CTA A	5	22
DV3R_5194-5216	GGA GTC CTT TCA GTG CTT CTT CC		23
DV3F_5115-5137	CAG CCA TCG TCA GAG AAG CCA CC	6	23
DV3R_6283-6305	AAT AAG TGC GGG CAT CAA GCC ACC		24
DV3F_6220-6244	GAG GAG AAC ATG GAT GTG GAA ATC	7	24
DV3R_7317-7339	TGC ACA CAG AAC CAA GAG CAT GA		23
DV3F_7238-7260	CCA ACG GTG GAC GGG ATA ATG AC	8	23
DV3R_8151-8174	GGG TTT CTC ACA AGC ATT CCT CC		23
DV3F_7791-7813	CTR TTG TGT GAC ATT GGA GAA TC	9	23
DV3R_8776-8789	TTR TGG AGT TCA CGT TCT CTG TCC		24
DV3F_8598-8620	TTY ACA GAG GAG AAC CAA TGG GA	10	23
DV3R_9482-9509	ATG AAA GTG GTG GGA GCA GAA AGG		24
DV3F_9468-9491	GAT TGG CAA CAR GTY CCT TTC TGC	11	24
DV3R_10451-10473	CTG GCG TTC TGT GCC TGG AAT GA		23
DV3F_9923-9943	CAC TTC CAG AGC AAC CTG GGC CC	12	23
DV3R_10451-10473	CTG GCG TTC TGT GCC TGG AAT GA		23

* Primer names with F indicate forward direction and R indicate reverse direction.

Table 2 (c). Oligonucleotide primers for genomic sequencing of DENV serotype-4.

Name*	Sequence (5' → 3')	Position	Length
DV4F_1-21	AGT TGT TAG TCT GTG TGG ACC	1	21
DV4R_836-856	TCC TGG GTT TCT GAG TAT CCA		21
DV4F_798-820	GGA AGG AGC CTG GAA ACA CGC CC	2	23
DV4R_1694-1716	TCA CAT CCT GTC TCT TGG CAT GAG		24
DV4F_1631-1651	GAA TTA CAA AGA GAG AAT GGT	3	21
DV4R_2519-2538	GTA CTG TTC TGT CCA AGT GT		20
DV4F_2479-2502	GGA AAG AGT TGA AAT GTG GAA GCG	4	24
DV4R_3482-3505	TAC CTG TGA TTT GAC CAT GTT CTC		24
DV4F_3428-3451	GGG AGA AGA TGG ATG TTG GTA TGG	5	24
DV4R_4446-4469	AGT TTC ACC AGG AGG GTA ATC ATG		24
DV4F_4376-4399	CAA GCC CAA TCA TAG AAG TGA AGC	6	24
DV4R_5504-5528	GTC GTT CCA GGA GGG GTT GCA GTC		24
DV4F_5468-5488	CCA GGG TGG AAA TGG GAG AGG	7	21
DV4R_6186-6206	TAG CTC AGC CAC ACC GGA AGG		21
DV4F_6138-6160	CAG AGG GGA GCA AAG GAA GAC CT	8	23
DV4R_7223-7245	GGC TTT GGC CTG CAA TCC TGG ACC		24
DV4F_6975-6994	AGA CAC ACC ATA GAA AAC AC	9	20
DV4R_7905-7922	GAA TCG GTT CTT CAT GTC		18
DV4F_7876-7898	GGC GAC ACT CAA GAA CGT GAC CG	10	23
DV4R_8872-8895	CGG CTG TCA TTC ACA GCT TCA CTG		24
DV4F_8813-8835	GTT AGG TCA AAT GCA GCC ATA GG	11	23
DV4R_9987-10010	GAT GAG CGT GGA TTG ACC ATG TTG		24
DV4F_9817-9839	AAG AGC CAG AAT CTC GCA GGG GG	12	23
DV4R_10662-10685	GAT CAA CAA CAC CAA TCC ATC TCG		24

* Primer names with F indicate forward direction and R indicate reverse direction.