

**DENGUE DIAGNOSTICS AND THERAPEUTIC
INTERVENTIONS IN VIET NAM**

by

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Abstract

Dengue is a major public health problem that affects tens of millions of people annually in tropical and sub-tropical countries. This acute viral infection happens to be severe and even life threatening but there is still no available drug or vaccine. Previous studies have noted early higher viral burden in patients who develop more severe symptoms suggesting that administration of a potent and safe antiviral may prevent progression to severe dengue. To verify this hypothesis, we have conducted the first RCT directed towards reducing the viral burden in vivo by administering chloroquine (CQ), a cheap and well-tolerated drug that inhibits DENV in vitro with concentrations achievable in vivo, to 307 Vietnamese adults with suspected dengue (257 of them were laboratory-confirmed cases). Unfortunately, we did not see an effect of CQ on the duration of infection. However in patients treated with CQ, we observed a trend towards a lower incidence of severe forms. We did not find any differences in the immune response that can explain this trend. We also found more adverse events, primarily vomiting, with CQ. In addition, we have explored the relationships between clinical features, antibody responses and virological markers in these patients. We found that the early magnitude of viremia is positively associated with disease severity and there are serotype dependent differences in infection kinetics. We found as well that DENV was cleared faster and earlier in patients with secondary infections. To complete this study, we have also evaluated 2 rapid lateral flow tests for the diagnosis of dengue in a panel of plasma samples from 245 RT-PCR confirmed dengue patients and 47 with other febrile illnesses. Our data suggest that the NS1 test component of these tests are highly specific and have similar levels of sensitivity (~60%). Both NS1 assays were significantly more sensitive for primary than secondary dengue. The IgM parameter in the SD Duo test improved overall test sensitivity without compromising specificity. All these findings are of major importance for further anti-viral drug testing.

Statement of contribution

Because the experimental part of this thesis is written as a series of chapters prepared for publication in peer-reviewed journals, several people other than myself have contributed to the work, and they deserve acknowledgement. These include:

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Abbreviations

(c)DNA	(complementary) deoxyribonucleic acid
(GAC/MAC) ELISA	(IgM/IgG antigen capture) enzyme linked immunosorbent assay
(m)Ab	(monoclonal) antibody
(p)DC	(plasmacytoid) dendritic cell
(pr)M	(precursor of) membrane
(RT)-PCR	reverse transcriptase polymerase chain reaction
ADCC	antibody-dependent cell cytotoxicity
ADE	antibody dependent enhancement
AE	adverse event
Ag	antigen
C	capsid
CD 4/8	cluster of differentiation 4/8
CI	confidence intervals
CQ	chloroquine
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin
DENV	dengue virus
DF	dengue fever
DHF	dengue haemorrhagic fever
DSS	dengue with shock syndrome
E	enveloppe
EAV	equine arteritis virus
Eq	equivalent
ER	endoplasmic reticulum
EQA	external quality assurance
FCT	fever clearance time
GM-CSF	granulocyte macrophage colony stimulating factor
HCMC	Ho Chi Minh City
Hct	hematocrit
HCV	hepatitis C virus
HLA	human leucocyte antigen
HR	hazard ratio
HRP2	histidine rich protein-2
HTD	Hospital for Tropical Diseases
ICT	immuno-chromatography
ICU	intensive care unit

IFN	interferon
IgM/G	immunoglobulin M/G
IL	interleukin
IQR	inter-quartile range
ISRCTN	international standard randomised controlled trial number
ITT	intention to treat population
JEV	japanese encephalitis
KM	Kaplan - Meyer
NK	natural killer
NS	non structural
OD	optical density
OR	odds ratio
OUCRU	Oxford University Clinical Research Unit
PGE2	prostaglandin E2
PP	per protocol
PRNT	plaque reduction neutralization test
RCT	randomized controlled trial
RDT	rapid diagnostic test
RNA	ribonucleic acid
RdRp	RNA dependent RNA polymerase
TGN	trans-Golgi network
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
UTR	untranslated region
WHO	World Health Organization

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General Introduction

The prevalence of dengue has increased dramatically in recent years. This viral disease is now the most important arbovirus infection globally. As well as the individual and direct health-care burden, work absenteeism during epidemics can have a substantial economic impact. There is still no approved specific treatment or licensed preventive vaccine. The only currently available way to prevent the transmission of its causative agent, the dengue virus (DENV), is to control its main vector: the mosquito *Aedes aegypti*. Fortunately, appropriate medical supportive care can save the lives of most patients suffering from the severe and potentially fatal hemorrhagic forms of dengue [1].

If a preventive vaccine is the ideal solution for low-income, high-burden endemic countries, an effective oral antiviral therapy could nonetheless be complementary to help reduce morbidity and mortality when a new epidemic outbreak occurs. The rationale for a dengue antiviral arises from studies that have noted higher viral burden early in the disease in patients who develop more severe clinical presentations compared with patients suffering from uncomplicated dengue. This observation suggests that administering a potent and safe compound that targets essential steps of the DENV replication early in the disease, thereby lowering the viral burden, may prevent progression to serious dengue disease. Nevertheless this hypothesis requires field-testing and the clinical trial detailed in this thesis is, to our knowledge, the first randomized controlled trial (RCT) directed towards reducing the DENV burden in vivo (previous RCTs have focused on supportive management only).

The first chapter of this thesis will review the state of knowledge on dengue and describe some recent progress in the fight against this disease. The second chapter will describe and discuss a RCT of chloroquine (CQ) to reduce the viral burden in dengue adult patients. The third chapter will summarize an evaluation of different commercial kits for the rapid detection of the DENV non-structural protein 1 (NS1) and discuss how these kits can contribute to better clinical trials and earlier diagnosis

of DENV infection. The fourth chapter will analyze and discuss different features of DENV kinetics in plasma and impart some lessons concerning the therapeutic window for antivirals. The fifth and last chapter will conclude and discuss more about organizational aspects of such trials.

All the studies described in this thesis were conducted at the Hospital for Tropical Diseases and the Oxford University Clinical Research Unit (OUCRU) in Ho Chi Minh City (HCMC), Vietnam. The Wellcome Trust supported this work.

Chapter 1 : Literature Review

1.1 Overview

Dengue is an acute infectious disease caused by dengue viruses. Infection by DENV may remain asymptomatic or cause a self-limiting febrile syndrome, often called dengue fever (DF) or get complicated in serious and potentially fatal dengue haemorrhagic fever (DHF) or dengue with shock syndrome (DSS). There are four serotypes of the virus (DENV-1 to DENV-4). Infection with one serotype provides long-lasting immunity against this serotype but not cross-immunity against the others. Sequential infections with different serotypes are then possible. *Aedes* mosquitoes transmit this virus and can be considered as the main reservoir. This disease is extremely widespread in tropical and subtropical countries especially in urban and semi-urban areas. This disease imposes an important burden on health-care systems, filling hospital beds during the rainy season and causing misery to millions of people. The World Health Organization (WHO) estimates that each year dengue affects more than 100 million people of the 2.5 billion living in the *A. aegypti* infested regions [1]. The number of annual deaths related to dengue is estimated at several tens of thousands [2]. The incidence of dengue has grown dramatically in recent decades. Due to the geographic extension of *A. aegypti* infested areas and the increasing number of serious cases in highly endemic regions, dengue is now regarded as a major public health problem in South-East Asia, South America and the Western Pacific.

1.2 Brief history and epidemiology

1.2.1 Origin of dengue

Major outbreaks of illnesses that could have reasonably been DF were reported in Asia, Africa and North America in 1779 and 1780: epidemics in Batavia (now Jakarta), Indonesia and Cairo, Egypt in 1779 and in Philadelphia, USA in 1780 [3-5].

However, diseases clinically compatible with DF were reported much earlier and it is likely that DF, or very similar diseases, had already a broad distribution at this time [6]. A Chinese encyclopaedia, published under the Chin Dynasty (265-420 AD), mentioned for the first time a disease very similar to DF [7]. This disease was called “poison from the water” and was thought to be linked with flying insects and water by the Chinese. Other names have been given to diseases very similar to DF: “knee disorder” in Egypt, “bone fever” in Indonesia, “3 days/7 days fever” in India or “ki-dinga pepo” (Swahili words that literally mean “a kind of sudden attack of cramp”) during an outbreak in 1823 in Zanzibar (today Tanzania) [8]. This term evolved into “dinga” or “denga”. In 1828, dengue was the word used to describe an epidemic in the Caribbean [9]. It could also be that the origin of the word is Spanish, since some letters of the Spanish royal family already mentioned this term in 1801 [10].

1.2.2 Emergence of dengue as a global public health problem

From the first documented epidemics until the middle of the 20th century, the disease pattern associated with DF was characterized by relatively infrequent but often large epidemics. However, it is likely that DF became endemic in many tropical urban centres during this time. Expansion in travel and trade has resulted in introduction of *Aedes* mosquitoes and DENV into many new areas. The World War II, because it disrupted ecosystems and provoked massive population displacements, likely contributed to the increased transmission of DENV in Southeast Asia and the Pacific. It was in this setting that the global pandemic of dengue began. In South-East Asia, hyperendemicity, i.e. the co-circulation of multiple DENV serotypes, appeared in urban areas and DF became endemic [11]. In the Americas, epidemics of dengue were rare until the 1970s because of a fairly successful *A. aegypti* eradication program as a method to control yellow fever (another viral disease also transmitted by this mosquito) [12-14]. But when this program was discontinued in the early 1970s and *A. aegypti* began to reinvade the continent (with the great success of yellow fever vaccination campaigns, maintaining the eradication program did not seem of utmost importance), epidemic activity increased [15]. By the 1990s, *A. aegypti* had nearly regained its geographic distribution it had before the eradication, and epidemics of

dengue invariably followed reinfestation by *A. aegypti* [16]. Nowadays, DF affects all continents especially Asia, the South Pacific Islands, Australia, and Central and Latin Americas [11, 17, 18].

Sporadic cases of DHF have been described since the 18th century but given their small number, DHF was not considered to be a public health problem. [13, 19, 20]. Likely because of the increased transmission and hyperendemicity, the first known epidemic of DHF occurred in Manila, Philippines in 1953-1954 [9]. Within the next decades, DHF in epidemic form had spread throughout Southeast Asia where it became a leading cause of hospitalization among children. Since then, epidemic DHF has expanded geographically from Southeast Asian countries west to India, Sri Lanka, the Maldives, and Pakistan and east to China [11]. Several island countries of the South and Central Pacific have experienced major or minor DHF epidemics [21]. Outbreaks have also been recorded in Australia [22]. In Central and Latin Americas, following the reintroduction of dengue many countries evolved from nonendemicity, i.e. no endemic disease, or hypoendemicity, i.e. one serotype present, to hyperendemicity, and epidemic DHF emerged, much as it had in Southeast Asia 25 years earlier [12, 13]. The first major American outbreak of DHF occurred in Cuba in 1981 [23, 24]. In Africa, outbreaks of DF have been increasingly frequent during the last decades. Major epidemics caused by all serotypes have occurred in East Africa and West Africa, and the Middle East [8, 25]. Major epidemics of DHF have not yet happened, even if sporadic cases have occurred [18, 26].

In 2011, DENV and *A. aegypti* mosquitoes have a worldwide distribution in the tropics, incidence of the disease is increasing and DHF is emerging in new countries (see Figures 1 and 2) [11, 27].

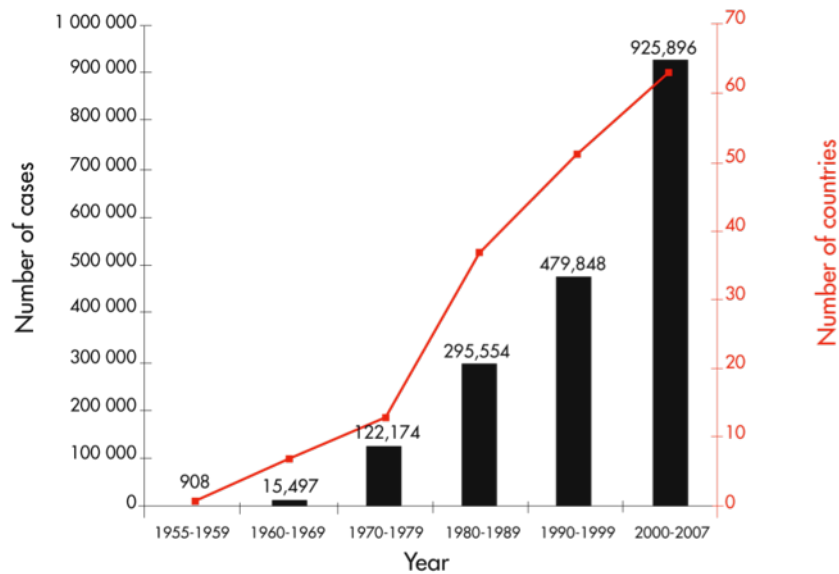


Figure 1: Prevalence and number of countries affected by dengue. The vertical bars indicate the average annual number of dengue cases and the red dots indicate the number of countries reporting cases of dengue fever [28]. Reprinted from *The Lancet*, Vol. 368, Kroeger, A. and M.B. Nathan, Dengue: setting the global research agenda, pages 2193-2195, Copyright (2006), with permission from Elsevier.

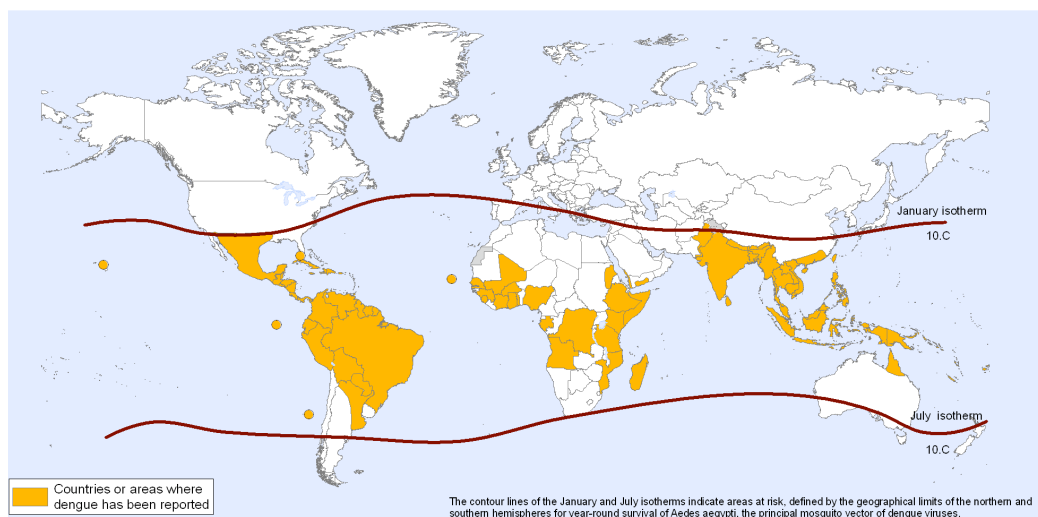


Figure 2: Map showing the countries or areas at risk of dengue transmission, as of 2010 [29]. In yellow are the countries or areas where dengue has been reported. The red lines are the isotherm 10°C in the Northern and Southern hemispheres, between is the area of the year-round survival of *A. aegypti*. Reprinted with the permission from the WHO.

1.3 Virus

1.3.1 Taxonomy and virus structure

DENV was first isolated in Hawaii (DENV-1) and New Guinea (DENV-2) in 1944 [30, 31]. DENV-3 and DENV-4 were then isolated in Manila, Philippines in 1956 [32]. DENV belongs to Group IV of the viruses (according to the Classification of Baltimore), i.e. the group of single-stranded positive RNA viruses, and falls in the family of *Flaviviridae* and the genus of *Flavivirus*. This genus includes more than 60 other viruses transmitted by arthropods such as the agents of the yellow fever, the West Nile disease, the tick-borne encephalitis and the Japanese encephalitis. DENV also belong to the arboviruses i.e. the viruses whose vectors are bloodsucking arthropods like mosquitoes, ticks and sandflies. This term is the contraction of "arthropod-borne viruses" but does not belong to the taxonomic classification of viruses. Arboviruses grouping is based on their mode of transmission and actually raises some problems because on the one hand it brings together morphologically heterogeneous viruses belonging to several distinct families: *Flaviviridae*, *Togaviridae*, *Rhabdoviridae*, *Bunyaviridae* and *Reoviridae*, and on the other hand, it excludes taxonomically close viruses with different mode of transmission and viruses belonging to other families (*Filoviridae*, *Arenaviridae*) but causing similar diseases (hemorrhagic fevers).

Like all *Flaviviridae*, DENV is an enveloped virus of ~50 nanometers diameter [33]. Its envelope is a lipid bilayer that contains 2 viral glycoproteins: the envelope (E) and the membrane (M) proteins [34]. Its genome is contained in an icosahedral nucleocapsid composed by the viral capsid (C) protein (Figure 3). The DENV genome is a linear non-segmented single-strand RNA of positive polarity and of ~11 kilobases length. The genomic RNA has a cap at its 5' end but no poly-A at its 3' end [35]. It has only one open reading frame that encodes a single polyprotein [36-40]. This precursor polyprotein is cleaved co- and post-translationally by viral and cellular proteases [41-44]. This cleavage generates 3 structural proteins (C, prM later proteolytically processed to produce M, E) and 7 nonstructural (NS) proteins (NS1, NS2a and b, NS3, NS4a and b, NS5) (Figure 4) [45-47]. The DENV genome has also 2

untranslated regions (UTR) at the 5' and 3' ends. The UTR in 5' has a large loop structure (SLA for stem-loop A) and a 5' upstream AUG region, which supports viral replication and expression, respectively [48]. The UTR in 3' plays a key role in regulating the RNA replication and increases the stability of RNA as a poly-A tail does [49, 50].

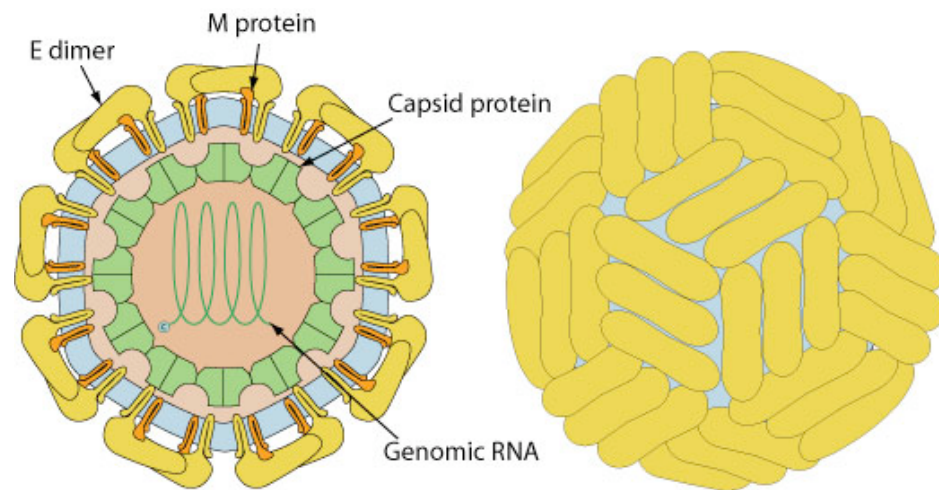


Figure 3: Diagram of a dengue virion showing its main structural parts [51]. Reprinted from ViralZone, Copyright (2011), with the permission of the Swiss Institute of Bioinformatics.

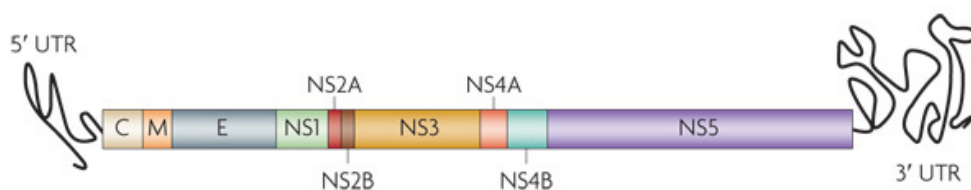


Figure 4: Schematic organization of DENV genome and its resulting protein [27]. Reprinted from *Nature Reviews Microbiology*, Vol. 8, Guzman, M.G. *et al.*, Dengue: A continuing global threat, pages S7-S16, Copyright (2010), with permission from Springer.

1.3.2 Viral proteins

1.3.2.1 C protein

The C protein is a small protein, rich in lysine and arginine residues and, therefore, strongly positively charged. With this property, this protein would neutralize the negative charges of the viral RNA, to which it is associated by its C-terminal extremity [52-54]. It would also play a key role in the assembly of new virions, ensuring the encapsidation of the viral RNA with the participation of NS proteins [55, 56]. It is assembled as a symmetric dimer [33, 53].

1.3.2.2 M protein

Two forms of the glycoprotein M have been characterized: prM (i.e. precursor of M) held in immature intracellular virions and M held in mature extracellular virions. In immature virions, which are formed at the endoplasmic reticulum (ER) and then migrate to the Golgi, prM is associated with E. Within this prM/E heterodimer, prM plays the role of a chaperone protein by preventing the acidic environment of the secretory pathway compartments to induce irreversible conformational changes of E protein [57, 58]. Shortly before the release of viral particles, a cellular furin-like protease cleaves prM to M in the trans-Golgi network [59]. For several flaviviruses, it was demonstrated that this cleavage ensures the infectivity of the virus particle. The absence of cleavage of prM to M would prevent changes of protein E that are necessary to the fusion process of the viral envelope with the cell membrane [60]. However it has been shown that some cell types are able to release dengue viral particles that are infectious but containing a high prevalence of prM [61, 62].

1.3.2.3 E protein

Found on the viral surface, the E glycoprotein is involved both in the initial attachment of the viral particle to host cell receptors and its entry into the host cell

[63-65]. Structurally conserved among flaviviruses, E protein consists of 3 distinct domains: domain I that participates in the conformational changes required for viral entry and nucleo-capsid escape from the endosomal compartment, domain II that contains the fusion loop, and domain III that has been suggested to bind cellular receptors [66-70]. In immature virions formed at the ER, the E protein is associated with prM in a heterodimer that gives a spiky exterior to the viral particle. This immature viral particle buds into the ER and eventually travels via the secretory pathway to the Golgi apparatus. As the virions pass through the trans-Golgi network (TGN), the low pH causes a conformational change of the E protein that disassociates it from prM and causes it to form E/E homodimers. These lay flat on the viral surface giving the maturing virion a smooth appearance. During this maturation the pr peptide is cleaved from the M peptide by host furin enzyme [33, 69, 71]. The M protein then acts as a transmembrane protein under the E protein shell of the mature virion. The pr peptide stays associated with E until the viral particle is released into the extracellular environment. It acts like a cap, covering the hydrophobic fusion loop of E until the viral particle has exited the cell [72]. Upon infection of new cells, acidification of the endosome induces the rearrangement of E/E dimers into E/E/E trimer to the virion surface [73]. This rearrangement leads to the exposure of the internal fusion peptide, which interacts with the endosomal membrane to initiate the fusion process [74, 75]. The E protein, and in particular its domain III, contains the main epitopes recognized by the virus neutralizing antibodies [76].

1.3.2.4 NS1 protein

NS1 protein exists in different forms, soluble or associated to cell membranes. Although it seems to have no anchor, as homodimer is capable of associating with cell membranes [77, 78]. Dimerization of NS1 is also required for its export along the secretory pathway, from the ER to the plasma membrane [79]. A portion of NS1 proteins is then associated with the plasma membrane on the surface of infected cells [80-82]. Another portion is released into the extracellular medium [78, 83]. In the extracellular medium, NS1 can be found in soluble form organized in oligomers or associated with microparticles [83-88]. The circulating form of NS1 can be detected in the serum of infected patients [89]. Because NS1 is associated with immature E

protein in the ER, the intracellular form of NS1 plays a role in the maturation of virions [88]. It could also be involved in early steps of viral replication in the replication complex but its precise role is still unclear [90-93].

1.3.2.5 NS3 protein

The NS3 protein is highly conserved among flaviviruses. In association with NS2b, the N-terminus of NS3 acts as a serine protease and is involved in the cleavage of several viral proteins [94-96]. The C-terminus exhibits a RNA helicase activity and is also involved in the cap addition and methylation of the viral RNA [97-99]. Finally, autoproteolytic cleavage of NS3 helicase domain has a regulatory effect on viral RNA replication [100]. Within the complex of viral RNA replication, NS3 interacts both with RNA dependent RNA polymerase (RdRp) NS5 and the 3'-UTR [101]. NS3 could also play a role in initiating the synthesis of the negative strand RNA [102].

1.3.2.6 NS5 protein

The NS5 protein is also highly conserved among flaviviruses. Its C-terminus plays an important role as RdPp [103, 104]. Its N-terminus has characteristics motifs of methyltransferases and may be involved in the cap addition to the viral RNA [105].

1.3.2.7 NS2a, NS2b, NS4a and NS4b proteins

Although the 4 small NS2a, NS2b, NS4a and NS4b proteins have variable sequences, their hydrophobic nature is conserved among flaviviruses suggesting an association with cell membranes [45]. NS2a appears to play a role in maturation of the C-terminal region of NS1 [106]. NS2b is associated with NS3 to form a complex that has a serine protease activity [107, 108]. Studies has showed that NS4b and, to a lesser extent, NS2a and NS4a are able to block the interferon (IFN) pathways, key elements of the anti-viral innate response, by blocking some signal transduction factors [109-111].

1.3.3 Viral cycle

The life cycle of DENV is depicted in Figure 5. The different steps are discussed in detail in the paragraphs below.

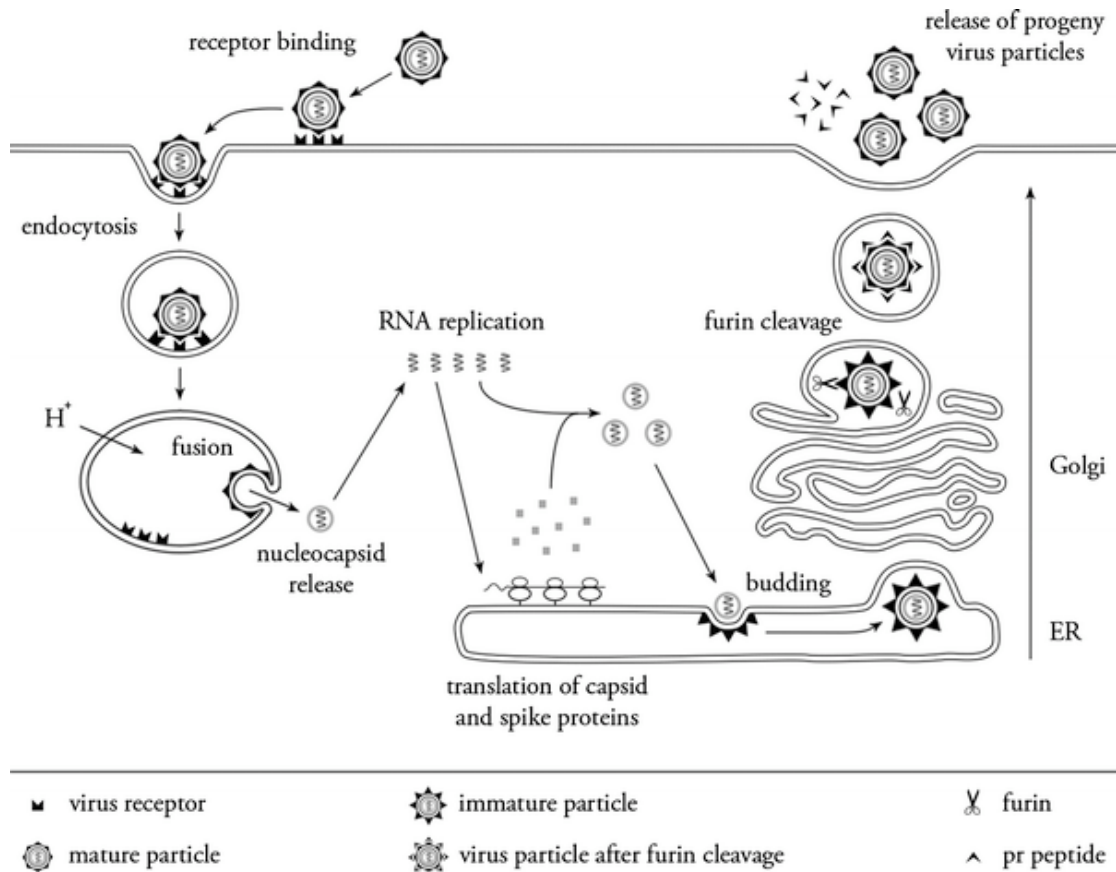


Figure 5: The infectious life cycle of DENV [112]. Reprinted from *Cellular and Molecular Life Sciences*, Vol. 67, Rodenhuis-Zybert, I.A. *et al.*, Dengue virus life cycle: viral and host factors modulating infectivity, pages 2773-2786, Copyright (2010), with permission from Springer.

1.3.3.1 Entry of the virus into the host cell

Several cell surface molecules have been shown to interact with the E protein: heparan sulfate, DC-SIGN (for Dendritic Cell-Specific Intercellular adhesion

molecule-3 Grabbing Non-integrin, a lectin expressed on the surface dendritic cells and macrophages), Rab5, GRP78 and the mannose receptor [113-118]. A receptor-dependent endocytosis then leads to internalization of the virus particle inside vesicles bearing clathrin [119]. The acidification of the endosome induced a series of rearrangements of the E protein, including its transformation from dimer to trimer [33, 63, 120]. These changes allow the fusion of the viral envelope and endosomal membrane and the release of the nucleocapsid into the target cell cytoplasm followed by uncoating of the viral RNA [74, 75, 121]. The hydrophobic fusion peptide of E protein is thought to play an important role in the endosomal fusion. In mosquito cell lines, the interaction of DENV with the target cell receptors is immediately followed by the fusion of the viral envelope with the plasma membrane [122, 123].

Because of the lack of good animal models, the cell tropism of DENV in human is still unclear. However, cells of the mononuclear phagocytic lineage such as dendritic cells, macrophages and monocytes appear to be major target cells [124, 125]. In human autopsy tissues, DENV antigen (Ag) has been detected in different cell lineages and various organs such as spleen, kidney, liver, lungs and central nervous system [126, 127]. In primary infections, DENV enters the monocyte-macrophage by the receptor-dependent endocytosis model described above but during a secondary infection, another mechanism may strongly potentiate the virus entry into certain cells. This phenomenon is called antibody dependent enhancement (ADE) and is linked to the presence of heterologous antibodies induced by a primary infection and present at sub-neutralizing levels upon a secondary infection with a DENV of a different serotype. These antibodies (Ab) form complexes with immature and mature virions and are thought to promote virus infection of Fc-bearing cells through Fc receptor mediated endocytosis (discussed in more detail in the immuno-pathogenesis section) [128].

1.3.3.2 Intracellular phase and release of the virus

Shortly after its release into the target cell, the viral RNA is translated into a polyprotein by host cell ribosomes at the ER membrane [34]. The polyprotein is

cleaved into its components, during and after the translation by cellular and viral proteases [41-44, 129]. Apart from its initial role of mRNA, viral RNA serves as a template for viral replication. Viral proteins (including NS3 helicase and the RdP NS5) and probably cellular proteins form a replication complex to synthesize the complementary negative RNA strands which are then used as a template for the positive RNA strands synthesis that are later translated or packaged into virions during maturation. The RNA replication takes place inside the ER. The positive RNA strands represent over 90% of the RNA produced in infected cells. The negative RNA strands are present mainly as double-strand replicative intermediates [46, 130, 131].

The C protein has an important role in translocation of viral proteins to the ER. Indeed, C is connected to prM by a hydrophobic stretch of amino acids that serves as signal to direct the viral proteins to the ER membrane to be internalized in its lumen. After the entry of C-prM into the ER lumen, the hydrophobic bond is cleaved by the viral protease NS3/NS2b. C, which is composed of α helices, matures after this cleavage. One of these α helices interacts with the viral RNA through basic residues. Then, the newly formed nucleocapsids bud into the ER lumen. E and prM together with the ER membrane (where they are anchored) form the envelope of immature virions. These are transported in secretory vesicles toward the Golgi. After the cleavage of prM in the TGN, the virion is considered as mature and capable of infecting new cells and finally released into the extracellular medium by exocytose [132, 133].

1.3.4 Molecular Diversity

Like all positive-sense RNA viruses, DENV has an error-prone RdRp and this generates sequence diversity in the virus population. Between the four serotypes, 60-70% of amino acids are conserved [134]. Phylogenetic analysis of DENV sequences has revealed that each serotype consists of a variety of sub-types, termed genotypes or clades [26, 135-137] (Figure 6). Classification of each serotype into genotypes often relates to the geographic region where particular genotypes have been found or were first found. Some genotypes are widespread while others remain confined to specific

geographic areas. The greatest molecular diversity in DENV is found in Southeast Asia [40]. However because the number of analyzed samples varies considerably between different areas, the observed extent of molecular diversity and geographical scattering could be due to sampling biases.

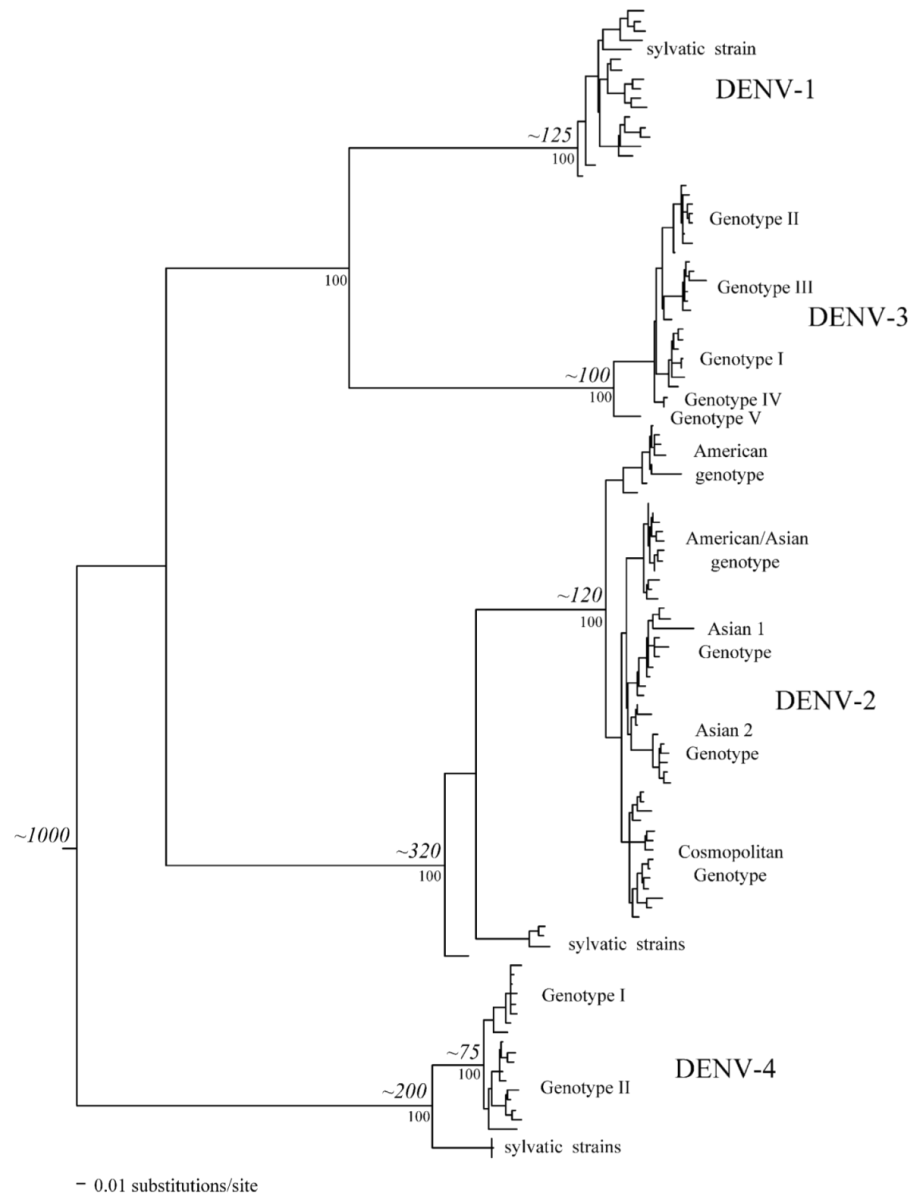


Figure 6: The genetic diversity of DENV. Phylogenetic tree of 120 sequences of E gene of DENV (constructed using the method of maximum likelihood) [138]. Reprinted from *Infection, Genetics and Evolution*, Vol. 3, Holmes, E.C. and S.S. Twiddy, The origin, emergence and evolutionary genetics of dengue virus, pages 19-28, Copyright (2003), with permission from Elsevier.

1.4 Vector

All DENV vectors are mosquitoes (i.e. insects of the *Diptera* order, *Nematocera* suborder and *Culicidae* family) that belong to the *Culicinae* subfamily and *Aedes* genus. The 2 main vectors are *Aedes (Stegomyia) aegypti* (L.) and *Aedes (Stegomyia) albopictus* (Skuse). Both took advantage of trade developments to spread throughout the tropics from their native area: *A. aegypti* originated from Africa and *A. albopictus*, commonly known as the “Asian tiger mosquito”, from South-East Asia [139, 140]. *A. aegypti* is thought to be responsible for most epidemics today while *A. albopictus* is causing mostly sporadic cases and local outbreaks [141, 142].

1.4.1 Transmission cycles

Results from serological and virus isolation studies have shown that DENV circulates in some monkeys in Southeast Asia and Africa [143]. Although it is impossible to exclude that circulation of DENV in these primates is due to an infection of human origin, these observations support the hypothesis of primitive sylvatic zoonotic transmission of DENV involving jungle monkeys and mosquitoes. Ancestral sylvatic strains of all serotypes would have then evolved independently since hundreds or thousands years ago and adapted to peridomestic vectors and human host [144]. Today, the outbreak dengue viruses appear clearly distinct from the strains transmitted in the sylvatic cycle involving monkeys of the rainforest canopy and some *Aedes* mosquitoes [26]. Only endemic or epidemic urban transmission occurring in large urban centres and involving *A. aegypti* and human host are significant in terms of public health.

1.4.2 *Aedes aegypti*

The role of *A. aegypti* in dengue transmission was first suspected by Graham in 1903 in Beirut (Lebanon) and later demonstrated by Bancroft in 1906 in Australia, both using experiments on volunteers [145, 146]. *A. aegypti* is the principal vector of both

yellow fever and dengue viruses (Figure 7). It has adapted well to human habitats, as it does not make noise when flying and does not attack the face but rather the feet and ankles. *A. aegypti* is a daytime mosquito that bites usually mid-morning and afternoon [147]. From its African origin, *A. aegypti* has been essentially spread by human activities. It reached the American continent being carried inside water storage jars in ships during the slave trade and also invaded Portugal and Spain before its proliferation elsewhere on European ships. It started to infest the Southeast Asia and the Pacific Islands during the 19th and 20th centuries [148]. If the species was eradicated in the Mediterranean and much of the South America in the 1950/1960s, it has since reinfested most of these areas. Today, it is established throughout the tropical and sub-tropical regions of the world. Dengue distribution is copying *A. aegypti* distribution and all areas where the vector is established are current or potential sites of outbreaks (Figure 8).

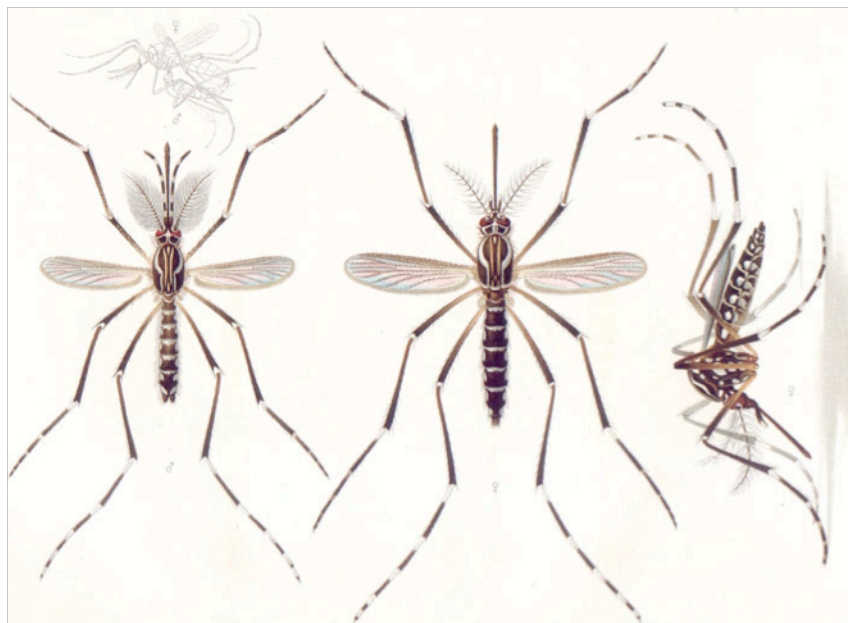


Figure 7: Colour print of *Aedes aegypti* (also called *Stegomyia aegypti*). To the left, the male, in the middle and on the right, the female, and above left, a flying pair *in copula* [149]. This image is in the public domain because its copyright has expired.

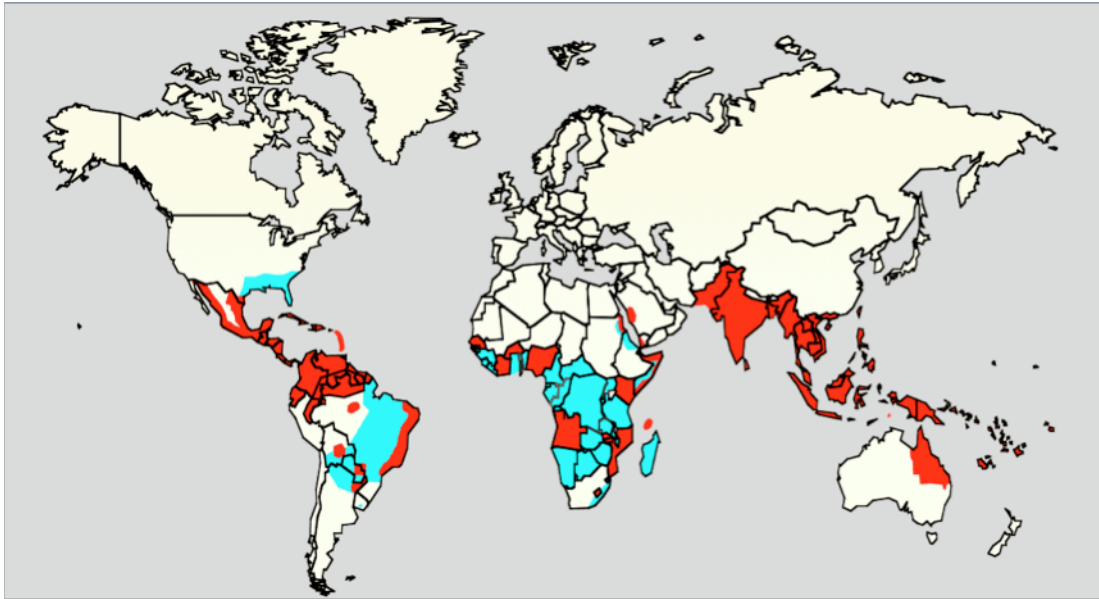


Figure 8: Map showing the distribution of DF in the world, as of 2006 (produced by the Agricultural Research Service of the US Department of Agriculture). Cyan: areas infested with *A. aegypti*. Red: areas with *A. aegypti* and recent epidemic DF [150]. This map is in the public domain in the United States because it is a work of the United States Federal Government.

A. aegypti is a polytypic species (i.e. having many subspecies and geographical variants) with a wide geographical distribution and high morphological, physiological and eco-ethological variability [151]. This species also has a large genetic heterogeneity [152, 153]. In 1967, Craig & Hickey had raised the possibility that the mosquito ability to transmit an arbovirus is determined genetically [154]. Variations in susceptibility to oral infection by DENV were then demonstrated experimentally on different colonies of *A. aegypti* [155-159]. This variability of vector competence among populations of *A. aegypti* of different geographic origins is intimately linked to the genetic heterogeneity of the species [160-162].

Genetic differentiation of *A. aegypti* populations is highly dependent of environmental factors related to human activities (e.g. insecticide treatment, methods of water storage, etc.) or not (e.g. rainy season) [163]. The impact of human activities on genetic differentiation of *A. aegypti* has been demonstrated in studies comparing the

susceptibility to oral infection and/or genetic differentiation of *A. aegypti* populations from city centres and from the outskirts [164, 165]. The findings implicate human activities associated with urbanization are shaping the genetic structure of *A. aegypti* population by affecting the dispersal of mosquitoes. The abundance of rainfall and duration of the rainy season may also influence genetic differentiation by affecting the mosquito density [166, 167]. Genetic variation in *A. aegypti* populations may result from alternation between extinction and recolonization processes during cycles of drought and flood associated with the pattern of rainfall (once the dry season begins, the number of available temporary breeding sites decreases and the mosquito population crashes).

1.4.3 *Aedes albopictus*

A. albopictus, the Asian tiger mosquito, is another possible vector [168]. The role of *A. albopictus* as a secondary vector has been identified in the Philippines in the 1920s [169, 170]. It originated from Southeast Asia and from islands of the Western Pacific and Indian Ocean where it is often found in peri-urban, rural and forested areas and feeds on a wide variety of mammalian and avian hosts and depositing its eggs in natural and artificial habitats [171-173]. *A. albopictus* has not the high degree of domestication and ecological specialization of *A. aegypti* and it is suspected to be responsible for introduction of sylvatic DENV strains into human populations [141]. Until the early 20th century, *A. albopictus* was mainly restricted to its native areas. This species has later spread to different regions around the globe, largely through ship-borne transportation of eggs and larvae in used car tires, which are traded around the world [139, 174]. It has become established in much of Americas (North and South), the mid-east, Africa and areas of Europe during recent decades after extending its range eastwards across Pacific islands during the early 20th century. [175-179].

A. albopictus has been involved in several outbreaks of dengue, essentially in Asia where it may play an important role in DENV transmission [168, 180]. In other regions, *A. albopictus* is probably only responsible for sporadic cases. As for *A. aegypti*, populations of *A. albopictus* from different geographic origins have

variations in their susceptibility to infection and a high genetic heterogeneity [181-183]. Furthermore, adaptation of this species to more temperate climates has been accompanied by biological evolution: the eggs of some strains established in regions further north are able to enter in diapause and withstand temperatures $\sim 0^{\circ}\text{C}$ [184].

Oral infection experiments have shown that wild strains of *A. albopictus* have a lower oral receptivity for DENV than wild strains of *A. aegypti* from the same geographic region [185, 186]. These results complement those of another study conducted on wild strains of Taiwan, showing that rates of infection and transmission of DENV are higher for *A. aegypti* than for *A. albopictus* [187]. If DENV transmission in Southeast Asia, where dengue is endemic, is mainly attributed to *A. aegypti*, *A. albopictus* may ensure the maintenance of DENV during inter-epidemic periods because of its more effective vertical transmission [188-190].

1.4.4 Mosquito life cycle and DENV transmission

Female *Aedes* need blood meals for maturation of their eggs. They can feed on various animals. *A. aegypti* feeds almost exclusively on humans and is described as being anthropophilic while *A. albopictus* is somewhat less discriminating in its species choice for biting and feeding. They breed mainly in artificial or natural containers in or around buildings. They lay eggs preferably in clear water where they hatch in 48 hours to yield larvae. These larvae then become pupae (a short resting, non-feeding stage of development) before becoming an adult mosquito. This cycle lasts 7-14 days. In nature, their lifespan is ~ 15 days but they can be kept alive much longer in laboratory [141].

Female mosquito infection takes place during a blood meal on a viremic person. After ingestion, the first organ encountered by DENV is the midgut. This organ can be a barrier against infection and against viral dissemination. This "digestive barrier" plays a major role in vector competency of mosquitoes, being more or less permissive to infection [155]. By the second day following the blood meal, DENV replicates in

epithelial cells of the stomach [191]. From the sixth day, after passing the digestive barrier, the virus infects and replicates in cells of adipose tissue then later in epithelial cells of salivary glands (and other tissues). The extrinsic incubation period between ingestion of the virus and its appearance in the salivary glands of the vector lasts ~10 days but several factors, including ambient temperature, may influence its duration (higher temperatures promote more rapid dissemination of DENV in the bodies of mosquitoes) [192]. When DENV is shed in saliva, the mosquito is potentially infective and DENV will be transmitted with saliva during the next blood meal. An infected mosquito is able to transmit DENV throughout its life. Although dispersal of adult mosquitoes from the breeding place is limited, train, bus, boat or airplane can transport infected mosquitoes over longer distances. Humans can also introduce DENV in new areas when travelling if appropriate vectors are present [193]. The ambient temperature also affects the breeding of mosquitoes and their lifespan [194].

1.4.5 Vertical transmission

Vertical transmission of DENV, or the ability for infected female mosquitoes to transmit DENV to their progeny, has been observed in the laboratory and to a much lesser degree in the field for both sylvatic species and *A. aegypti* [195-197]. Studies have shown that the vertical transmission of dengue virus can persist over several generations and *A. aegypti* females infected by transovarial means are infective but the importance of vertical transmission in the epidemiology of dengue is poorly understood [188, 198-200].

1.5 Disease syndrome

1.5.1 DF

DF is an acute, often debilitating illness that affects infants, young children and adults, but seldom causes death. DF is characterised by a high fever (above 38°C), sudden onset of extreme fatigue, severe headache, myalgia, arthralgia, retro-orbital pain and rash. It is also noticed, especially among children, the presence of sore throat

and abdominal pain with nausea and diarrhoea. Leukopenia and thrombocytopenia may also be observed. DF is self-resolving and rarely causes death [1]. The clinical features of DF vary according to the age of the patient. Infants and young children may have a fever with rash. Older children and adults may have either a mild fever or the classical incapacitating disease described above [201].

1.5.2 DHF

On the contrary, DHF can be very severe and even life threatening. In the first few days of illness the clinical signs and symptoms of DHF are indistinguishable from DF. The clinical complications of DHF occur between day 3-6 of illness and are characterised by- a) the leakage of water and small molecules from capillaries into interstitial spaces in tissues, b) thrombocytopenia, c) hemorrhagic manifestations. Indeed, loss of plasma volume and abnormal hemostasis are the major pathophysiological features of DHF and the basis of differential diagnosis with the DF. Pleural effusion, ascites and/or an increase of hematocrit (i.e an hemoconcentration) are the signs of plasma leakage into the extravascular space. Hepatomegaly is often present but fulminant hepatitis is rare [202]. Vascular shock may also occur and can be rapidly fatal without rapid fluid resuscitation to restore the intravascular volume. DHF with cardiovascular collapse is called dengue shock syndrome (DSS). This is manifested by a rapid and weak heartbeat, a low blood pressure and a diminished peripheral perfusion. Mortality in DSS varies from 20% without appropriate medical care to less than 0.5% with careful fluid management. A hallmark of these complications is that they occur when the viremia is in steep decline and the patient is nearly or already afebrile.

1.6 Risk factors for dengue haemorrhagic fever

Host and viral factors responsible for the severity of dengue are not well known (Figure 9) [2]. Indeed, beside an inappropriate immune response of the host, intrinsic factors of DENV also appear to contribute to the disease severity.

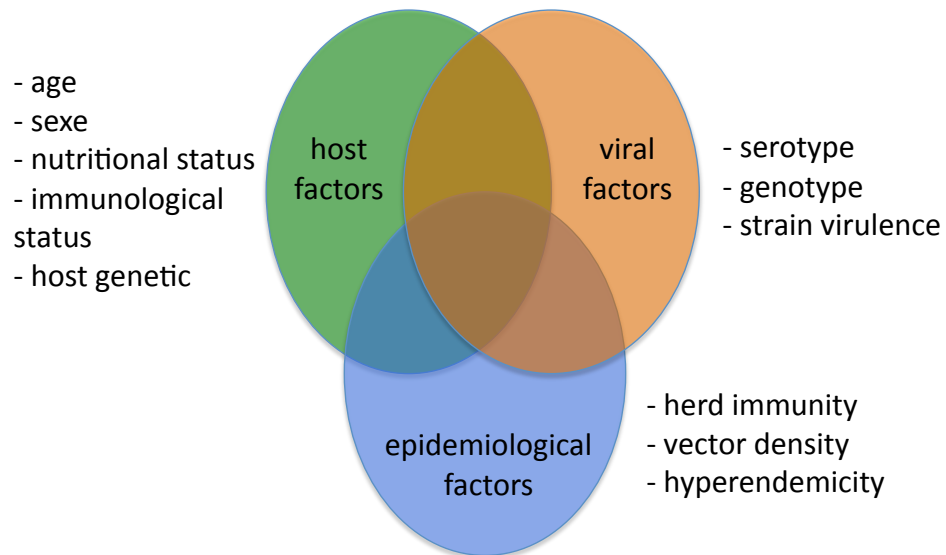


Figure 9: Risk factors for DHF/DSS: an integral hypothesis [2].

1.6.1 Patient immune status

Several epidemiological studies have shown an increased risk of DHF in children and adults during sequential infections, with secondary infection caused by a DENV serotype different from the primary infection [203-206]. Epidemiological studies in infants have shown that DHF occurs during primary infection in infants born to dengue-immune mothers [207-211]. A mechanism called “antibody-dependent enhancement” of the infection has been proposed by Halstead et al. in 1977 to explain these observations [212]. DHF may occur during secondary infection in children and adults or during the primary infection in infants because sub-neutralizing levels of DENV-reactive Abs acquired during the first infection or by trans-placental may increase sufficiently the infectivity of DENV and lead to higher viremia [213, 214]. Its mechanism is discussed in the section about the immuno-pathogenesis of DHF.

1.6.2 Host genetic

Results from epidemiological studies suggest that people of African ancestry are less

susceptible to DHF [2, 215]. Several allelic variants of multiple gene loci involved in both acquired and innate immune responses have been proposed to contribute to clinical outcome. Major histocompatibility complex (HLA) alleles have been associated with disease outcome and/or specific serotypes [216-219]. Various single nucleotide polymorphisms in different genes have also been associated with susceptibility or protection to severe dengue [220-223]. However, these data are from case-control association studies that present several limitations: small sample size, multiple testing, unknown population stratification and variable case definitions. With the exception of DC-SIGN, no association in an HLA allele or candidate gene has ever been replicated in an independent study. Alternatively, it is expected that genome-wide association approach would soon bring new insights in understanding of genetic susceptibility to severe dengue. Genome-wide association studies have power to detect relatively small contributions to genetic risk whilst also discounting any population stratification in cases or controls [224].

1.6.3 DENV virulence

Reduced and shorter viremia in monkeys (or human volunteers) has been used as a marker of attenuated virulence for vaccine candidates (when compared to the parent virus). However, the absence of good animal models of DHF makes it difficult to measure directly the virulence of naturally occurring DENV strains. Data from epidemiological studies suggest that some DENV strains are associated with lower viremia and generally cause milder illness [225-227]. Although DHF may be caused by the 4 serotypes, it appears that the most severe forms are often caused by DENV-2 followed by DENV-1 and DENV-3 [203, 204, 228-232]. DENV-4 is generally associated with milder clinical symptoms although it can also cause severe dengue [233]. In addition, virulence varies among genotypes. Epidemiological studies have shown that DENV-2 of Asian/American genotype were able to produce DHF during secondary infections while those of American genotype were unable to do so. [234-236]. In Sri Lanka, the emergence of DHF in 1989 coincides with the introduction of DENV-3 genotype different from those that circulated previously [231].

1.6.4 Relationship between the viral burden and the disease severity

Host factors related to severity and viral factors of virulence are not mutually exclusive and both predict that higher extent of viral replication would be seen in patients with more severe forms, the increase of the virus burden being thought to lead to DHF and DSS. Indeed, many studies have shown a positive correlation between viremia and disease severity. During the febrile and early convalescent periods, Taiwanese adults with secondary DENV-3 infections and DHF had higher levels of plasma viral RNA than did patients with DF [237]. An association between higher peak viremia and increased disease severity was observed in Thai children with acute DENV-1 and -2 infections [238]. DHF was associated with higher viremia early in illness in Thai children with secondary DENV-3 infections [239]. In contrast, some studies, have failed to confirm any association between viremia levels and disease severity [240]. NS1 antigenemia, another marker of the host viral burden, is also higher in patients with DHF or DSS [241].

1.7 Protection against dengue and immuno-pathogenesis of severe disease

1.7.1 Immune response in dengue

During primary and secondary infection, anti-DENV immunity is provided initially by the innate immune system. The immune response in dengue is characterized by a robust neutralising antibody response, that provides a lifelong homotypic protection but a short-term heterotypic protection of several months only, while the cellular immunity is playing a critical role in the viral clearance [31, 242-244].

1.7.2 Humoral immunity

Ab can neutralize DENV by preventing virus-cell binding and fusion [213]. Experiments in animals have shown that passive transfer of Ab against structural proteins, and to a lesser extent against NS proteins are protective against a lethal

injection of DENV [245-247]. Abs of maternal origin also provide protection against dengue in newborn infants. In case of secondary infection, heterotypic Ab can increase viral replication in monocytes and macrophages, and then aggravate the disease (see next section about the immuno-pathogenesis). Ab can also participate in the lysis of infected cells induced by the complement and in the “antibody-dependent cell cytotoxicity” (ADCC). Recognition by Ab of Ag on the cell surface causes recruitment of natural killer (NK) cells bearing a receptor fixing the Fc fragment of Ab that induce ADCC. NK cells are equipped with cytoplasmic granules containing perforin and granzymes whose release causes the target cell to die by apoptosis.

Anti-E Ab are the major protective Ab, they are made early in the disease course and are among the most abundant Ab after infection [248]. Abs to E protein domain III were either serotype specific or cross-reactive and potently neutralized DENV infection [249]. In contrast, monoclonal Abs that bound to domains I/II of E or prM protein neutralized poorly and showed broad cross-reactivity with the four DENV serotypes [250-253]. All mAbs enhanced infection at subneutralizing concentrations [249]. Results from recent studies suggest also that some anti-prM mAbs can augment infectivity of poorly infectious immature virions [128].

1.7.3 Cell-mediated immunity

The role of dengue-specific cellular immunity is less clearly defined. T cells might limit the expansion of infection by killing infected cells and secreting inflammatory cytokines, and are likely to have a role in viral clearance [243, 244]. An important characteristic of the cellular response is a high avidity, homologous response of type 1 helper T cells secreting IFN- γ [254, 255]. Proteins NS1, NS3 and E are recognized by CD8⁺ cytotoxic T cells [256-259]. Anti-DENV CD4⁺ CD8⁺ T cells produce IFN- γ , TNF- α and chemokines (chemotactic cytokines) and are effective in lysing DENV infected cells *in vitro* [255]. NK cells, that are major components of the innate immune system against viruses, play an important role in the lysis of DENV infected cells via ADCC (see above) [260]. Regulatory T cells have also been studied in DENV infection and, although being fully functional, able to suppress vasoactive

cytokines production and expand, this expansion is likely to be insufficient or happen too slowly to control the immune response in patients with severe disease [261].

1.7.4 Secretion of cytokines

Expression of cytokine genes is increased in patients with dengue compared to healthy volunteers [262-264]. *In vitro* and *ex vivo* studies have shown that infection with DENV may induce secretion of several cytokines and chemokines such as TNF- α , IFN- γ , interleukin (IL) IL-6, IL-8 and IL-10 or RANTES [265-267]. Increased production of pro-inflammatory and anti-inflammatory cytokines has also been observed in Vietnamese infants with dengue [210].

1.8 Immuno-pathogenesis of severe dengue

The pathogenesis of DHF remains poorly understood but it is now well accepted that some aspects of the immune response are involved in the immuno-pathogenesis of severe clinical presentations. However, the fact that DHF/DSS pathogenesis is a complex, multifactorial process involving cocirculation of various serotypes and the interplay of host and viral factors should be kept in mind (Figure 8).

1.8.1 ADE

The current prevailing model but still controversial to explain the development of severe dengue is ADE. The underlying mechanism is the facilitation, by viruses complexed with heterologous cross-reactive non-neutralizing IgG or present at sub-neutralizing concentrations, of the infection of cells expressing receptors for the constant fragment of the immunoglobulins such as monocytes and macrophages [238, 268, 269]. These pre-existing IgG are believed to promote opsonization, or simple concentration at the cell surface, of DENV and lead to increased virus uptake by

monocytes and macrophages thus increased replication, viremia and finally consequent disease severity [214]. Described for other viruses, the ADE model is supported in dengue by several prospective and retrospective epidemiological studies, and *in vitro* and *in vivo* experiments. [270]. ADE in dengue has been shown *in vitro* using dengue immune sera or monoclonal antibodies and cells of monocytic and B-lymphocytic lineages bearing Fc receptors [250, 268, 271-273]. For example, *in vitro* viral replication in human peripheral blood mononuclear phagocytes is increased in presence of diluted flavivirus antiserum (Figure 10) [19]. ADE has also been demonstrated in animal models. Viremia was significantly higher in monkeys infused with human dengue immune serum (from cord blood) and in monkeys that received a small amount of anti-DENV monoclonal Ab [268, 274]. A mouse model of DENV ADE has been recently described in which sublethal infection is enhanced by passive transfer of anti-DENV antibodies, leading to lethal disease characterized by plasma leakage, elevated serum cytokines, and thrombocytopenia [275, 276].

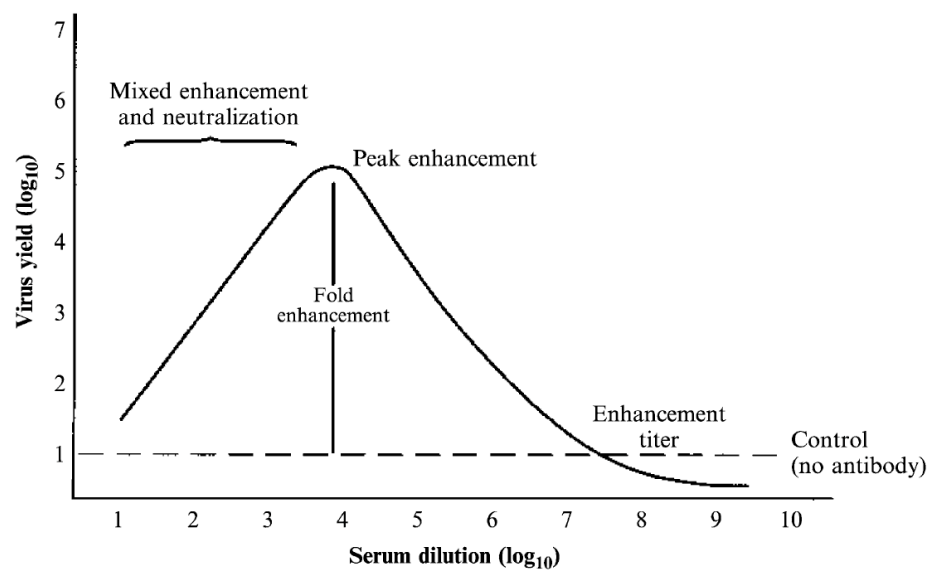


Figure 10: Illustration of the phenomenon of ADE *in vitro* [277]. At low serum dilutions, there is no (or very weak) viral replication, for greater dilutions, viral replication is increased (peak facilitation) but for higher dilutions, the facilitating effect is progressively lost. Reprinted from *Advances in Virus Research*, Vol. 30, Halstead, S.B., Neutralization and antibody-dependent enhancement of dengue viruses, pages 421-467, Copyright (2003), with permission from Elsevier.

In humans, many studies have shown that DHF occurs significantly more frequently during sequential infections by different serotypes in children and adults, and also in infants due to the presence of maternal antibodies [277]. This latter situation represents the strongest evidence of the ADE relevance. In endemic areas, the majority of mothers have Ab against several DENV serotypes and their infants acquire passively these Ab during the last trimester of pregnancy [210-213]. Prospective studies have shown that these Ab decrease over the time, with neutralizing Ab significantly dropping several months before the IgG that can bind intact DENV virions and then leaving a window between 6 and 12 months of age where virion-binding but non-neutralizing IgG might facilitate enhanced viral infection and predispose some infants to symptomatic or possibly severe disease [212-217]. A prospective study in Thai children has shown that ability of pre-illness sera to facilitate infection of primary human monocytes is positively correlated with later development of severe forms, i.e. sera with higher facilitating activity are found in children who later present a more severe form of the disease [278]. Kliks et al. showed a correlation between age of disease onset in Thai infants and dilution at which serum from cord blood maximally enhances infection of primary monocytes [279]. Infection-enhancement assays have shown that sub-neutralizing levels of maternally derived dengue antibody are a crucial risk factor for severe dengue in Vietnamese infants [265]. Chau et al. have found a strong temporal association between the Fc-dependent, DENV infection– enhancing activity of neat plasma from healthy infants born to dengue-immune mothers and the age-related epidemiology of severe dengue [280]. However Laoprasopwattana et al. while observing enhancing activity in undiluted preillness plasma from children who subsequently had secondary DENV infection but this enhancement did not correlate with subsequent disease severity or peak viremia levels in either secondary DENV-2 or DENV-3 infections [281].

The sequence homology between the 4 DENV serotypes (they are ~60-70% identical) that is responsible for a certain “antigenic closeness” between all the DENV makes the ADE model relevant at a molecular level [128, 134, 236]. Shared epitopes may generate a polyclonal serum that could be temporally cross-neutralizing but that become facilitating at sub-neutralizing concentrations. Furthermore, ADE has been

linked to increased production of cytokines and chemokines that have roles in vascular dysfunctions associated with severe dengue and also to a caspase-dependent apoptosis of target cells [282-286].

However, not all patients with secondary infection develop DHF and not all cases of severe disease result from secondary infection [2, 287]. ADE is then neither sufficient nor entirely necessary for severe disease and other factors must then play a part in pathogenesis. Indeed, facilitating antibody activity in pre-illness sera was not correlated in a recent study with subsequent onset of severe forms in Thai children when they were further exposed to DENV [281]. Other aspects of the humoral response have been explored. ADCC may contribute to dengue pathogenesis since it has been observed in sera of patients with DHF/DSS but not in patients suffering from DF [288]. Activation of complement by virion/Ab complexes may also affect the vascular permeability.

1.8.2 T cells “original antigenic sin”

It has been hypothesized that T cells may also contribute to the pathogenesis of secondary infection [289-291]. Following the ADE of viral replication in monocytes and macrophages, viral Ags might be presented to memory T cells sensitised during a previous infection. Recognition of serotype cross-reactive epitopes by these T cells leads to a selective expansion of lower avidity cross-reactive memory T cells that might dominate the naïve T cells with higher avidity for the infecting serotype [292, 293]. A study with Thai children has indeed shown that the affinity of these activated T cell was paradoxically low for the infecting serotype and higher for the serotype presumed responsible for primary infection, thus mimicking the “original antigenic sin” phenomenon [292]. This would be responsible for ineffective clearance of the virus and could lead to increased virus replication and more severe disease [292, 294]. In addition, these cross-reactive T cells have been shown to release pro-inflammatory cytokines such as TNF- α implicated in dengue pathogenesis (Figure 11) [295]. If the extent of T-cell activation has been related to the disease severity in some studies [265, 286], a recent study has found no evidence of increased CD8⁺ T cell activation

prior to the commencement of resolution of viremia or h emoconcentration [258]. It is then possible that other mechanisms independent of CD8+ T cells are responsible for early triggering of capillary leakage in children with DHF [258].

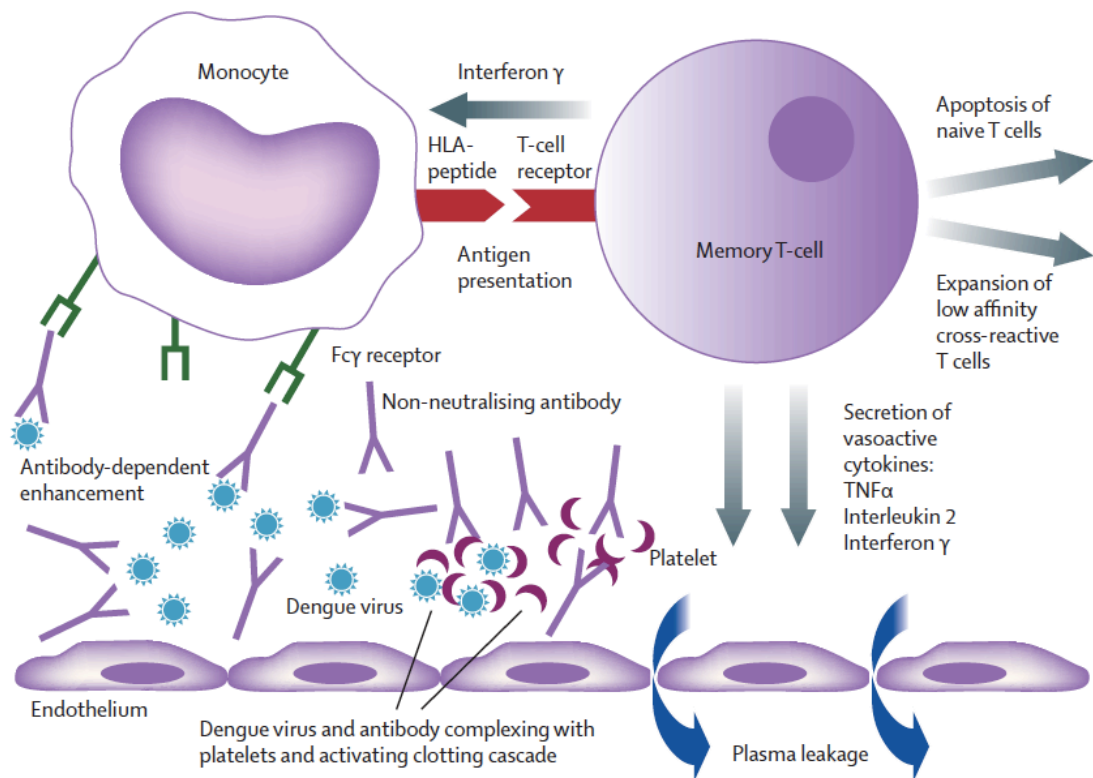


Figure 11: Schematic representation of the immuno-pathogenesis of severe dengue [296]. Reprinted from *The Lancet Infectious Diseases*, Vol. 9, Webster, D.P. *et al.*, Progress towards a dengue vaccine, pages 678-687, Copyright (2009), with permission from Elsevier.

It has also been hypothesized that the expansion of regulatory T cells is insufficient or happens too slowly to control the immune response in patients with severe disease [261]. The role of ADCC in the pathogenesis of dengue is not yet well understood, but early activation of NK cells was found in patients with DHF/DSS [256, 297-299]. It has been proposed that original antigenic sin is also present in the neutralizing antibody responses to dengue with 2 potential adverse outcomes for the infected host [300]. First, by driving the production of low-avidity antibodies, viral clearance may

be delayed. Second, original antigenic sin has the potential to affect ADE. At present, ADE is proposed to be driven predominantly by pre-existing antibody. However, as there are substantial increases in antibody during the early stages of secondary infection, boosting of poorly neutralizing, low-avidity responses during the course of the secondary infection may amplify this effect still further [301]. However, the link between the infection (even facilitated) of monocytes and macrophages, and all the pathophysiological changes leading to capillary leakage and bleeding remains poorly understood.

1.8.3 Cytokines

Studies have suggested that the capillary permeability resulting from dengue infection is caused by the malfunction of vascular endothelial cells induced by cytokines or chemokines [76, 254, 302]. Patients with severe disease have higher concentrations of cytokines including IFN- γ , TNF- α , and IL-1, -2, -6, -8, and -10 and chemokines such as MCP-1 IP-10 [76, 303]. These findings have been supported by recent studies of sera from severe dengue patients in Vietnam, India, and Cuba [304-306]. This over-secretion of cytokines or chemokines may be explained by the previously cited non-exclusive hypotheses of ADE and “original antigenic sin”. Indeed, the massive lysis of monocytes and macrophages, and activation of cross-reactive memory T cells due to the large number of antigen-presenting cells that express viral antigens may induce a sudden secretion of cytokines and chemokines. These cell-signalling molecules would then cause increased vascular permeability (causing hemoconcentration, plasma loss and blood pressure drop), thrombocytopenia and the coagulopathy observed in severe forms. Nevertheless in case of a primary dengue in infants, the immediate response of the host cannot be due to DENV reactive memory T cells. However, high levels of inflammatory cytokines have been described in Vietnamese infants with primary dengue [210]. It is important to note that most of these studies are only descriptive and not really designed to conclude about potential causative links. In addition, differences of patient populations, DENV serotypes, illness durations, etc. make comparison across studies very difficult. It is also very likely that a bias toward publications that show differences between patients exists [224].

1.9 Laboratory diagnosis

The laboratory diagnosis of dengue is essential to confirm DENV infection in patients with clinical signs of the disease. A distinction can be made between early tests, such as the polymerase chain reaction (PCR) and the NS1 Ag detection that can be positive from the first day of symptoms, and later tests detecting IgM and IgG that appear after 5 days but can persist for months or even lifetime for IgG (Figures 12 and 13). The combination of these tests can provide a definitive diagnosis, but relies on appropriate timed sample collection for best sensitivity.

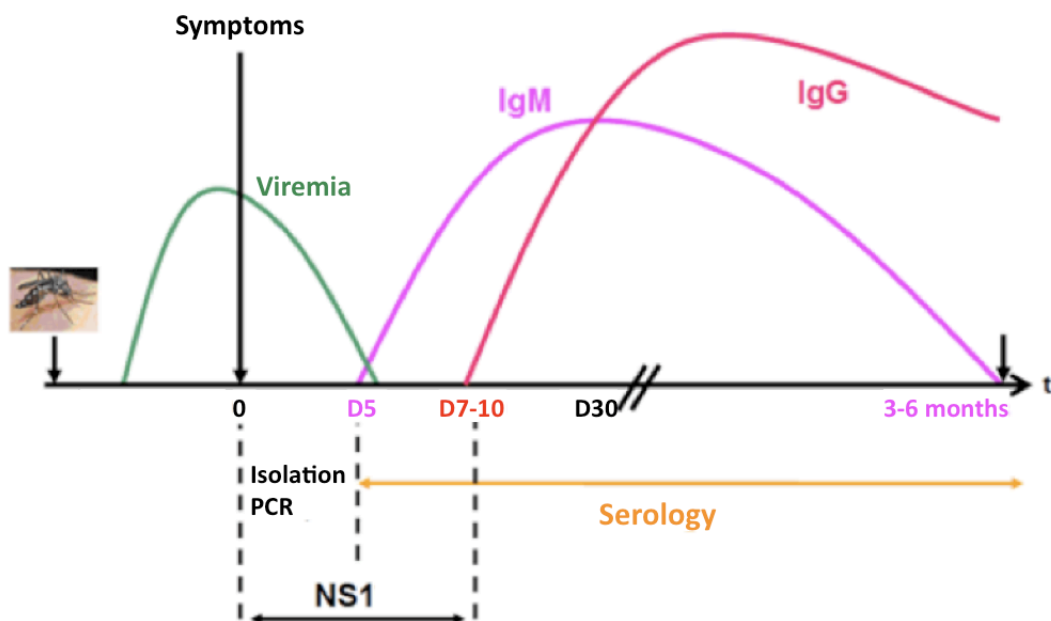


Figure 12: Kinetics of Ag and Ab during a primary dengue infection (adapted from the Centre National de Référence des arbovirus, Institut Pasteur). Detection by gene amplification is possible until several days after the onset of symptoms (day 0). Detection of NS1 can be positive up to day 7-10. Detection of IgM / IgG is possible respectively from day 5 and around day 10. IgM persist for several months.

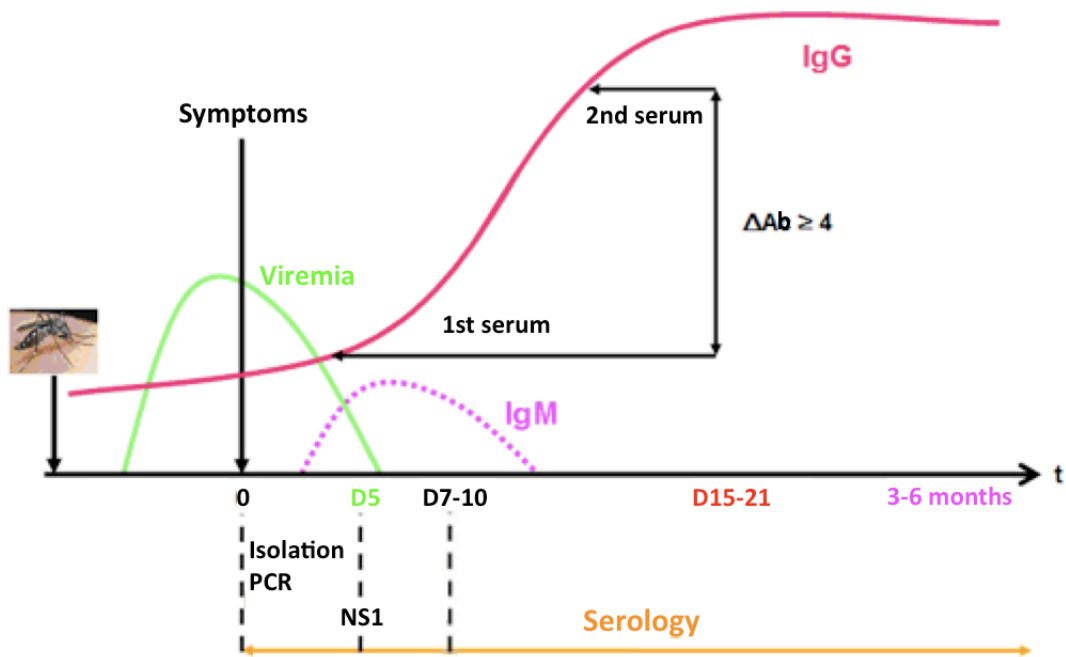


Figure 13: Kinetics of Ag and Ab during a secondary dengue (adapted from the Centre National de Référence des arbovirus, Institut Pasteur). The viremic phase is similar than during primary dengue. The IgM response may exist but remain discreet, fleeting or undetectable. Titre of IgG increases between two samples taken at 10 days interval (of a factor of 4 minimum).

1.9.1 Isolation and characterization of DENV

DENV isolation is the gold standard. It is done by inoculation of clinical specimens into certain cell lines, cell culture and detection of viral Ag by indirect immunofluorescence. C6/36 cell line (from *Aedes albopictus*) is the most commonly used due to its higher sensitivity to infection than most of the mammalian cell lines (Vero, BHK) [307, 308]. Serotyping is done by immunological (using serotype specific monoclonal Ab) or molecular (PCR or sequencing) techniques [1]. This technique is only possible in the early days of the disease when DENV is abundant enough in the blood. It requires a good preservation of the sample (express shipping or freezing) until inoculation. This technique is not suitable for routine diagnosis and is in fact

only very rarely used in diagnosis because it is long and difficult to implement (more than 5 days for cultures to become positive) [309].

1.9.2 Detection of DENV nucleic acids

Several methods of viral RNA detection by classic reverse transcriptase - PCR (RT-PCR) were developed in the 90s [310, 311]. The technique described by Lanciotti et al. is still used and can detect and identify all the 4 serotypes according the size of the amplicon. In the last 10 years, several methods using real-time RT-PCR have been developed [312-315]. RT-PCR is a rapid technique that allows early diagnosis. It can be used during all the viremic phase (Figures 12 and 13) [1, 238] and is able to quantify DENV viral load that might be an indicator of the disease severity. However it is expensive (it requires trained staff and expensive equipment and reagents) and its routine use in developing countries is at an early stage. A recent external quality assurance (EQA) program did not reveal any significant difference in performance methods and a low sensitivity was appreciated in the general results of this EQA, even when reliable real time procedures were used with higher risk of false negatives in the diagnostic results. The most important factor in the reliability of the results for dengue diagnosis is the individual performance of each laboratory as demonstrated by the different score values reached by different laboratories that used the same published protocols [316].

1.9.3 Detection of NS1 antigen

The NS1 protein is secreted from virally infected cells. It can be detected in peripheral blood during acute infection and up to 14 days after the illness onset, i.e. even when DENV RNA is not anymore detectable by RT-PCR (Figures 12 and 13) [317, 318]. NS1 detection is used for early diagnosis. The specificity of the commercially available tests is generally high but the sensitivity is variable [317, 319-323]. An immuno-enzymatic technique and more recently a technique by immuno-chromatography (ICT) have been developed (Table 1). The sensitivity of NS1 tests

depends on the day of the disease [320, 324, 325]. These tests have a higher sensitivity during the first 3 days after illness onset [320]. They are generally more sensitive in primary infection. Secondary infection and presence of IgG and IgM Ab are associated with a reduced sensitivity [320, 326]. There is some correlation between NS1 and the viremia levels in patients with DHF [327, 328]. One of the limitations of these tests is they cannot determine the DENV serotype [329].

The commercial ELISA (Enzyme Linked ImmunoSorbent Assay) tests available in 2010 were essentially the Platelia NS1 ELISA®, the pan E Early ELISA® and the SD Dengue NS1 Ag ELISA® marketed by Bio-Rad, PANBIO and Standard Diagnostic respectively. These kits are fast (a few hours) and easy to use. The required equipments are a microplate washer, an incubator and a spectrophotometer for reading. ELISA is usually used to test several samples simultaneously. It is perfectly suited for large series i.e. during epidemic periods. The NS1 ELISA Platelia® (Bio-Rad) has been reported as being more sensitive than the pan-E Early ELISA® (PanBio) [320]. The technique for rapid detection of NS1 Ag by ICT is faster (30 minutes) and easier to implement than ELISA. This technique is a bit less sensitive [319, 320]. The sample is placed at one end of a nitrocellulose membrane mounted on a support. If NS1 Ag is present in the sample, it binds an Ab present in the test. The Ag/Ab complexes then migrate by capillary and are stopped by capture Ab fixed on the membrane. A positive result is seen by the appearance of a coloured line. The available rapid diagnostic tests (RDTs) in 2010 were Bio-Rad Dengue NS1 Ag Strip® and Standard Diagnostics Dengue Duo®. These are the two tests evaluated in Chapter 3. These tests are sold unit by unit and are then very suitable to the inter-epidemic period when there are only a few cases at the same time.

1.9.4 Detection of IgM / IgG

Detection of dengue specific IgM and/or IgG is done by ELISA or ICT (Table 1). Detection of IgM seroconversion in paired samples allows diagnosis of dengue: IgM can be detected from the 5th day after onset of clinical symptoms and persist for 1-2 months. IgG appears at the same time in secondary infection or later in primary

infection but can persist throughout life (Figures 12 and 13). Several MAC ELISA (e.g. Dengue IgM Capture ELISA® of PANBIO) and rapid tests for the IgM detection are commercially available. Some of them have been independently evaluated [326, 330-333]. The Ags used are usually inactivated viral particles [334]. The sensitivity and specificity of detection of DENV-reactive IgM and IgG depend on the Ag used. A cross-reactivity with other flaviviruses such as Japanese encephalitis virus is possible but can be controlled [335]. The IgM/IgG ratio is commonly used to differentiate primary and secondary dengue [334, 336]. In this context, the term of secondary dengue rather describes the serological profile of the immune response, and it is impossible to know if it is the second, third or fourth exposure [337]. The advantage of the MAC ELISA test is that it is inexpensive and simple to perform [334, 336]. The main limitation of these serological tests is that they are not sensitive enough in the early days of the disease and strictly, requires paired samples for laboratory confirmation of dengue.

Table 1: List of commercially available and independently evaluated test kits for the diagnosis of dengue.

Test name	Company	Method of detection	Analytes	Volume of sample
Platelia Dengue NS1 Ag Test	Bio-Rad	ELISA	NS1	50µL
Dengue Early ELISA	Inverness	ELISA	NS1	100µL
SD Dengue NS1 Ag	Standard diagnostic Inc.	ELISA	NS1	50µL
Dengue NS1 Ag STRIP	Bio-Rad	ICT	NS1	50µL
Dengue Early Rapid	Inverness	ICT	NS1	50µL
SD BIOLINE Dengue Duo	Standard diagnostic Inc.	ICT	NS1/IgM/IgG	110µL
Dengue Duo Cassette	Inverness	ICT	IgM/IgG	10µL
Dengue Fever IgG & IgM Combo Device	Merlin	ICT	IgM/IgG	100µL
Immunoquick Dengue Fever IgG & IgM	Biosynex	ICT	IgM/IgG	1µL
Dengue IgM Capture ELISA	Inverness	ELISA	IgM	10µL
SD Dengue IgM Capture ELISA	Standard diagnostic Inc.	ELISA	IgM	10µL
Dengue IgG Capture ELISA	Inverness	ELISA	IgG	10µL
Dengue IgG Indirect ELISA	Inverness	ELISA	IgG	10µL
SD Dengue IgG Capture ELISA	Standard diagnostic Inc.	ELISA	IgG	10µL
MP diagnostics ASSURE	MP Biomedicals	ICT	IgA	25µL

1.10 Treatment

There is currently no antiviral treatment. Vector control and personal protection against bites of *Aedes* mosquitoes are the only way to reduce the risk of infection. Once the person is infected, symptomatic management can relieve the patient and prevent clinical worsening. However, current efforts to develop vaccines and specific drugs may bear fruit and change this situation.

1.10.1 Symptomatic treatment

It is recommended to use painkillers such as paracetamol. Salicylates are not recommended because their anti-platelet aggregation properties may exacerbate a clinical picture of DHF or DSS. Early plasma loss replacement by oral or intravenous solutions (crystalloid, Ringer lactate or colloid solutions such as dextran 40) can reduce severity and prevent occurrence of vascular shock. Different therapeutic approaches have been evaluated [202, 338-340]. The WHO has developed guidelines for the management of DHF and DSS [1]. Mortality can be greatly reduced by regular monitoring, good oral and/or intravenous rehydration and careful attention to the warning signs of severe dengue.

1.10.2 Vaccines

If the naturally acquired immunity (i.e. robust neutralising Ab response develops after infection) has provided optimism for the feasibility of a dengue vaccine, its development has proven to be difficult because, amongst other things, this vaccine has to be tetravalent to provide immunity against all the serotypes. However significant progress has been made recently and clinical trials have grown in number [341-344]. The development programs are now sufficiently advanced to expect a first generation vaccine on the market within the next 5-7 years [343]. In addition, several promising candidates but at earlier stages of development could be ready in the next decades and

represent the next generation of vaccines. To facilitate this development, the WHO has made recommendations for the clinical evaluation and the Paediatric Dengue Vaccine Initiative (PDVI) has helped the implementation of trials [345].

1.10.2.1 A difficult development

The main mechanism of protection against DENV is believed to be neutralization by specific Ab and all vaccine candidates currently in development seek to induce high levels of neutralizing Ab. The co-circulation of 4 serotypes requires a vaccine that protects against the 4 serotypes be tetravalent. Moreover, the induction of a protective response against the 4 serotypes must be simultaneous and well balanced to avoid the theoretical risk of ADE. Indeed, there is a theoretical risk that a vaccine causes severe dengue (DHF and DSS) in vaccinated people if a satisfactory immunity against all the serotypes has not been established. It is even possible that these people are prone to less severe secondary infections than the control group although this is based on a study of a few volunteers [346]. Anyway, it is essential that the risk of ADE, even theoretical, is evaluated by extended follow-ups of vaccinated cohorts.

An additional challenge is the lack of validated criteria on which to rely to assert that protective immunity has been achieved. It is however widely accepted that the main effectors of this immunity are neutralizing Ab and their titer is commonly used to estimate the protection induced by the vaccine candidates. This is determined by a seroneutralization technique by reducing the number of plaques or PRNT (for “Plaque Reduction Neutralization Test”) using a virus of the same serotype of the vaccine strain. This titer is expressed as the serum dilution causing 50% reduction in the number of the plaques observed in the control containing the virus but not the serum. This is called PRNT50. It is generally admitted that seroconversion is obtained from a naive subject if its PRNT50 titer is >10 (i.e. higher dilution than a 1:10 serum dilution reducing 50% of plaques) [342, 347]. However, no specific titer of Ab or neutralizing epitopes has been precisely defined and the contribution of the other immune mechanisms is still not totally assessed. Moreover, it has been shown that PRNT results could vary significantly depending on the assay conditions [348]. Further

studies are needed to determine which parameters are associated with protection and would show whether the vaccine candidate induces a protective immune response or not [349]. Another challenge is the lack of animal models that faithfully reproduce the disease or the immune response in humans. Despite possible discrepancies between preclinical and clinical tests, experiments on animals are necessary to evaluate new vaccine candidates [350]. However, significant progress has been recently made in the development of murine and primate models that develop measurable viremia and can be used as models of infection.

1.10.2.2 Vaccines in development

Among various vaccine candidates in development, many are prepared from live viruses attenuated empirically or by genetic engineering. These vaccines are now in clinical development. They have been first developed as monovalent vaccines (i.e. directed against a single serotype) and were later combined into tetravalent mixtures. Vaccines derived from live attenuated viruses generally induce a very sustainable humoral and cellular response because they mimic very closely the natural infection. The most advanced vaccine is currently the chimeric tetravalent vaccine dengue / yellow fever from Sanofi-Pasteur (the ChimeriVax® technology has been originally developed by Acambis) in which prM and E genes of each of the 4 serotypes replace those of the yellow fever virus in 17D vaccine strain. The NS protein genes of yellow fever virus allow the virus replication. The attenuation is due to the yellow fever part of the chimera. Each monovalent version and several polyvalent mixtures have been tested in phase I and phase II clinical trials in volunteers of different age groups and in endemic areas or not. At least 2 doses are necessary to achieve high levels of neutralizing Ab. It is currently entered in phase III. Others live attenuated vaccines are in clinical development, one developed by GlaxoSmithKline and the Walter Reed Army Institute of Research, uses a strains of each serotype attenuated by serial passages on primary cells of dog kidney (PDK) and another one developed by the National Institute of Allergy and Infectious Diseases (NIAID) uses live viruses attenuated by 2 different methods: the removal of 30 nucleotides in the 3'-UTR of DENV-1 and -4, and construction of intertypic chimeric viruses based of the DENV-4

strain obtained by the removal 30 nucleotides in the 3'-UTR. In these chimeras, the structural protein genes of DENV-4 have been replaced by those of DENV-2 and -3. Vaccine prepared from inactivated viruses or from subunits and nucleic acids are also in development but at a preclinical stage. DNA based vaccines have been designed to deliver the viral structural protein genes into the host cells. The vaccine candidates consisting of recombinant protein(s) or subunit(s) are generally combined with adequate adjuvant(s) [351]. Once the license obtained, introduction of the vaccine will be adapted to the situation of each country. The WHO has set out guidelines that will help national authorities in making their decision in order to maximize the impact of the vaccination on the population but also on the transmission of the virus among the mosquito vector [352].

1.10.3 Antiviral drugs

Antiviral research is relatively recent in dengue. It has accelerated in recent years due to the increased interest in dengue and the significant progress made in understanding the structural biology of DENV. In recent years, several new structures of DENV and other flaviviruses proteins have been resolved. This research has also benefited from the efforts to develop drugs against HCV, particularly the work on anti-HCV molecules targeting the RdRp (HCV and DENV belong to the same family and their RNA polymerase are very similar in structure) [353]. Several studies have shown association between high viral loads and disease severity. Similar differences in viral load were observed in animal models of ADE [204, 268, 274]. Some have then suggested that the progression to severe forms could be avoided by administering at an early stage of the disease a small molecule, sufficiently active, targeting one or more essential step(s) of DENV replication and thus reducing the viral load. This hypothesis remains to be validated by a proof of concept but the life cycle of DENV readily shows that steps involved in cell entry, fusion with the cell membrane, RNA replication, virions assembly and release can be targeted by small molecules [354]. *In silico* and high throughput screening of libraries of molecules have led to the discovery of several lead compounds [355]. Some of the molecules with an anti-DENV activity are listed in the Table 2.

Table 2: Compounds with anti-DENV activity and their target.

Compound	Target	Model system	Reference
Substrate-based peptidomimetics	Viral protease NS2B/NS3	Enzymatic assay	[356-359]
Aprotinin	Viral protease NS2B/NS3	Enzymatic assay	[360]
ARDP0006 and ARDP0009	Viral protease NS2B/NS3	Enzymatic and cell-based assays	[361]
Palmitine	Viral protease NS2B/NS3	Cell-based assay	[362]
(BIP)(2)B	Viral helicase NS3	Enzymatic assay	[363]
Beta-d-2'-ethynyl-7-deaza-adenosine triphosphate (nucleoside analogue)	Viral RdRp NS5	Enzymatic assay	[364]
N-sulfonylanthranilic acid derivatives (non-nucleoside inhibitors)	Viral RdRp NS5	Enzymatic assay	[365, 366]
adenosine analogues (NITD008, NITD449 and NITD203)	Viral RdRp NS5	Enzymatic, cell-based assays and animal experiment	[367-369]
Iminosugar deoxynojirimycin and derivatives	Host α -glucosidases	Cell-based assay	[370]
CM-9-78 and CM-10-18 (imino sugars)	Host α -glucosidases	Enzymatic, cell-based assays and animal experiment	[371, 372]
Castanospermine	Host α -glucosidases	Cell-based assay and animal experiment	[373, 374]
Iminocyclitol compounds	Host α -glucosidases	Cell-based assay	[375, 376]
Carbohydrate-binding agents (such as the plant lectins)	Viral E protein (inhibition of DENV binding on DC-SIGN expressing cells)	Cell-based assay	[377]
Dasatinib	Host Kinase	Cell-based assay	[378]
Ribavirin	Host nucleoside biosynthesis	Cell-based assay but inactive in animal model	[379-382]
ETAR (ribavirin analogue)	Host nucleoside biosynthesis	Cell-based assay	[383]
IM18 (ribavirin analogue)	Host nucleoside biosynthesis	Cell-based assay	[383]
Brequinar	Host nucleoside biosynthesis	Cell-based assay	[384]
Mycophenolic acid	Host nucleoside biosynthesis	Cell-based assay	[382, 385, 386]
Zosteric acid and devired compounds	Viral entry	Cell-based assay	[387]
Sulfated polysaccharides	Virus entry	Cell-based assay	[388-393]
WSS45 (sulfated polysaccharides)	Virus entry	Cell-based assay	[394]
PI-88 (sulfated polysaccharides)	Virus entry	Cell-based assay	[391]
PM-1001 (vanadium substituted polyoxotungstate)	Virus entry	Cell-based assay	[395, 396]
SA-17 (doxorubicin derivate)	Virus entry	Cell-based assay	[397]
Carbohydrate-binding agents (such as the plant lectins)	Viral E protein (inhibition of DENV binding on DC-SIGN expressing cells)	Cell-based assay	[377]
DN57opt and IOAN1 (peptides)	Viral E protein	Cell-based assay	[398]
Peptides from the E-protein fusion domain	Viral E protein	Cell-based assay	[399]

N-octyl-beta-D-glucoside competitors	Viral E protein (fusion inhibitor)	Enzymatic and cell-based assay	[400, 401]
Compounds with a central thiophene ring	Viral E protein (fusion inhibitor)	Cell-based assay	[402]
Neutralizing monoclonal antibodies	Viral E protein	Cell-based and animal model	[70, 403-413]
Glutathione	Reversal of intracellular factor- κ B activation	Cell-based assay	[414]
Ribavirin 5'-Triphosphate	Viral methyltransferase NS5	Enzymatic assay	[415]
ATA (aurintricarboxylic acid)	Viral methyltransferase NS5	Enzymatic assay	[416]
S-adenosyl-homocysteine derivatives	Viral methyltransferase NS5	Enzymatic assay	[417]
Small interfering RNA	Viral RNA	Cell-based assay	[418]
Arginine-rich antisense phosphorodiamidate morpholino oligomers	Viral RNA	Cell-based assay and animal experiment	[419-421]
Geneticin (aminoglycoside)	Unknown	Cell-based assay	[422]
Flavanoid-derived compounds	Unknown	Cell-based assay	[423]

The NS3/NS2b protease and the NS5 RdRp are currently the major targets. Other targets such as the E protein, NS3 helicase and NS5 methyltransferase are under study, and recent advances in understanding the mechanism of membrane fusion has opened new possibilities [424, 425]. The use of infectious clones and replicons has facilitated screening in cell culture [426, 427]. Preclinical development is facilitated by the existence of murine models of infection [380]. For example, the tropism of DENV in cells and tissues is similar in AG129 mice (deficient in interferon α / β and γ receptor) and humans [428, 429]. Another approach is to target the clinical manifestations of dengue. To develop such compounds, it is possible to use animal models that reproduce, albeit with many limitations, some of the symptoms of dengue. For example, AG129 mice infected with the D2S10 DENV-2 strain present early vascular leakage and die quickly [430]. ADE phenomenon can be observed in this model that is currently used for testing compounds that target vascular leakage [429]. It has also been proposed to target some elements of the host that are necessary for the virus replication, especially for the final stages of viral proteins modification and virions assembly [431]. Using a combination antiviral / anti-inflammatory, to both fight the virus and modulate the exacerbate inflammatory response thought to be at the origin of some of the complications of severe dengue, could also be an interesting approach as suggested by Ralf Altmeyer for influenza infection [431].

The major obstacle to the antiviral strategy is the relatively narrow interval between the symptoms onset and the disappearance of the virus from the circulation. Indeed, to be effective an antiviral drug should be administered early enough after the onset of symptoms, yet the vast majority of patients present to health care providers 2-3 days after the fever onset, and sometimes after. As children are seriously affected by dengue in endemic countries, as adults in some areas, drugs should therefore target all age groups, but because of difficulties in implementation of pediatric clinical trials, the first clinical trials will be conducted in adults (> 15 years).

1.11 Prevention

Prevention of dengue is primarily to fight against the mosquito vector. There are 2 ways to control the vector: the control of larvae and adult mosquitoes and the prevention of bites.

1.11.1 Collective prevention and vector control

The most common historical approach to limiting dengue was control of *A. aegypti*. Physical destruction of the breeding sites and elimination of larvae and adult mosquitoes by chemical or biological means are the main methods to fight against the mosquito vector. Although such programmes are capable of achieving significant reductions in number of vectors, systematic eradication programs are labour-intensive, expensive to sustain and appear to be less effective than expected at preventing dengue transmission [432]. With the intention to develop a more cost-effective vector control, Singapore has adopted a case-reactive approach to vector control but after past success of initial method, this new approach has proved to contribute to the increased dengue incidence. For Ooi et al., Singapore needs to return to a vector control program that is based on carefully collected entomologic and epidemiologic data [433].

1.11.1.1 Destruction of breeding sites

Modern *A. aegypti* control programmes such as those in Singapore and Cuba are also based on locating and eliminating both domestic breeding sites and residual eggs, but growing prosperity in many areas has led people to resent the invasion of their homes by control officials [434-437]. Furthermore, short-term success generally lowers public perceptions of disease risk, and generates increased hostility to continued control attempts. *Aedes* mosquitoes can lay their eggs in any container - natural or artificial - containing stagnant water. The destruction of these breeding sites is an efficient way to avoid the proliferation of *Aedes*. For this, it is necessary to eliminate stagnant water and, if possible, to remove all the containers and other objects that can retain some water (plastic containers, used tires, broken bottles, flower pots). Given the mosquitoes development cycle duration, destruction of breeding sites should be performed 2 or 3 times a week. This simple method is essentially based on the active participation of local people. If its implementation is not always effective, this method is promoted in all affected countries (Figure 14).



Figure 14: Panel encouraging Vietnamesees to fight *Aedes aegypti* (near Bao Loc).

1.11.1.2 Chemical insecticides and larvicides

Treatment with chemical larvicides is an effective way to fight the DENV vectors. There are non-toxic for humans and treated water is suitable for drinking. They are long-term effective regulators of insect growth with long duration of action such as the pyriproxyfen. To fight against the adult mosquitoes, fumigations with insecticide help to reduce number of vectors but are not able to stop widespread of dengue. Anyway, these techniques are heavy to implement at a collective level and are mostly used by the richest affected countries (e.g. Singapore).

1.11.2 Individual prevention measures

At an individual level, prevention is also possible by using different mechanical protective measure. Experimental data indicate that personal protective measures such as the use of mosquito nets and repellents are associated with a reduction of the risk of infection [438-443]. Many entomological studies have shown that insect repellents, such as the N, N-diethyl-m-toluamide (DEET), can prevent bites of dengue vectors and should reasonably reduce the risk of infection. Their use is recommended to all the travelers in areas at risk of DF with specific precautions to pregnant women and young children [444-448]. For newborns, insect repellents are not recommended and the priority should be given to impregnated mosquito nets.

1.12 Scope and objectives of this thesis

DENV has recently become the most important arboviral pathogen in tropical and subtropical regions throughout the world. It is today a matter of urgency to find better ways to treat infected patients and control its transmission. In addition to vaccine and vector control, antiviral might be a relevant and complimentary approach (see previous sections). The hypothesis that states progression to serious dengue disease and adverse morbidity may be reversed by administering potent and safe small molecule compounds that target essential steps in virus replication early during the

disease, thereby lowering the viral load requires field-testing [1]. In addition, both trial and future utilization of antivirals will require cheap, easy and rapid methods for early diagnostic of infected patients. Identically, new knowledge on relationship between disease severity, patient immune status, infecting serotype and virological parameters is needed to develop and use antivirals against dengue and would be useful for a better understanding of dengue pathogenesis.

The specific objectives of this work are as follows:

- 1) Evaluate chloroquine as an antiviral to treat dengue in Vietnamese adults. To the best of our knowledge this is the first random controlled trial of a putative anti-dengue virus compound.
- 2) Evaluate NS1 rapid diagnostic tests as early diagnostic tests for dengue. To the best of our knowledge, this is the first side-by-side assessment of different NS1 rapid diagnostic tests.
- 3) Evaluate the usefulness of NS1 rapid diagnostic tests to enrol patients in randomized controlled trial and compare 2 available rapid diagnostic tests and evaluate IgM/IgG parameters of the SD Duo test.
- 4) Characterize the relationships between virological features of dengue virus infection and its clinical manifestations in the Vietnamese adults who participated in the chloroquine randomized controlled trial.
- 5) Make some observations and recommendations regarding the rational use of intervention therapies in dengue, e.g. antiviral drugs.

A more detailed rationale for each objective is given at the beginning of chapters 2, 3 and 4.

Chapter 2 : A randomized clinical trial of chloroquine for the treatment of dengue in Vietnamese adults

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2.1 Background

There is currently no treatment for dengue, only supportive care. High viremia has been associated with severe dengue disease (DHF and DSS) (see chapter 1). Leading from this observation, medicines that reduce the viral burden may lower the morbidity and mortality associated with dengue and also the rate of transmission of the virus. In recent decades, new utilizations of CQ, a cheap, widely available and well-tolerated lysosomotropic 4-amino-quinoline derivative introduced into medicine in the 1940s and used for treatment and prevention of malaria, have been investigated (see in annexes the Investigator's Brochure for an exhaustive inventory of properties and clinical uses of CQ). De Duve et al. introduced the term “lysosomotropic agent” in 1974 to designate substances that are taken up selectively into lysosomes [449]. CQ is able to inhibit the growth of several intracellular bacteria and fungi and it appears to have a modest anti-viral activity on viruses from diverse taxonomic families [450]. This has led to speculation that CQ could have a therapeutic role in the treatment of viral diseases where there are limited or no other therapies [450, 451].

Several mechanisms of action have been proposed to explain this broad-spectrum antiviral activity. Although not well elucidated and presumably different according to viruses, these are related to a pH increase induced by CQ within intracellular organelles that affect viral replication. Briefly, CQ has been shown to inhibit several viruses requiring an acidification step for entry into their host cell [452-455]. Another possible mechanism is the effect of CQ on post-translational modifications of newly

synthesized viral proteins by impairing some proteases or transferases that require a low pH for their activity [456, 457]. CQ might also have an indirect antiviral effect by interfering with the biosynthesis of cellular receptors [458, 459].

In the context of DENV, an alkalization of endosomes due to CQ might interfere with the low pH-dependent structural changes involving the E fusion loop central to successful virion fusion with the endosomal membrane and release of infectious viral nucleic acid into the cytoplasm [75]. The lysosomotropic and weak base properties of CQ could also exert anti-viral activity by interfering with furin-dependent virus maturation, which require low pH environments in the lumen of the trans-Golgi network [133]. Treatment of DENV-2 infected mammalian cells with CQ reduces the infectivity of the produced virus by six- to eightfold, possibly by inhibiting the normal proteolytic maturation process of the virus [62]. Treatment of mammalian-expressing cells with chloroquine inhibits DENV infection [460]. The 50% inhibitory concentration of chloroquine for DENV is achievable inside human cells following ingestion of standard doses of CQ [461, 462].

In addition to these mechanisms, CQ has shown immuno-modulatory and anti-inflammatory properties of CQ that might play an advantageous role in viral infection. Recognition of DENV by plasmacytoid dendritic cells (pDCs) occurs through a TLR-dependent pathway that requires endosomes acidification [463, 464] and chloroquine-mediated blocking of this process partially inhibited West Nile virus-induced IFN- α production by pDC cultures [465]. CQ could also modulate antigen processing via an increased export of soluble antigens into the cytosol of DCs [466]. CQ could modulate the host response to virus infection. CQ also attenuates inflammatory cytokine responses [467, 468] and this may in part explain why CQ is used as a 2nd line therapy in the treatment of inflammatory and autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus [469-472]. Possibly related to its anti-inflammatory properties, CQ exerts an antipyretic effect equal to paracetamol during treatment of uncomplicated *P. falciparum* malaria [473].

Moreover, CQ fits to the requirements of WHO for the development of dengue antivirals [1]. The minimal target profile includes a low toxicity that premises prophylactic and/or paediatric use, an oral route of administration, a frequency of dosing of once per day, a stability to heat and humidity, a long shelf-life, and low/reasonable production costs to allow a reasonably priced product.

Given this, the purpose of this study was to evaluate CQ as a potential active treatment against all serotypes able to reduce the symptoms and the incidence of severe dengue and with the hypothesis that CQ decrease viral replication and therefore may confer a clinical advantage in a randomized, double-blind placebo-controlled trial of adolescents and adults with dengue. To the best of our knowledge this is the first RCT of a drug used to fight DENV infection. Previous randomized controlled trials in dengue have focused on supportive management and there has never been a trial directed towards reducing the virus burden. The choice of the primary endpoints has been driven by the fact that measurable viremia is typically present for the duration of the febrile period, with the first 48-72hrs characterized by relatively high viremia levels that then rapidly decline as acquired humoral and cellular immune responses resolve infection [238] and NS1, a non-structural protein secreted by virus-infected cells, can be detected in the peripheral blood in some, but not all, symptomatic individuals [317, 324]. Both viremia and NS1 levels have been found higher in patients with more severe clinical patterns of disease [474].

2.2 Materials and methods

2.2.1 Study setting, participants and treatment allocation

We performed a randomized (allocation ratio 1:1), double blind, placebo-controlled parallel-group study in 307 adults hospitalized for suspected DENV infection (see in annexes the protocol). Study participants were recruited from the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam. Patients were eligible if they were ≥ 15 yrs, had a self-reported illness history of 72hrs or less and were suspected of having dengue. Patients were excluded if they were pregnant or receiving therapy for

other chronic disorders, had a history of hypersensitivity to CQ, or written consent from either the patient or a parent was not obtained. Physicians in the Hospital for Tropical Diseases were responsible for enrolment. Patients were randomly assigned to receive CQ (Mekophar Chemical-Pharmaceutical Joint-Stock Company, Ho Chi Minh City, Viet Nam) or placebo. The regimen for CQ was 600mg base (4 x 150mg tablets) on enrolment to the study, then 600mg on day 2 and 300mg on day 3 (following the World Health Organization recommended treatment regimen for CQ susceptible *P. vivax*) [475]. Patients in the placebo arm received the same regimen of tablets (identical color and size). All treatment courses were contained in identical pre-packed bottles that were randomly assigned to patients via a computer-generated sequence of random numbers in blocks of 20 patients. A pharmacist generated the random sequence and was the only person who knew the content of each bottle. All patients, care providers and study investigators were blinded to treatment assignments. Physicians in the Hospital for Tropical Diseases were responsible for ensuring that the correct sequence of study codes, and therefore the treatment allocation, was followed. The study medication was given within 1 hr of a baseline blood sample being collected. Clinical care, including other treatments such as parenteral fluid therapy was at the discretion of the attending physician and following hospital guidelines. Case classification was according to 1997 WHO classification criteria and was applied to each case after review of study notes [476]. The Scientific and Ethical committee of the HTD and the Oxford Tropical Research Ethical Committee approved the study protocol and all patients gave written informed consent. The trial was registered with the ISRCTN Register (ISRCTN38002730).

2.2.2 Investigations

2.2.2.1 Clinical and laboratory investigations

Clinical history and examination findings were recorded daily into case record forms. An ultrasound was performed in all patients within 24hrs of defervescence. Venous blood samples were collected at hospital admission (prior to study drug administration), then twice daily (around 9am and 3pm) for a minimum of 5 days after starting treatment (defined as study day 1) and again 10–14 days after discharge

from the hospital. Plasma, for use in diagnostic investigations, was stored frozen in multiple aliquots at -80°C until use. A complete blood count, including hematocrit (Hct) and platelet measurements, was performed daily for all patients. Hct measurements were performed more frequently if clinically indicated. The extent of hemoconcentration during symptomatic illness was determined by comparing the maximum Hct recorded during hospitalization with either the value recorded at follow-up when available or against a sex-matched population value.

2.2.2.2 Adverse events (AEs)

AEs were defined as any unfavorable and unintended abnormal laboratory finding, symptom or disease that occurred during the course of the study, regardless of whether it was considered to be related to the intervention. AEs were classified as mild (grade 1), moderate (grade 2), severe (grade 3) and life-threatening (grade 4) according to the Common Terminology Criteria for Adverse Events from National Cancer Institute (Table 3). The relatedness of the AEs to study drug was investigated and graded as definitely, probably, possibly, unlikely to be, or not related.

Table 3: different AEs grades.

Mild or grade 1	Transient or mild symptoms; no limitation in activity; no intervention required. The AE does not interfere with the participant's normal functioning level.
Moderate or grade 2	Symptom results in mild to moderate limitation in activity; no or minimal intervention required. The AE produces some impairment of functioning, but it is not hazardous to health.
Severe or grade 3	Symptom results in significant limitation in activity; medical intervention may be required. The AE produces significant impairment of functioning or incapacitation.
Life-threatening or grade 4	Extreme limitation in activity, significant assistance required; significant medical intervention or therapy required; prolongation of hospitalization.

2.2.2.3 Dengue diagnostics

A diagnosis of “confirmed acute dengue” was based on results from either: 1/ capture IgM and IgG ELISA using DENV/JEV antigens and mAb reagents; 2/ internally-controlled and serotype-specific real time RT-PCR assay; 3/ NS1 ELISA and reached using several a diagnostic algorithm described in Fig. 15. The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA Units in early convalescent plasma samples taking into account the day of illness. The cut-off in IgG ELISA units for distinguishing primary from secondary dengue by day of illness was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed in the laboratory of Dr Sutee Yoksan using a reference IgM and IgG antigen capture ELISA described previously (the “AFRIMS ELISA”) [336].

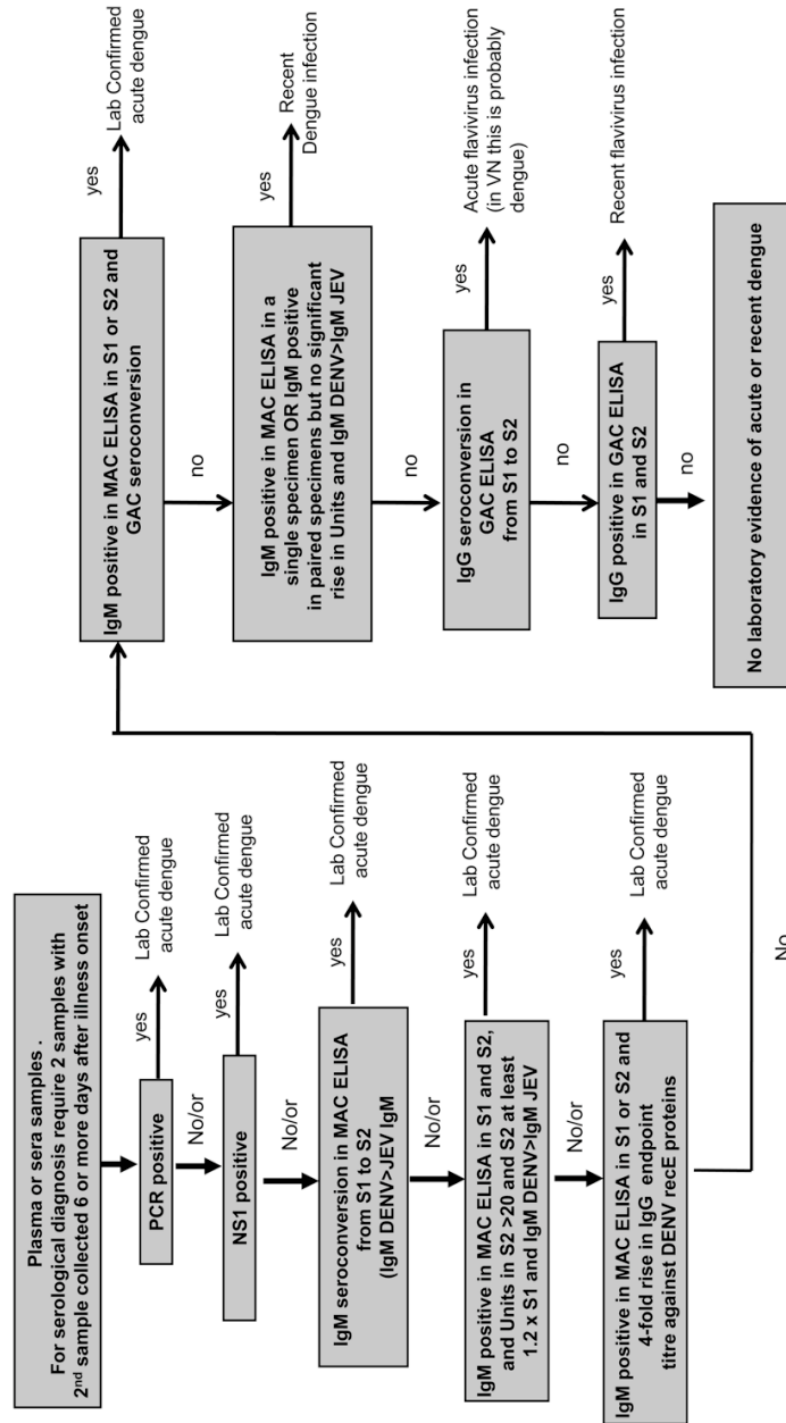


Figure 15: diagnostic algorithm used at OUCRU (HCMC, Viet Nam) to confirm dengue diagnosis [320]. A positive result in any of the first 6 tests is sufficient for a laboratory diagnosis of confirmed dengue. Abbreviations: JEV, Japanese encephalitis, recE recombinant DENV E proteins, MAC ELISA, IgM antigen capture ELISA; GAC ELISA, IgG antigen capture ELISA.

2.2.2.3.1 Detection of DENV-reactive IgM/IgG by MAC/GAC ELISA

Supernatant from DENV-1 to -4 or JEV infected C6/36 cells and anti-DENV or JEV mouse mAbs were supplied by Venture Technologies (Sarawak, Malaysia). Briefly, ELISA plates (Maxisorp, Nunc) were coated with anti-human IgM (A0425-Dako Company) or anti-human IgG (I2136-Sigma Company) with the dilution at 1:2000 overnight at 4°C. Plates were washed and then blocked with phosphate buffer saline (PBS) 3% Bovine Serum Albumin (BSA) for at least 2hrs at room temperature. Samples, positive and negative controls were diluted at 1:100 in PBS 0.05% Tween 20. After the 2nd wash, 100µl of diluted plasma and controls were added and incubated for 2hrs at room temperature. After washing five times, 100µl of antigen (pooled supernatants from C6/36 cultures of DENV-1 to -4 or of JEV) was added to each well and incubated at 4°C overnight. After washing, the assay was continued with a 1hr incubation of a cocktail of mouse mAbs to DENV-1 to -4 and JEV E proteins and then bound mAb detected by 100µl of a 1:2000 dilution of anti-mouse Ig Horseradish Peroxidase (HRP) (P260-DAKO Company). After washing, substrate o-phenylenediamine dihydrochloride (OPD) was used to develop and the colourimetric reaction was stopped by adding 10% H₂SO₄. Optical Density (OD) was read at 490nm. The IgM and IgG positive control was a pool of acute plasma from Vietnamese dengue patients. The negative control was a mixture of plasma collected from healthy adult Vietnamese blood donors with no evidence of having dengue at the time of blood collection. Wells with PBS in place of DENV antigen were used to define the background absorbance value. The cut-off value was defined as being 5 times higher than the mean OD of negative controls after subtracting background OD. The result of test samples was interpreted based on OD sample / cut-off ratio as negative if ratio < 0.8, equivocal if 0.8 ≤ ratio < 1.2 and positive if ratio ≥ 1.2.

2.2.2.3.2 DENV serotype identification and viremia quantification

Serotype was determined and DENV viremia in plasma was measured using an internally controlled, serotype-specific, real-time RT-PCR assay adapted from Laue et al and described in Simmons et al [263, 477]. Briefly, a diluted suspension of equine

arteritis virus (EAV) was added to each plasma sample prior to RNA extraction. The EAV served as an internal control for both RT and real time PCR. RNA extraction was automated (NucliSens easyMAG, BioMerieux, Marcy l'Etoile, France) and performed according to manufacturer's instructions. For each sample, we used 100µl plasma specimen and approximately 20µl EAV diluted suspension and then harvested 60µl stock of concentrated DENV/EAV RNA. RNA was then reverse transcribed to cDNA. The DENV serotype and EAV specific primers/probes used in the real-time PCR are given in Table 4. A linearized plasmid containing the cloned target amplicon was used to make a standard curve for each assay. Results were expressed as cDNA equivalents per ml of plasma. Sample measurements were only valid when there was a detectable signal from the internal control amplicon (Ct value range from 30-35) and were considered as positive if above the assay limit of detection defined as the last dilution of standard that gave a specific signal. The detection rates and the intra-experimental reproducibility of our qPCR assay are given in Table 5.

Table 4: Dengue and EAV specific primer sequence and probes.

DENV	Sequences (5'-3')	
	Primer Forward	Primer Reverse
1	ATCCATGCCCACCAAYCAATG	GATCARTGGTGTGGATCCCTG
2	ACAAGTCGAACAACCTGGTCCAT	GAGAAGACCAATGGTGCGGC
3	TTTCTGCTCCCACCACTTTCAT	AGACTRGCATCCAACGCCA
4	GYGTGGTGAAGCCYCTRGAT	ATGAAGGATGGCCGYTCACT
EAV	CATCTCTTGCTTTGCTCCTTA	AGCCGCACCTTCACATTG
	Probes	
1	6-FAM TCAGTGTGGAATAGGGTTTGGATAGAGGAABHQ-1	
2	6-FAM GTT+T+Tg+T+CT+TC+CA+TCCA-BHQ-1 ^a	
3	6-FAM AAGAAAGTTGGTAGTTCCTGCAGACCCCA BHQ-1	
4	6-FAM ACTTCCCTCCTCTTYTTGAACGACATGGGA BHQ-1	
EAV	Cy5 GCGCTCGCTGTCAGAACAACATTATTGCCACAGCGCG BHQ-1	

^a The plus sign indicates that the subsequent nucleotide is a locked nucleic acid.

Table 5: Detection rates and intra-experimental reproducibility of the qRT-PCR assay.

Standard dilution (in cDNA Eq/mL)	DENV-1		DENV-2		DENV-3		DENV-4	
	Ct mean (SD)	Detection Rate (%)	Ct mean (SD)	Detection Rate (%)	Ct mean (SD)	Detection Rate (%)	Ct mean (SD)	Detection Rate (%)
1.5E+9	20.73 (0.71)	100	17.53 (0.51)	100	19.98 (1.10)	100	22.34 (1.63)	100
1.5E+8	24.55 (0.75)	100	21.28 (0.50)	100	24.06 (1.01)	100	26.21 (0.95)	100
1.5E+7	28.14 (0.74)	100	25.02 (0.67)	100	28.15 (0.94)	100	30.05 (1.87)	100
1.5E+6	31.23 (0.58)	100	28.56 (0.58)	100	31.84 (0.90)	100	33.48 (2.10)	100
1.5E+5	33.81 (0.51)	100	32.02 (0.64)	100	35.11 (0.68)	100	36.48 (1.49)	100
1.5E+4	36.28 (1.06)	100	35.12 (0.73)	100	38.02 (0.73)	100	38.45 (N/A)	50
1.5E+3	38.09 (1.12)	24	37.77 (1.06)	88	39.59 (1.26)	44	N/A	0

2.2.2.3.3 DENV NS1 detection

NS1 was detected by using the NS1 Platelia assay from BioRad (Hercules, CA) according to the manufacturer's instructions. Plasma was diluted 1:2 in assay diluents before processing. A 100µl of diluted plasma was mixed with conjugate and then added to pre-coated wells for 30 minutes incubation at 37°C. Assay was developed with TMB subtract. The optical density was read with dual wavelength at 450/620 nm within 30 minutes after stopping the enzymatic process.

2.2.3 Outcome assessment

2.2.3.1 Primary outcomes

The primary endpoints were the time to resolution of viremia and the time to resolution of NS1 antigenemia. Time to resolution of viremia was defined as the time from the start of treatment until the first of two consecutive RT-PCR negative plasma samples. Time to resolution of NS1 antigenemia was defined as the time from the

start of treatment until the first of two consecutive NS1 ELISA negative plasma samples. Patients who did not reach viremia or NS1 antigenemia clearance were treated as censored at their last date of viremia or NS1 antigenemia measurement.

2.2.3.2 Secondary endpoints

The fever clearance time (FCT) was defined as the time from the start of treatment to the start of the first 48hrs period during which axillary temperature remained below 37.5°C. Other pre-defined secondary endpoints were- a) the median nadir platelet count, b) the mean maximum % hemoconcentration (calculated as (maximum Hct recorded during the inpatient period) – (Hct at follow-up when available or a sex-matched population value) / (Hct at follow-up when available or a sex-matched population value) x 100), c) the proportion of patients who were treated with intravenous fluid (the decision was based in clinical signs and conducted according to HTD protocols ; briefly, intravenous fluids were given if the attending physician believed treatment was necessary because of persistent vomiting, gastrointestinal bleeding, hemoconcentration or hypotension), d) the proportion of patients in each arm classified as having DHF, e) the proportion of patients in each arm with grade 3 or 4 AEs that were probably or definitely related to the intervention (Table 1), f) the proportion of patients in each arm with one or more episodes of vomiting and g) the proportion of patients in each arm with bleeding that required blood transfusion.

2.2.4 T cell and cytokine investigations

To determine the optimal time point for cytokine measurement, levels of IL-1 β , IL-6, IL-8, IL-10, IL-12p70, and TNF- α were measured on serial plasma samples from 39 patients by using a CBA Human Inflammatory Cytokines kit (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions (except that all samples were fixed in 4% paraformaldehyde before being analyzed). Subsequently, a luminex-based Bio-Plex system (Bio-Rad Laboratories, Hercules, CA) was used according to the manufacturer's instructions to measure simultaneous plasma levels of IL-2, IL-4, IL-6,

IL-8, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF), INF- γ , and TNF- α in one plasma sample from each patient. Flow-cytometric analysis of whole-blood samples stained with fluorochrome-conjugated monoclonal antibodies (CD3-Cy, CD4-PE-Cy7, CD8-PE, CD38-FITC, HLA-DR-PerCP and Ki67-FITC) was performed by use of a FACScalibur flow cytometer (Becton Dickinson (BD)). Cell-surface staining was routinely performed on 150 μ L of fresh whole blood. All antibodies were purchased from BD. Whole-blood samples from healthy volunteer subjects were used as group control.

2.2.5 Sample size calculation

Assuming a median time from enrolment to resolution of viremia or NS1 antigenemia in the placebo group of 72hrs and a reduction of this time by 24hrs due to CQ treatment (corresponding to a hazard ratio (HR) of 0.67 assuming an exponential distribution of the resolution times), we would need to observe viremia or NS1 antigenemia resolution in 191 patients to show such an effect with 80% power at the two-sided 5% significance level. Assuming sufficient follow-up to observe viremia or NS1 antigenemia resolution in 90% of patients, we would need to include at least 213 patients with confirmed dengue.

2.2.6 Statistical methods

The statistician was unblinded for the data analysis. Data stayed blinded until the database was cleaned and locked ready for data analysis. All statistical analyses were performed using Intercooled STATA version 9.2 (StataCorp, TX). A two-sided p-value ≤ 0.05 was considered significant for all parameters. The intention-to-treat (ITT) population was defined as all subjects who were randomized regardless of whether or not they began the treatment regimen. All laboratory confirmed dengue patients completing the expected number of days of treatment who fulfilled the inclusion/exclusion criteria of the protocol and who did not leave before the end of the study drug course formed the per-protocol (PP) population. Secondary endpoints

(except the FCT) were compared between the 2 groups and analyzed using the Kruskal-Wallis test for continuous variables and the Fisher's exact test for categorical variables. For the primary endpoints and the FCT, the null hypothesis is that CQ has no effect on duration of DENV viremia, NS1 antigenemia and fever. Survival analysis using the Kaplan-Meier (KM) method and log-rank test was used for all time-to-event outcomes. Cox regression was used to quantify the difference in risk between treatment groups and to adjust for all the following baseline variables: time since illness onset at enrolment, serological status, serotype (DENV-1 vs other), viremia and temperature. Because these covariates were thought to influence the time to resolution of viremia, the time to resolution of NS1 antigenemia and the FCT, all were retained in the final adjusted models. The proportional hazards assumptions were checked using a test based on Schoenfeld residuals.

2.3 Results

2.3.1 Baseline characteristics of enrolled patients

Between May 2007 and July 2008, 307 adults with suspected dengue were randomized to CQ or placebo (Fig. 16). Of these 307 patients, 257 had laboratory confirmed dengue and 50 had no evidence of recent or acute dengue. All patients recovered fully. The baseline characteristics of the study population are summarized in Tables 6 and 7. Baseline characteristics were generally well-balanced between the two groups except for baseline viremia which tended to be higher in the CQ group (median 9.04 vs 8.52 Log₁₀ copies/mL) and the proportion of DENV3 infected patients, which was lower in the CQ arm (11.3% CQ vs 21.8% placebo).

2.3.1 Primary endpoints

2.3.1.1 DENV viremia clearance times

There were 248 patients viremic at enrolment in the ITT population and 239 in the PP population (Tables 6 and 7). Viremia clearance times were not significantly different

in the CQ arm compared to the placebo arm for either the ITT or PP analysis (Fig. 17A and B) (ITT HR 0.80, 95% confidence intervals (CI) 0.62-1.05, log rank test P=0.10 and PP HR=0.80, 95% CI 0.61-1.05, log rank test P=0.11). Median times to resolution of DENV viremia were similar in the ITT and PP population: ITT 77.5hrs and PP 78hrs (ITT inter-quartile range (IQR) 53-100hrs and PP IQR 66-100.5hrs) for CQ arm and both ITT and PP 71hrs (IQR ITT 48-94.5hrs and PP 48-95.5hrs) for Placebo arm. Adjusting for baseline covariates did not alter these findings (ITT HR=0.95, 95% CI 0.72-1.26 and PP HR=0.94, 95% CI 0.71-1.25). Of the 24 patients still qRT-PCR positive at discharge (median discharge time for those patients = 5 days since enrolment) only 14 presented at follow-up and none were qRT-PCR positive (median follow up time for those patients = 13 days after enrolment).

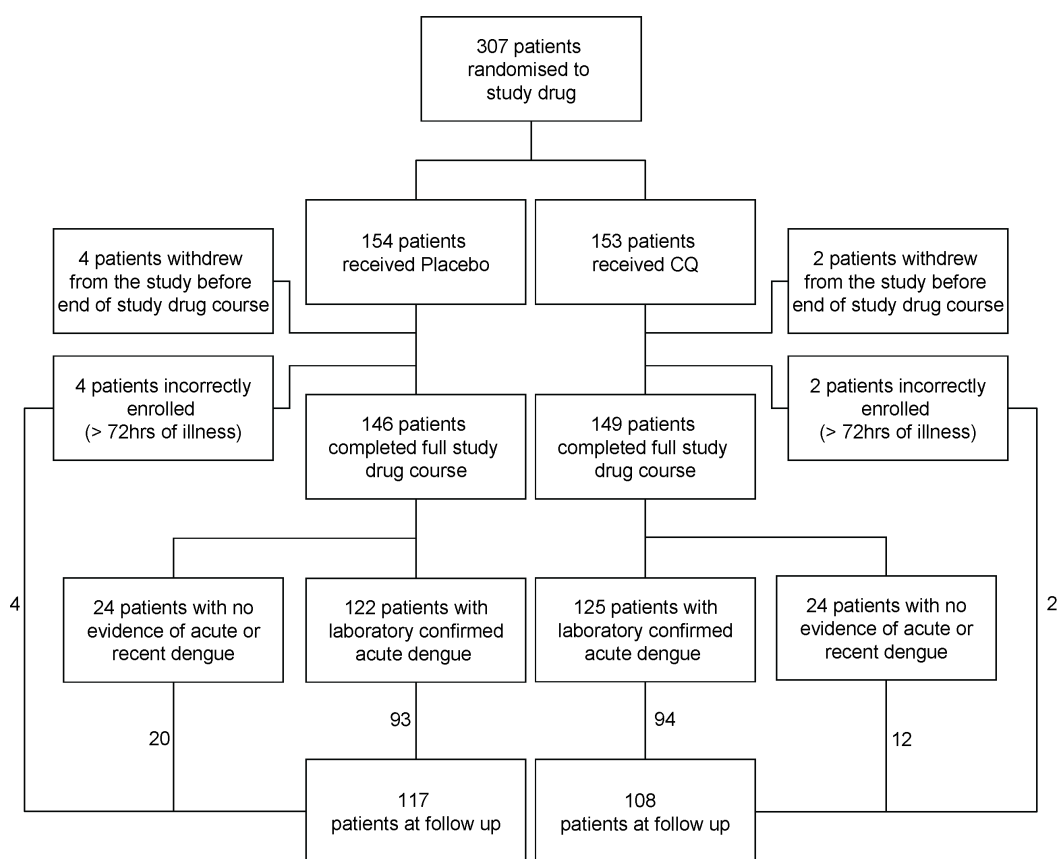


Figure 16: Participant flow in the randomized controlled trial of CQ vs Placebo.

Table 6: Baseline characteristics in the intention-to-treat population.

Variables	CQ group (N=153)	Placebo group (N=154)
	N (%) or Median (interquartile range)	
Age (years)	22 (18-27)	22 (19-28)
Male sex	104 (68.0%)	106 (68.8%)
Dengue confirmed	128 (83.7%)	129 (84.3%)
Viremic	124 (81.0%)	124 (80.6%)
Infecting serotype:		
DENV-1	80 (64.5%)	67 (54.0%)
DENV-2	26 (21.0%)	27 (21.8%)
DENV-3	14 (11.3%)	27 (21.8%)
DENV-4	4 (3.2%)	3 (2.4%)
Viremia (log10 copies / mL of plasma):		
All serotypes	9.0 (8.0-9.6) ^a	8.5 (7.6-9.3)
DENV-1	9.2 (8.3-9.8)	8.9 (7.9-9.7)
DENV-2	8.3 (7.0-9.4) ^a	7.7 (6.3-8.6)
DENV-3	8.3 (7.6-9.1)	8.2 (7.4-9.3)
DENV-4	7.7 (6.9-8.7)	7.7 (6.8-8.1)
Serological status:		
Primary	21 (16.4%)	12 (9.3%)
Secondary	105 (82.0%)	110 (85.3%)
Ambiguous	2 (1.6%)	7 (5.4%)
NS1 ELISA positive	113 (73.9%) ^b	110 (71.4%)
Febrile	150 (98.0%) ^c	147 (95.5%) ^c
Temperature (°C)	39.3 (38.7-40)	39.5 (38.8-40)
Time since illness onset (hrs)	49 (40-56)	48 (40-57)
Platelet count at enrolment	129,000 (95,000-168,000)	125,000 (91,000-167,000)

^a Baseline viremia value missing for 1 patient in the CQ arm.

^b Two patients NS1 negative at enrolment became positive later (both in the CQ arm).

^c Three patients afebrile at enrolment later developed fever (1 in the CQ arm and 2 in the Placebo arm).

Table 7: Baseline characteristics in the per-protocol population.

Variables	CQ group (N=125)	Placebo group (N=122)
	N (%) or Median (interquartile range)	
Age (years)	21 (15-45)	21 (15-54)
Male sex	82 (65.6%)	83 (68.0%)
Viremic	121 (96.8%)	118 (96.8%)
Infecting serotype:		
DENV-1	78 (64.5%)	66 (55.9%)
DENV-2	25 (20.7%)	24 (20.3%)
DENV-3	14 (11.6%)	25 (21.2%)
DENV-4	4 (3.3%)	3 (2.5%)
Viremia (log₁₀ copies / mL of plasma):		
All serotypes	9.0 (8.0-9.6) ^a	8.5 (7.6-9.3)
DENV-1	9.2 (8.3-9.8)	8.9 (7.9-9.6)
DENV-2	8.5 (7.4-9.4) ^a	7.7 (6.8-8.6)
DENV-3	8.3 (7.6-9.1)	8.2 (7.4-9.3)
DENV-4	7.7 (6.9-8.6)	7.7 (6.8-8.1)
Serological status:		
Primary	21 (16.8%)	12 (9.8%)
Secondary	103 (82.4%)	110 (86.9%)
Ambiguous	1 (0.8%)	7 (3.3%)
NS1 ELISA positive	111 (88.8%) ^b	104 (85.2%)
Febrile	123 (100%)	117 (95.9%) ^c
Temperature (°C)	39.3 (37-41)	39.5 (37-41)
Time since illness onset (hrs)	49 (14-72)	48 (9-72)
Platelet count at enrolment	128,000 (25,000-345,000)	117,000 (19,000-234,000)

^a Baseline viremia value missing for 1 patient in the CQ arm.

^b Two patients NS1 negative at enrolment became positive later (both in the CQ arm).

^c Two patients afebrile at enrolment later developed fever (both in the Placebo arm).

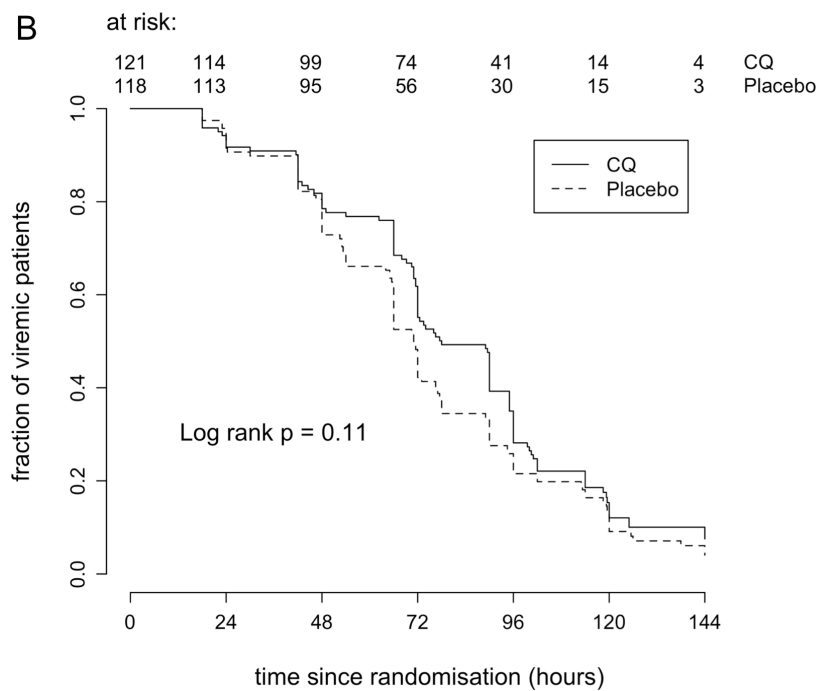
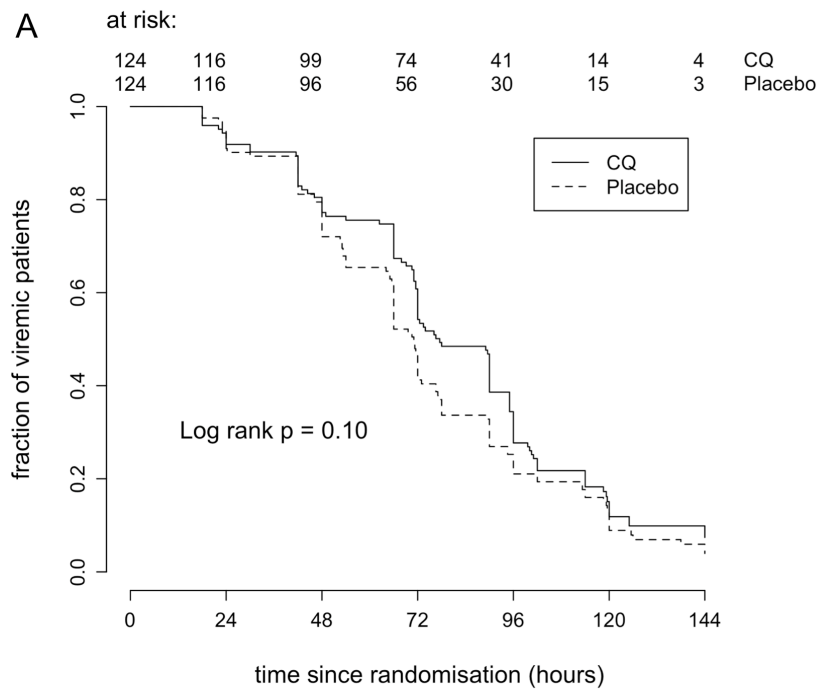


Figure 17: Time to resolution of viremia. Kaplan – Meyer survival analysis of time to resolution of plasma viremia by treatment group (CQ or placebo) and population; **A)** Intention to treat population and **B)** Per Protocol population.

2.3.1.2 Time to negative NS1 antigenemia

In the ITT population, there were 223 (72.6%) patients NS1 positive at the time of enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42hrs later) (Table 6). Time to resolution of NS1 antigenemia was not significantly different between CQ and placebo arms (Fig. 18A) (HR=1.07, 95% CI 0.76-1.51, log rank test $P=0.70$). Adjusting for baseline covariates did not alter these findings (HR=1.18, 95% CI 0.82-1.68). Median times to resolution of NS1 antigenemia were 96hrs (IQR 65.5-115hrs) in the CQ arm and 94.5hrs (48-120hrs) in the placebo arm. There were 96 patients still NS1 ELISA positive at discharge. This suggests that NS1 antigenemia is relatively long lived. Moreover 17 patients (27% of patients NS1 positive at discharge and with a follow-up sample) were still NS1 positive at follow-up (time range: 10.7-14 days after enrolment). In the PP population, there were 215 (87.0%) patients NS1 positive at the time of enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42hrs later) (Table 7). Time to resolution of NS1 antigenemia was not significantly different between the 2 arms (Fig. 18B) (HR=1.14, 95% CI 0.80-1.63, log rank test $P=0.47$). Adjusting for baseline covariates did not alter these findings (HR=1.19, 95% CI 0.83-1.71). Median times to resolution of NS1 antigenemia were 96hrs (IQR 66-116hrs) and 96hrs (54-120hrs) respectively for CQ and placebo arms.

2.3.1 Secondary endpoints

2.3.1.1 Fever clearance times

In the ITT population, there were 297 patients febrile at enrolment (plus 3 afebrile who developed fever soon after) (Table 6). FCTs were significantly shorter in the CQ arm compared to the placebo arm (HR=1.37, 95% CI 1.08-1.74, log rank test $P=0.01$ but there was a trend that the hazards were non-proportional $P=0.07$) (Fig. 19A). However, when adjusted for baseline covariates, the rate of fever clearance among patients who received CQ was not different from patients who received placebo (HR=1.16, 95% CI 0.89-1.51, $P=0.28$). Median FCTs were 69hrs (IQR 45-93hrs) and 75hrs (IQR 36.5-99hrs) respectively for CQ and placebo arms.

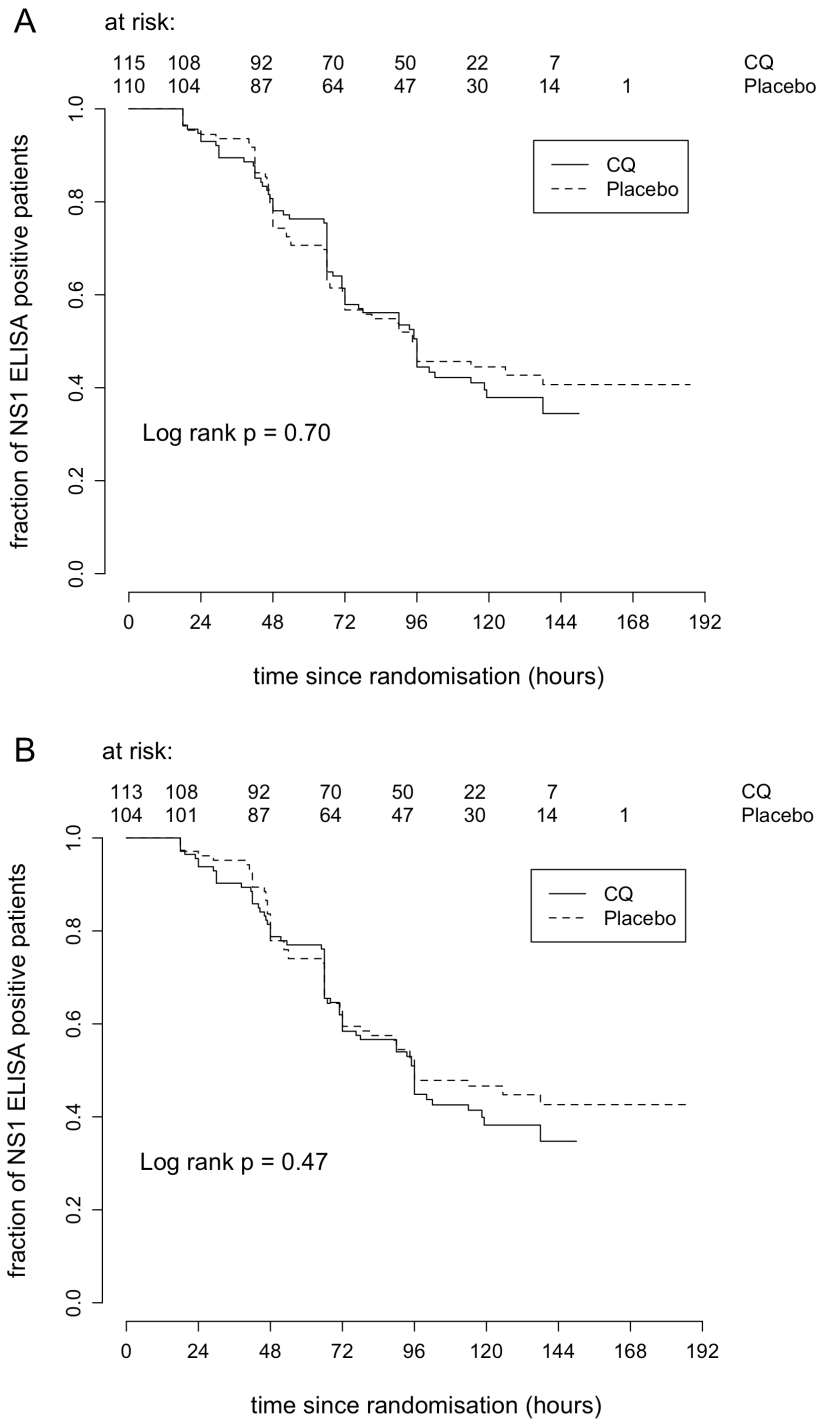


Figure 18: Time to negative NS1 antigenemia. Kaplan – Meyer survival analysis of time to resolution of NS1 antigenemia by treatment group (CQ or placebo) and population; **A**) Intention to treat population and **B**) Per Protocol population. Two patients NS1 negative at enrollment were later positive (both in the CQ arm) and for the purposes of analysis were considered positive at the time of enrolment.

There were 240 patients febrile at enrolment (plus 2 afebrile who developed fever soon after) in the PP population (Table 7). FCTs were not different between the 2 groups (HR =1.24, 95% CI 0.96-1.60, log rank test P=0.10) (Fig. 19B). Adjusting for baseline covariates did not alter these findings (HR=1.28, 95% CI 0.98-1.68, P=0.07). Median FCTs were 69hrs (IQR 45-93hrs) and 76hrs (IQR 46-99hrs) respectively for CQ and placebo arms.

2.3.1.1 Platelet nadir and maximum hemoconcentration

The median nadir platelet count was the same in the CQ and placebo arms 45,000 (IQR 25,000-60,000) (Mann-Whitney P=0.61, PP population). There were also no significant differences between the two arms in the mean level of hemoconcentration detected (10.8% (8.9-12.7) and 12.2% (10.3-14.1) for the CQ and placebo respectively (Mann-Whitney P=0.27, per protocol).

2.3.1.2 DF vs DHF in each arm

There was a trend, though not significant (P=0.09), towards fewer patients with DHF in the CQ arm. There were 29 patients (23.2%) with DHF in the CQ arm compared to 41 (33.6%) in the Placebo arm (odds ratio (OR) 0.60, 95% CI 0.34 – 1.04, P=0.07). As the infecting DENV serotype might influence clinical severity, and at baseline the two arms differed in the prevalence of each serotype, the analysis of the effect of CQ on disease severity was also adjusted for serotype by logistic regression, but this did not alter these findings.

2.3.1.3 Adverse events

Two patients in the CQ arm developed severe adverse events (both grade 3) that were possibly related to CQ. One patient with hematemesis was admitted to the ICU for 3 days, with stable vital signs. The 2nd patient was anorexic and vomiting with a narrow pulse pressure (100/80 mmHg) and was admitted to the ICU for 3 days. There were no severe AEs in the placebo arm. Significantly more adverse events occurred in the CQ arm: 18 patients reported a total of 33 AEs versus 6 patients with 8 AEs in the placebo arm (Fisher's exact test $P=0.01$). The most common adverse event was vomiting (~51% of all grade adverse events).

2.3.1.4 Number of patients requiring fluid and blood transfusion

Thirty-two patients (21 in CQ arm and 11 in placebo arm) required parenteral crystalloid fluid therapy during their hospitalization (for rehydration, and/or maintenance) but none required blood transfusion. There was no significant difference between the 2 groups in the need for fluid therapy ($P=0.11$ in the PP population and $P=0.06$ in the ITT population).

2.3.2 T cell activation in peripheral blood of study participants

Given the clinical experience of using CQ therapy in inflammatory autoimmune disorders, we investigated whether CQ was associated with a measurable attenuation of the T cell response. To this end, the activation state of peripheral blood $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was assessed in fresh whole-blood at the time of enrolment, on illness day 6, and again at follow-up in 172 consecutive patients enrolled in the study between September 07 and June 08 (85 in CQ arm, 87 in placebo arm), amongst whom there were 147 laboratory-confirmed dengue patients. The activation markers used were CD38, HLA-DR and Ki-67. As a reference, we also phenotyped T cells in fresh whole blood from 9 healthy adult volunteers. Strikingly, in dengue patients we observed a large population of surface-activated ($CD38^+$ or $HLA-DR^+$) and

proliferating (Ki-67⁺) CD8⁺ T cells at early convalescence that were mostly absent at the time of enrolment and follow-up (Fig. 20). There was no evidence however of a significant difference in the proportion of activated T cells in patients treated with CQ or placebo.

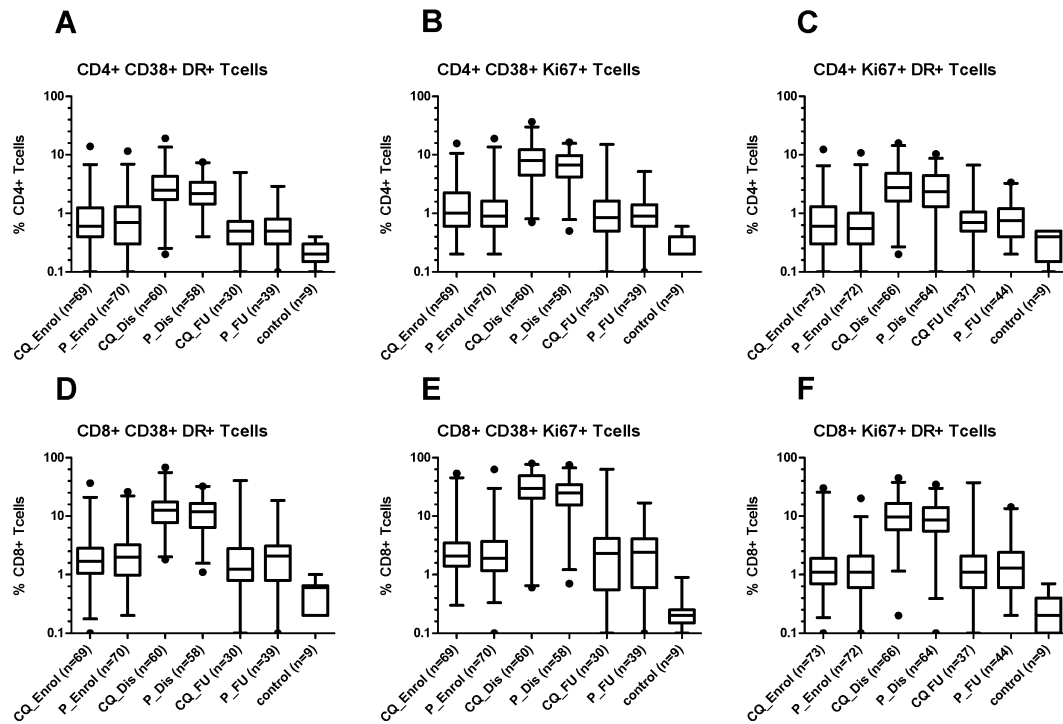


Figure 20: Surface phenotypes of CD4⁺ and CD8⁺ T cells in laboratory-confirmed dengue patients randomized to placebo or CQ. The Box and Whisker plots show the median number and range (2.5–97.5 percentile) of percentages of surface-activated T cells in peripheral blood from CQ (n = 74) and Placebo (n = 73) treated laboratory-confirmed dengue patients at different time points. The median illness day (range) for enrolment samples was 2 (0–3) days, for hospital discharge was 6 (4–8) days and for follow-up was 15.5 (13–30) days. Shown are percentages of peripheral blood CD4⁺ T cells that were A) CD38+HLA-DR+, B) CD38+Ki67+, and C) Ki67+ HLA-DR+. Also shown are percentages of CD8⁺ T cells that were, D) CD38+, HLA-DR+, E) CD38+Ki67+, and F) Ki67+ HLA-DR+. The labels below the graphs indicate the time at which sample collection occurred.

2.3.3 Plasma concentrations of cytokines/chemokines

To understand if CQ modulated the cytokine response to DENV infection, plasma concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF- γ , and TNF- α were measured in plasma from 234 laboratory-confirmed dengue patients (121 in CQ arm, 113 in placebo arm) 2 or 3 days after randomization (Fig. 21). However, there was no significant difference in plasma concentrations of any of these cytokines between CQ or placebo treated patients (Mann-Whitney $P > 0.1$).

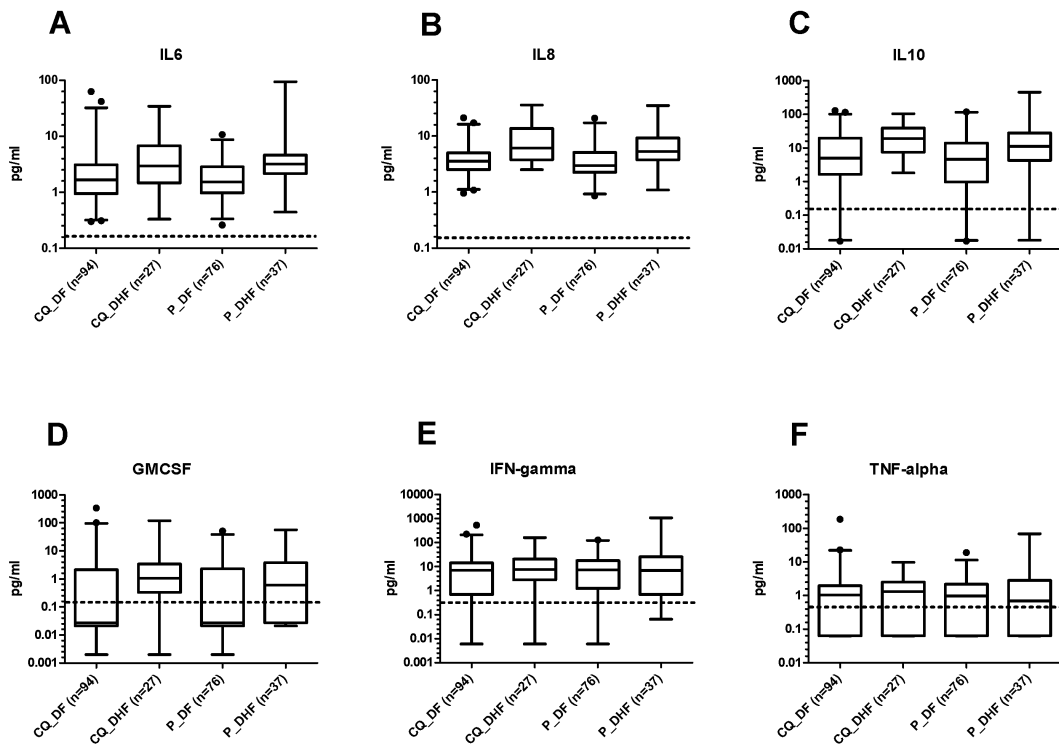


Figure 21: Plasma concentrations of pro- and anti-inflammatory cytokines in laboratory-confirmed dengue patients randomized to placebo or CQ. The Box and Whisker plots show the median and range (2.5th–97.5th percentile) of plasma concentrations of A) IL-6, B) IL-8, C) IL-10, D) GM-CSF, E) IFN- γ , and F) TNF- α from DF and DHF patients treated with CQ or Placebo. The dashed line represents the assay limit of detection. Concentrations of IL-2 and IL-4 are not shown because they were below the limit of detection.

2.4 Discussion

There are no specific therapies for treating dengue. This controlled trial was conducted to determine if CQ could reduce the viral burden in dengue patients. We found no evidence that CQ reduced the duration of viremia or NS1 antigenemia in adult dengue patients, but did observe a modest anti-pyretic activity of CQ in the intention to treat population, but not in dengue laboratory-confirmed cases. CQ was associated with a higher frequency of adverse events compared to placebo, but these were generally mild. There was no evidence that CQ reduced the magnitude of cytokine or T cell responses to DENV infection.

To our knowledge the only previous therapeutic trial of CQ for an acute viral infection has been in a small number of patients with Chikungunya virus infection [478], in which CQ had no impact on either duration of febrile arthralgia or viremia. Several possible reasons could explain the lack of measurable activity in this study of CQ against virological markers of DENV infection in vivo. Although the C_{max} of CQ inside cells approximates the IC_{50} value of CQ against DENV in vitro, it is possible that CQ does not achieve inhibitory concentrations inside the reticuloendothelial cells where DENV replication is believed to occur [479]. Furthermore, it may not achieve the same pH modulation in vivo that is postulated to explain its activity on cultured virus in vitro.

Alternative trial designs and protocols, such as increasing the therapeutic dose, dosing patients earlier in their illness or increasing the sample size substantially might increase the chances of observing an in vivo effect by CQ on the duration of DENV viremia and NS1 antigenemia. The importance of treating early is highlighted by the fact that in this trial the median duration of illness prior to treatment was relatively short (~48 hrs) and the median viremia clearance times after treatment were ~3.75 days in the CQ arm and ~3 days in the placebo arm. Strikingly however, the duration of NS1 antigenemia was relatively long, with as many as 92/243 (38%) of dengue patients still NS1 positive at the time of discharge from hospital, although most of this antigen is probably generated in the first few days of illness and its prolonged

clearance simply reflects its large, oligomeric structure [480, 481]. The time to resolution of NS1 antigenemia may therefore not be an optimal endpoint and an alternative approach could have been to compare the proportion of patients that were positive at a single post-therapy timepoint (e.g. study day 5). Collectively, these data underscore that there is only a brief therapeutic window of opportunity to improve upon the host's virus-eliminating immune response. Encouragingly however, strategies to diagnose patients very early in their illness are available in the form of NS1 rapid diagnostic tests [320, 482-484] and these could in principal guide rational treatment with an anti-viral or other intervention as early as 24-48hrs into the illness course. Of additional value, but not yet identified, would be early prognostic markers of severe outcome, so that interventions can be delivered to those patients at higher risk.

A CQ-mediated anti-pyretic effect equal to paracetamol has been shown during treatment of uncomplicated *P. falciparum* malaria [473, 485, 486]. This effect may be explained by CQ's anti-inflammatory properties, including CQ effects on TLR signaling [487, 488]. Fever during an infection is thought to be initiated by virtually immediate cyclooxygenase-2, prostaglandin E2 (PGE2) production, activation of hypothalamic PGE2 receptors and then cytokines and TLR ligand activity [489]. It is reasonable to believe that CQ mediates an anti-pyretic effect by altering the levels and balance of these pyretic mediators during infection. Accordingly, we found a small reduction in fever clearance median times (~6 hrs) amongst CQ patients in the intention-to-treat patient population, and whilst a similar trend was observed amongst the dengue confirmed patients, it was not statistically significant. CQ might be a better anti-pyretic in non-dengue patients in this study because these patients had milder infections, albeit of unknown origin.

Fewer patients receiving CQ developed DHF. The intriguing possibility that CQ mediated an anti-disease effect, but not a measurable anti-viral effect in this trial is plausible given the literature on CQ as a pleotropic immune-modulatory drug. To find support for this possibility we measured pro- and anti-inflammatory plasma cytokine concentrations and T cell activation markers in dengue patients. Of particular interest were vasodilatory and pyretic cytokines such as TNF- α that have been identified as

susceptible to CQ modulation [467, 490] and important in the pathogenesis of the dengue capillary leak syndrome [491]. Similarly, the magnitude of T cell activation has been postulated to be associated with dengue severity [492]. Whilst robust T cell activation and cellular proliferation was indeed present around the time of defervescence, there was no evidence of a difference between CQ and placebo arms for the cellular markers we investigated nor in the cytokines that were measured. The absence of a measurable impact by CQ on these elements of the host response might suggest any trend towards less DHF in the CQ arm is simply chance or reflects our inability to identify and measure true immunological correlates of disease. Only further large trials, with clinical endpoints, will determine if CQ has a disease modulating effect.

Our study had several limitations. The study was hospital-based and therefore the patient population, although presenting early in their illness, may not reflect that seen in primary health care settings where milder infections might be expected. The study was performed in adults, who generally compensate well for capillary permeability, and it's plausible that different findings might be observed in children, who in most endemic settings carry much of the disease burden. We measured viremia by quantitative RT-PCR as a surrogate and well-characterised marker of infection though we recognise this is not that same as a quantitative biological assay of infectious virus. Finally, we did not formally conduct pharmacokinetic analysis of CQ in treated patients and this could have aided the interpretation of the final outcomes.

There is growing interest in the potential for anti-viral therapies for dengue [493, 494]. This study illuminated several important issues in the design of anti-viral interventions trials. Most striking is the rapid decline in the DENV viremia beginning ~72hrs into the illness, highlighting the fact that anti-viral interventions will likely need to be delivered very early and aggressively, preferably guided by cheap, sensitive and specific diagnostics. NS1 is a useful and easily assayed biomarker of DENV infection and in the context of a trial it conceivably provides a slightly different insight into virus infection than is given by measurement of viral RNA in plasma. In early phase trials, measurement of virological and clinical markers at multiple time-points per day is strongly recommended given the speed of viral

clearance and evolution of disease. In later phase trials, the choice of clinical endpoints will depend on the target patient population and the setting. In children, single or combination endpoints around dengue shock syndrome, the most common life-threatening complication in children, should be considered. In adults, other complications such as severe bleeding may also be relevant.

In summary, this study suggests CQ has no measurable impact on virological or immunological parameters of DENV infection in young adults. We also found no convincing evidence that CQ reduces the time to fever resolution in adults with dengue. Interventions with either more potent anti-viral molecules and/or immunomodulatory drugs are needed to improve clinical outcomes for patients in endemic settings.

Chapter 3 : Comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viremia and antibody responses

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3.1 Background

There are several reasons why early and accurate diagnosis of dengue is important. First, an early and accurate diagnosis can assist in patient management by directing clinical attention to the appearance of major warning signs of severe or even life threatening complications, e.g. rapidly rising Hct, poor peripheral perfusion. Second, an accurate dengue diagnosis prevents unnecessary and possibly expensive antibiotic usage. Third, prompt diagnosis of index cases can facilitate vector control activities in the community so as to mitigate further transmission. Fourth, the expanded use of accurate dengue diagnostics provides important data on the epidemiology and health burden of dengue and in doing so can inform and guide public health policy, particularly as dengue vaccines and anti-virals make their way through development pipelines. Lastly, early detection will be necessary prior treatment by anti-viral compound(s) when available. Commercial ELISA tests that detect the DENV NS1 protein in plasma/sera have provided a new avenue for diagnosing dengue [319, 324-326, 495-498]. The detection of NS1 on rapid lateral flow point-of-care tests offers an even faster route to a presumptive dengue diagnosis [320]. As more point-of-care RDTs for dengue, particularly those targeting NS1, reach the marketplace their prices will likely drop to the point they are affordable for use in even resource limited health-care settings. The example of RDTs for malaria that target HRP2 provides a useful example of a point-of-care antigen-detection test where a large number of manufacturers in the market have resulted in cheaper tests such that they are being promoted in even the most resource limited settings [499].

In the context of RCTs of dengue antivirals and after the study described in chapter 2, we were looking for a cheap, easy and fast but accurate way to include higher proportions of dengue laboratory-confirmed cases. Therefore, it has been decided to evaluate how NS1 rapid tests can contribute to better RCTs. Indeed, if specific and reasonably sensitive, they would make possible the laboratory diagnosis of dengue at the same time than patient inclusion allowing consequently the inclusion of only laboratory-confirmed cases. They would also make the inclusion easier then facilitating larger RCTs targeting non-hospitalized patients. In 2009, the available RDTs for the detection of NS1 Ag were Bio-Rad Dengue NS1 Ag Strip and Standard Diagnostics (SD) Dengue Duo. We thus evaluated the sensitivity and specificity of these 2 tests for the detection of NS1 in a panel of plasma samples from dengue patients with different viral serotypes and viremia levels. As The SD Dengue Duo is distinguished from the Bio-Rad NS1 Ag Strip in that it also tests for DENV IgM and IgG, we also took this opportunity to evaluate the contribution of the IgM/IgG testing part to the diagnosis of dengue. These 2 RDTs were also compared because the SD Dengue Duo was much cheaper than the Bio-Rad tests and, in case of similar performances, the SD tests would have been preferred.

3.2 Materials and methods

3.2.1 Patient samples

The panel of plasma samples used in this study was from patients enrolled in the DENCO study, a multi-centre descriptive study of dengue conducted at the Hospital for Tropical Diseases, Paediatric Hospital #1 and Paediatric Hospital #2 in Ho Chi Minh City, Viet Nam from August 2006 to May 2007. Following written informed consent by the study participant, or a parent/guardian in the case of children, patients above 6 months of age with clinically suspected dengue and fever for less than 7 days were enrolled in the study. Ethical approval was obtained from the Ethics Review Committee of the Hospital for Tropical Diseases, Paediatric Hospital #1 and #2. Two plasma or sera samples were collected from each patient, one at day of enrolment and the second 7-14 days after fever onset.

3.2.2 Dengue diagnostics and the reference algorithm

No single diagnostic assay can diagnose all dengue patients at the various times they may present with symptoms. Consequently, a diagnosis of “confirmed acute dengue” was reached using an algorithm previously described [320] and based on 3 assays; RT-PCR detection of DENV RNA in plasma, and changes in DENV-reactive IgM and IgG levels in paired plasma specimens (see chapter 2). In brief, a diagnosis of “lab-confirmed dengue” was made if there was a clinical suspicion of dengue and, a) the RT-PCR assay was positive, b) DENV-IgM seroconversion (i.e. from negative to positive) occurred between paired specimens, c) levels of DENV reactive IgM increased significantly between paired specimens and were very high in the 2nd sample (at least 20% increase in DENV-IgM ELISA Units from 1st to 2nd sample and 2nd sample has at least 20 ELISA Units), d) there was a four-fold rise in IgG titre to recombinant DENV E proteins measured in indirect ELISA in the presence of significant DENV IgM levels or e) IgG seroconversion was demonstrated in the IgG capture ELISA in the presence of significant DENV IgM levels. MAC and GAC ELISA, using DENV/JEV antigens and mAb reagents provided by Venture Technologies (Sarawak, Malaysia), were performed as previously described [500]. The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA units in early convalescent plasma samples taking into account the day of illness. The cut-off in IgG ELISA units for distinguishing primary from secondary dengue by day of illness was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed at the Centre for Vaccine Development, Mahidol University, Bangkok, Thailand using a reference IgM and IgG antibody capture ELISA described previously [336]. DENV loads in plasma were measured using an internally-controlled, serotype-specific, real-time RT-PCR assay that has been described previously [263]. Results were expressed as cDNA equivalents per milliliter of plasma.

The Bio-Rad NS1 Ag Strip and SD Dengue Duo rapid tests were provided by Bio-Rad (Hercules, CA) and Standard Diagnostics (Kyonggi-do, Korea) respectively and were performed according to the manufacturer’s instructions. Each plasma sample for assessment was tested on both rapid tests in parallel. Each assay strip was

independently assessed after the incubation time suggested by the manufacturer by the technician conducting the test and by a 2nd analyst who was blind to the first assessment. Discordant results were referred to a 3rd analyst whose decision was final. The analysts performing and scoring the assays were blind to the reference assay results and to any clinical information on the patients.

3.2.3 Analysis and Statistics

Concerning the SD Dengue Duo rapid test and for comparative purposes only, the IgM and IgG parameters were included in the interpretation of the test in some analyses. The statements “NS1 or IgM” and “NS1 or IgM or IgG” were then used and should be understood as if at least one of these parameters (i.e. NS1, IgM or IgG) is positive the sample is considered as positive. However, the detection of IgM and/or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue. All statistical analysis was performed using Intercooled STATA version 9.2 (StataCorp, TX). Significance was assigned at $P < 0.05$ for all parameters and were two-sided unless otherwise indicated. Uncertainty was expressed by 95% CI. Categorical variables between groups were compared by Fisher’s exact test. The *t*-test was used for continuous variables.

3.3 Results

3.3.1 Characteristics of the study population

The characteristics of the study population (n=292 cases) that contributed acute plasma to the test panel are shown in Table 8. The panel of dengue cases (n=245) were consecutively enrolled, qRT-PCR positive patients in the DENCO study (see M&M). The median duration of illness prior to the test plasma sample being collected was 3 days (range: 1-6). There were 47 patients in whom there was no virological or serological evidence of acute or recent dengue in paired plasma specimens.

3.3.1 Sensitivity and specificity of NS1 tests versus qRT-PCR

Bio-Rad and SD Duo NS1 rapid tests were equally sensitive for the diagnosis of acute dengue relative to the reference qRT-PCR test (Table 9; Bio-Rad 61.6% vs SD Duo NS1 62.4%, $P=0.93$). The specificity of both NS1 tests was 100%, albeit the number of patients who had no evidence of acute or recent dengue was small ($n=47$). Inclusion of the IgM parameter in the interpretation of the SD Duo test significantly increased its diagnostic sensitivity (75.5% vs 62.4%, $P=0.0024$). Inclusion of the IgM and the IgG parameters in the interpretation of the SD Duo test further increased the diagnostic sensitivity over NS1 alone (83.7% vs 62.4%, $P<0.0001$) but came at a cost of reduced specificity (100% for NS1 alone or NS1/IgM versus 98% for NS1/IgM/IgG).

Table 8: Baseline table summarizing the characteristics of the patient population that contributed to the sample panel.

Variable	Confirmed dengue (n=245)	Other febrile illness (n=47)
	N (%) or Median (range)	N (%) or Median (range)
Male sex	141 (57.8%) ^a	25 (55.6%) ^b
Age (years)	12 (1-49) ^a	9 (2-53) ^b
Day of illness	3 (1-6)	2 (1-6) ^b
Infecting serotype and serological status		
DENV-1	138 (56.3 %)	
Primary	49 (35.6 %)	
Secondary	87 (63.0 %)	
Indeterminate	2 (1.4 %)	
DENV-2	91 (37.61%)	
Primary	9 (9.9 %)	
Secondary	81 (89.0 %)	
Indeterminate	1 (1.1 %)	
DENV-3	16 (6.6 %)	
Primary	8 (50.0 %)	
Secondary	8 (50.0 %)	

^a sex and age was unknown in 1/245 acute dengue patients

^b sex, age and day of illness was unknown in 2/ 47 patients with other febrile illness

3.3.1 Sensitivity of NS1 tests by day of illness

The sensitivity of NS1 tests alone was not significantly different between test samples collected within 3 days of illness onset versus those collected at a later time (Table 10). However, if the result of SD IgM and IgG parameters were included in the interpretation of the SD test then sensitivity was significantly improved in test samples collected after 3 days of illness onset (Table 8; P=0.05).

Table 9: Sensitivity and specificity, positive and negative predictive values of each assay against the gold standard algorithm.

Assay parameter	Patients (n=)	Acute Dengue cases (n=)	Positive in rapid test (n=)	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV (%) (95% CI)
BR NS1	292	245	151	61.6 (55.2 - 67.8)	100 (93.8 – 100) ^a	100 (98.0 - 100) ^a	33.3 (25.6 - 41.8)
SD NS1 alone	292	245	153	62.4 (56.1 - 68.5)	100 (93.8 – 100) ^a	100 (98.1 - 100) ^a	33.8 (26.0 - 42.3)
SD NS1 or IgM	292	245	185	75.5 (69.6 - 80.8)	100 (93.8 - 100) ^a	100 (98.4 - 100) ^a	43.9 (34.3 - 53.9)
SD NS1 or IgM or IgG	292	245	206	83.7 (78.4 - 88.1)	97.9 (88.7 - 99.9)	99.5 (97.3 – 100)	53.5 (42.4 – 64.3)

^a one-sided, 95% CI

Table 10: Sensitivity of the dengue RDTs in samples collected within 3 days of illness onset versus those collected at a later time.

Status	Total (n=)	BR-NS1	SD-NS1	SD NS1 or IgM	SD NS1 or IgM or IgG
		% Sensitivity (95% CI)			
Test sample ≤ 3 days of illness	156	60.9 (52.8 – 68.6)	62.2 (54.1 – 69.8)	73.1 (65.4 – 79.9)	80.1 (73.0 – 86.1)
Test sample > 3 days of illness	89	62.9 (52.0 – 72.9)	62.9 (52.0 – 72.9)	79.8 (69.9 – 87.6)	89.9 (81.7 – 95.3)
<i>P</i> value ^a		0.7860	1.0000	0.2809	0.0498

^a Fisher's exact test

3.3.2 NS1 sensitivity in primary or secondary infection

NS1 detection rates with both RDTs were significantly lower in patients with secondary dengue than primary dengue (Table 10). Interestingly, inclusion of the IgM and IgG parameters in the interpretation of the SD Duo test significantly increased the diagnostic sensitivity over NS1 alone in patients with secondary dengue (Table 11). The overall difference in sensitivity between primary and secondary dengue was not associated with the illness day at the time of testing (primary dengue, mean day of illness: 3.30 days versus secondary dengue: 3.26 days, $P=0.71$). Reduced sensitivity was also not associated with viremia levels between primary and secondary dengue cases (log₁₀ mean viremia for primary: 7.04 versus for secondary 6.78, $P=0.29$). In a more stratified analysis, NS1 sensitivity was also higher in DENV-1 infected patients (where the sample size was highest) with primary dengue compared to secondary dengue at all time points of acute illness (Figures 22A and B).

Table 11: Sensitivity of dengue RDTs in patients with primary and secondary serological profiles

Status	Total (n=)	BR-NS1	SD-NS1	SD NS1 or IgM	SD NS1 or IgM or IgG
		% Sensitivity (95% CI)			
Primary dengue	66	80.3 (68.7 – 89.1)	80.3 (68.7 – 89.1)	83.3 (72.1 – 91.4)	83.3 (72.1 – 91.4)
Secondary dengue	176	55.1 (47.4 – 62.6)	56.3 (48.6 – 63.7)	72.7 (65.5 – 79.2)	84.1 (77.8 – 89.2)
<i>P</i> value ^a		0.0003	0.0006	0.0951	0.8472

^a Fisher’s exact test

A possible basis for reduced sensitivity in secondary dengue is that NS1, along with other viral antigens, is less likely to be available for detection when a substantial level of DENV-reactive IgG is present. To explore this further, we analyzed NS1 detection sensitivity in the context of DENV-reactive IgG and IgM status as defined by the reference ELISA in the test sample. The presence of measurable DENV-reactive IgG (in the GAC ELISA) in the test sample was associated with a significant reduction in

NS1 sensitivity in the SD assay (Table 10). On the contrary, the presence of measurable DENV-reactive IgM (in the MAC ELISA) but not IgG (in the GAC ELISA) in the test sample was associated with a significant increase in NS1 sensitivity relative to seronegative test samples (Table 12).

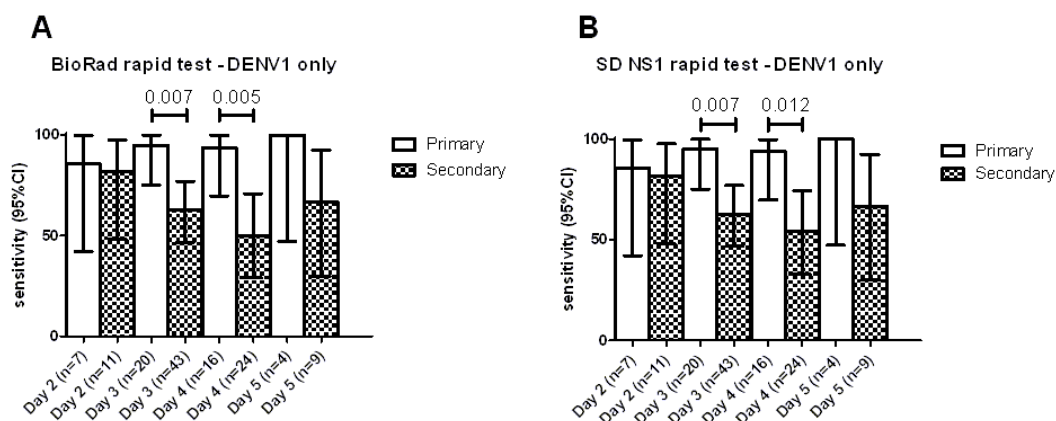


Figure 22: NS1 sensitivity of Bio-Rad and SD rapid tests in relation to primary and secondary serological status and day of illness in DENV-1 infection. Shown is the sensitivity of NS1 detection in the test sample according to primary and secondary serological status and day of illness for A) Bio-Rad NS1 test and B) SD NS1 test.

Table 12: Sensitivity (95% CI) of SD NS1 RDT assay in the presence or absence of measurable DENV-reactive IgG or IgM detected by ELISA in the test sample

Status ^a	IgG+ & IgM-	IgG+ & IgM+	IgG- & IgM-	IgG- & IgM+
% Sensitivity (95% CI)	14.3 (4.0 - 32.7)	31.7 (18.1 - 48.1)	67.9 (58.2 - 76.7)	91.4 (82.3 - 96.8)
Confirmed acute Dengue cases (n=)	28	41	106	70
<i>P</i> value ^b		0.1546	0.0001	0.0002

^a according to the MAC and GAC reference ELISAs.

^b Fisher's exact test

3.3.3 NS1 sensitivity in relation to viremia levels

We hypothesized that viremia levels would be associated with the detection of plasma NS1. Accordingly, in DENV-1 patients with equivalent illness durations (3 and 4 days of illness) viremia levels were significantly higher ($P=0.0005$) in patients who were NS1-positive versus those who NS1 negative in both tests at day 3 (with Bio-Rad and SD rapid test: \log_{10} mean viremia=7.71 versus \log_{10} mean viremia=6.11 respectively) (Figures 23A and B). To place NS1 detection in the context of both viremia and serological response, individual test results for the Bio-Rad NS1 rapid test in DENV-1 infected patients were plotted against corresponding viremia and serological responses in the same test sample (Figures 24A and B). These graphs illustrate the dynamic relationship between the detection of NS1, time since illness onset and in particular, the interfering effect of DENV-reactive IgG in the test sample.

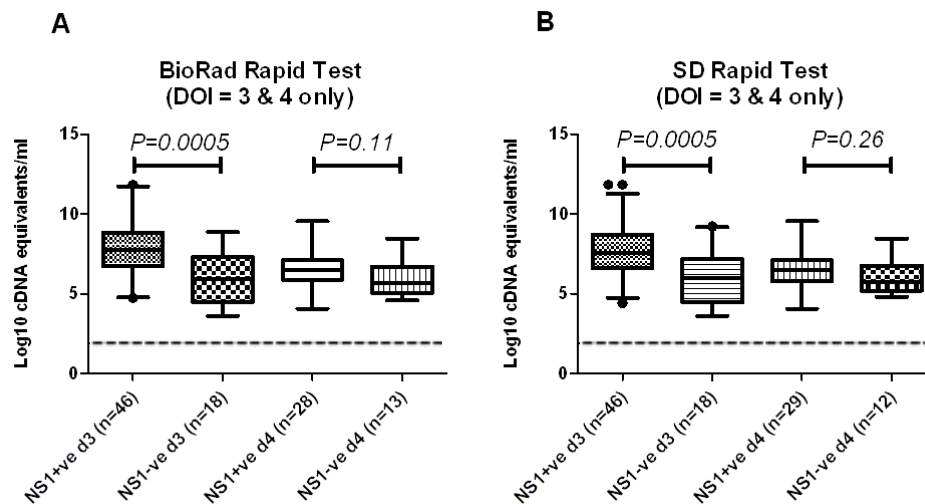


Figure 23: Viremia by NS1 status in DENV-1 infected patients samples at 3 and 4 days after illness onset. Shown is the median (interquartile range, 95% range of data, and outliers) viremia level in NS1 positive and NS1 negative patients with the same illness duration (3 and 4 days) tested by **A**) Bio-Rad NS1 test or **B**) SD Duo test. The limit of detection of the assay is shown with a dashed line. Viremia levels were significantly higher in NS1 positive patients relative to NS1 negative patients (t -test) at day 3 of illness.

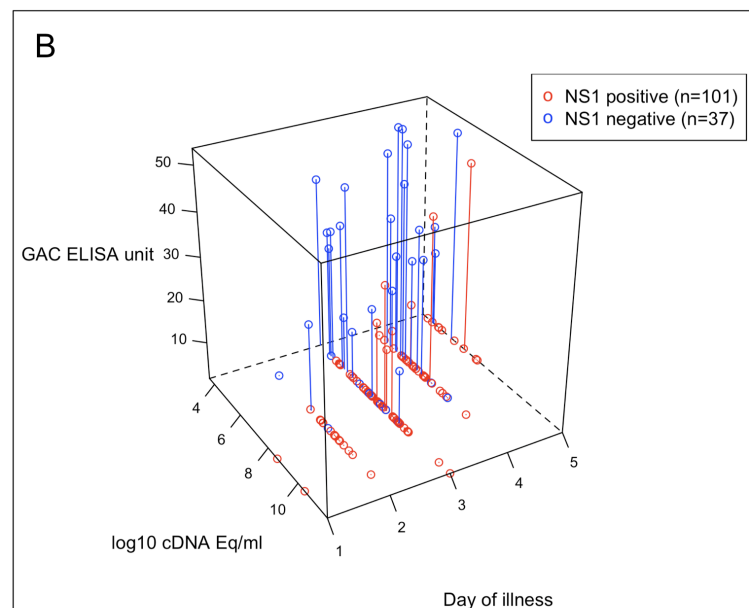
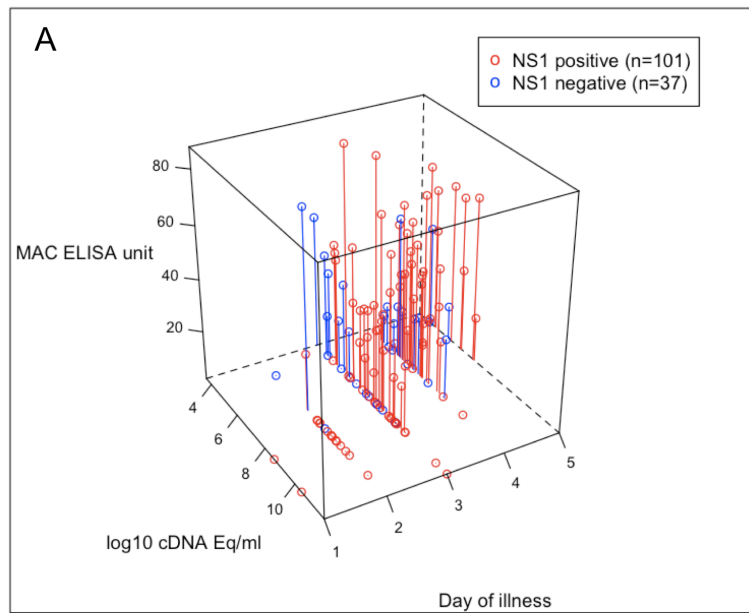


Figure 24: NS1 status (with Bio-Rad rapid test) in relation to MAC and GAC ELISA results, viremia and day of illness in DENV-1 infection. Shown are A) MAC or B) GAC ELISA units, viremia level and day of illness in NS1 positive and NS1 negative patients.

3.4 Discussion

In the present study we showed that two different commercially available lateral flow RDTs, the Bio-Rad NS1 Ag Strip and SD Dengue Duo, have similar sensitivities (61.6% vs 62.4% respectively) for the detection of NS1 in plasma from RT-PCR positive patients. The factors negatively influencing the detection of NS1 included the presence of DENV-reactive IgG in the test sample and the presence of secondary infection. The inclusion of the IgM test in the SD Dengue Duo provides for greater sensitivity (75.5%) without compromising specificity. The reasonable sensitivity and specificity of the SD Dengue Duo suggests it warrants additional prospective evaluations.

There is, as yet, no dengue RDT that could be considered as providing a definitive diagnosis of dengue. At best, current tests provide a presumptive diagnosis for a patient with a dengue like illness. A recent, TDR/WHO sponsored multi-centre evaluation of anti-dengue virus IgM rapid tests highlighted the very modest specificity of several commercially available IgM rapid tests, particularly in samples from patients with other infections, with malaria being a major confounder [501]. As acknowledged by the authors of the evaluation study, specificity issues must be considered in the context of the setting in which a particular test is to be used [501]. For example, in SE Asia there is no Yellow Fever virus transmission and in many urban settings there is no malaria transmission that could serve to limit the accuracy of a dengue RDT. The challenge then is to identify the best test for a particular setting and this is best done by accumulating more systematic data across a range of patient populations in different health care settings. Decision-tree based algorithms, as suggested for selecting malaria rapid tests, could be helpful in this regard for dengue RDTs [502].

To the best of our knowledge this is the first side-by-side assessment of different NS1 RDTs. Our finding that they were equally sensitive for NS1 detection is encouraging and suggests other points of difference should be considered, e.g. ease of use, cost and stability at room temperature. The strength of the SD Dengue Duo is that the DENV IgM and IgG test windows provides an additional diagnostic investigation that complements NS1 detection. The inclusion of the DENV-IgM result in this current

evaluation improved sensitivity without a reduction in the very high specificity associated with NS1 detection (100%). The inclusion of the IgG test result modestly further improved sensitivity. Caution is needed however as a positive IgM or IgG result alone could also represent infection anytime in the previous few months and should therefore be considered a presumptive diagnosis. Indeed, detection of IgM or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue. The potential for reduced specificity was highlighted in that one patient with no laboratory acute evidence of dengue had a positive IgG test result alone. Nevertheless the use of IgM and IgG test parameters in a NS1 RDT is rational, as it will likely provide improved presumptive diagnostic coverage towards the end of the acute illness when NS1 levels are declining but the DENV-specific IgM and IgG titres are climbing rapidly. The sensitivity and specificity of the IgM and IgG test components of the SD Dengue Duo have been described previously as part of the TDR/WHO assessment of dengue RDTs [501].

The presence of DENV-reactive IgG in the test sample, a relatively low viremia and secondary dengue were the major factors associated with a negative NS1 finding on both tests. The bias of these RDTs towards patients with higher viremia levels is probably a positive feature of these tests in that they are likely biased towards patients at risk for complications during their illness [503]. Somewhat surprisingly we did not replicate our previous observations in a different patient population that NS1 sensitivity by Bio-Rad rapid test was higher in the first 3 days of illness than at later times [320]. Differences exist between the two studies and these include- a) the first study included adults and b) the first study was done prospectively in real time with fresh plasma rather than frozen stored plasma. It is possible these factors might account for the differences between the study findings. It may also reflect chance differences associated with relatively small sample sizes. Similarly, we found greater NS1 sensitivity in samples where there was a measurable DENV-reactive IgM level, a finding inconsistent with our previous observations using ELISA based NS1 detection where the presence of IgM was not associated with higher or lower sensitivity [320]. The different findings might reflect chance differences in the patient population or differences between ELISA versus RDT.

A weakness of this study was that it was performed using a panel of stored plasma specimens and was heavily biased towards DENV-1, the most common serotype in circulation in Viet Nam since 2006. Similarly, assay performance and interpretation were performed by experienced lab analysts and not by clinicians “at the bedside”. Different results may also have been obtained if an outpatient population, rather than a hospitalized set of patients, were used to generate the assessment panel used here. The evaluation panel is also biased in that all samples from dengue patients were RT-PCR positive. Despite these limitations, the current study provides a baseline in terms of sensitivity and specificity of these two RDTs for Vietnamese dengue patients and highlights virological and immunological factors associated with assay performance. Further prospective evaluations of both tests are warranted.

Chapter 4 : Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with dengue - implications for therapeutic interventions

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4.1 Background

In a context of particular interest in therapeutic against DENV infections, a clear and consistent picture of relationships between viremia kinetics, disease severity and patient immune status is essential for the rational design of RCTs. The literature describing the overall relationship between plasma/serum viral burden, disease severity and immune status generally supports the hypothesis that there is a positive correlation between markers of viral burden in the first 2-3 days of fever and the severity of clinical outcomes. This has been described in the chapter 1. In adults, where the risk of clinically apparent disease occurring in primary infection is possibly greater [504, 505], there is less evidence relating virological features of infection to immune status or clinical outcome. Kuberski et al. reported in 1977 that the magnitude of viremia in young adult patients was higher in primary than secondary DENV-1 infections [225]. More recently, DENV viremia levels from Taiwanese adult patients were reported to be lower in secondary than in primary DENV-2 infections [506].

The dynamics of virus clearance might also be relevant to clinical outcome. In Thai children, Vaughn and others have shown that the slope of the descending portion of the viremia curve was steeper for patients with secondary infection versus those with primary infection and viremia decreased more quickly for patients with DHF than for

patients with DF at defervescence [238]. The accelerated clearance of viremia in secondary infection most likely reflects the contribution of anamnestic humoral and cellular immune responses, which themselves have been implicated in the pathogenesis of capillary leakage. Conversely however, Wang et al suggested clearance of the virus and virus-containing immune complexes was slower in adult DHF patients [507].

A better understanding of the relationship between biomarkers of virus infection, the immune response and disease evolution is critical for the rational use of intervention therapies in dengue, e.g. antiviral drugs or immune-modulating therapies. To this end, this study describes the kinetics of viremia and NS1 in an intensively investigated cohort of Vietnamese adults with dengue and less than 72hrs of fever.

4.2 Methods

4.2.1 Study setting, participants and ethical considerations

A double blind RCT of CQ in 307 adults hospitalized for suspected DENV infection was conducted at the Hospital for Tropical Diseases (Ho Chi Minh City, Vietnam) between May 2007 and July 2008. Information on recruitment, inclusion criteria, treatment and randomization have been published previously [508]. The Scientific and Ethical committee of the HTD and the Oxford Tropical Research Ethical Committee approved the study protocol and all patients gave written informed consent. The trial was registered with the ISRCTN Register (ISRCTN38002730). Herein we describe the clinical and virological features of 257 patients with laboratory confirmed dengue.

4.2.2 Dengue diagnostics and detection of DENV RNA and NS1 in plasma

A diagnosis of laboratory confirmed dengue was reached using serological, antigen detection and molecular methods [320]. In brief, RT-PCR detection of DENV RNA in

plasma was performed using an internally controlled, serotype-specific, real-time RT-PCR TaqMan assay that has been described previously [263]. RNA extraction from plasma samples was automated (NucliSens easyMAG, BioMerieux, Lyon, France). Results were expressed as cDNA equivalents per mL of plasma. A capture IgM and IgG ELISA (MAC and GAC ELISA) using DENV/JEV antigens and mAb reagents provided by Venture Technologies (Sarawak, Malaysia), was performed as previously described [509]. NS1 was detected by using the NS1 Platelia ELISA assay from BioRad (Hercules, CA) according to the manufacturer's instructions. Samples defined as equivocal in the NS1 Platelia ELISA assay were repeated and if they were still equivocal they were regarded as being negative.

4.2.3 Host immune status

The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA units in early convalescent plasma samples taking into account the illness day. The cut-off in IgG ELISA units for distinguishing primary from secondary dengue by illness day was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed at the Centre for Vaccine Development, Mahidol University, Bangkok, Thailand using a reference IgM and IgG antibody capture ELISA described previously [336].

4.2.4 Clinical and laboratory investigations

Clinical history and examination findings were recorded daily into case record forms. An ultrasound was performed in all patients within 24hrs of defervescence. Venous blood samples were collected at hospital admission, then twice daily (around 9am and 3pm) for a minimum of 5 days after hospital admission and again 10–14 days after discharge from the hospital. Plasma, for use in diagnostic investigations, was stored frozen in multiple aliquots at -80°C until use. A complete blood count, including Hct and platelet measurements, was performed daily for all patients. Hct measurements were performed more frequently if clinically indicated. The extent of

hemoconcentration during symptomatic illness was determined by comparing the maximum Hct recorded during hospitalization with either the value recorded at follow-up when available (191/248 i.e. 77% of the patients) or against a sex- and age-matched population value. The day of fever onset was designated day 1 of illness.

4.2.1 Case definition

DF and DHF were diagnosed according to 1997 WHO classification criteria and was applied to each case after review of study notes [202]. The 1997 definitions were used for this study because at the time of clinical assessment the 2009 WHO Guidelines and revised classification scheme was not available. DF was defined as a laboratory confirmed dengue case with no evidence of haemorrhagic signs and no evidence of capillary permeability as defined for a DHF case. DHF was defined as laboratory confirmed dengue case with thrombocytopenia ($<100,000$ platelets/mm³), any hemorrhagic manifestation, and evidence of plasma leakage (as denoted by a $>20\%$ increase in the Hct from the baseline value or by the presence of pleural or abdominal effusions).

4.2.2 Analysis and Statistics

The data used in this analysis was taken from a randomised controlled treatment trial of dengue. Since the intervention (i.e. CQ) had no measurable impact on virological or immunological outcomes, for the purposes of this analysis we did not distinguish between patients in the CQ or placebo arms of the study. All statistical analysis was performed and figures designed using the software R (version 2.10.1). Significance was assigned at $P<0.05$ and were two-sided unless otherwise indicated. Uncertainty was expressed by 95% CI. The Kruskal-Wallis rank sum test was used for continuous variables and the Fisher's exact test for categorical variables. Survival analysis using the Kaplan-Meier method and log-rank test was used for all time-to-event outcomes. Time to resolution of viremia or NS1 antigenemia was defined as the time from the start of symptoms until the first of two consecutive plasma samples below the RT-

PCR limit of detection or NS1 ELISA negative. The FCT was defined as the time from the start of symptoms to the start of the first 48hrs period during which axillary temperature remained below 37.5°C.

For the viremia kinetics analysis, when the RT-PCR signal was below the assay limit of detection (defined as the last dilution of standard that gave a specific signal), a value equal to concentration of the last dilution of standard that gave a specific signal / 10 was assigned. The maximum viremia level was defined as the highest plasma viremia level measured during illness. The maximum viremia level was considered to be a peak viremia level only in cases in which viremia rose after the enrolment specimen. To compare kinetics of viremia between patients with different serological status, disease severity and serotype, the means of log-transformed viremia measurements made on the same illness day were used as a summary measure of the viremia on that day. To estimate the maximum daily rate of DENV clearance, the slope of the viremia curve was calculated for each illness day as the change in the means of log-transformed viremia measurements made on the same illness day. Only the maximum decreasing daily rate of each patient was used for analysis.

4.3 Results

4.3.1 Characteristics of the study population

Of the 307 adults with suspected dengue enrolled in the CQ RCT between May 2007 and July 2008, 257 had laboratory-confirmed dengue including 248 patients with a defined serological and clinical classification and 9 patients with ambiguous or unknown clinical outcomes or serology (mainly because they left the study prematurely). The characteristics of the study population are summarized in Tables 13 and 14. DENV-1 (57.3%) was the commonest serotype detected in this population of patients, then DENV-2 (20.6%), DENV-3 (15.7%) and DENV-4 (2.8%). DHF was significantly associated with secondary infection compared with primary infection (65/215 vs 4/33 i.e. 30.2% vs 12.1%, $P=0.04$, OR 3.13, 95% CI 1.04 – 12.75). DHF resulting from secondary infection was more commonly associated with DENV-2

(21/45 (46.7%)) than for other serotypes (DENV-1: 33/124 (26.6%), DENV-3: 10/33 (30.3%) and DENV-4: 1/7 (14.3%) (DENV-2 vs DENV-1, -3 and -4 P=0.02, OR=2.38, 95% CI 1.14 - 4.96) (Table 14).

Table 13: The characteristics of the study population ^a.

Variables	N (%) or Median (interquartile range)	
	Primary (N=33)	Secondary (N=215)
Age (years)	19 (17-25)	22 (18-27)
Male sex	20 (60.6%)	147 (68.4%)
Viremic	30 (90.9%)	209 (97.2%)
Infecting serotype:		
DENV-1	18 (60.0%)	124 (59.3%)
DENV-2	6 (20.0%)	45 (21.5%)
DENV-3	6 (20.0%)	33 (15.8%)
DENV-4	0 (0.0%)	7 (3.4%)
NS1 ELISA positive	28 (84.8%)	186 (86.5%)
Febrile	31 (93.9%)	209 (97.2%)
Time since illness onset (hrs)	51.5 (43.5-68.0)	49.0 (41.3-55.0)
Disease severity:		
DF	29 (87.9%)	150 (69.8%)
DHF	4 (12.1%)	65 (30.2%)

^a Were omitted from this table 9 patients with ambiguous or unknown clinical outcomes or serology (mainly because they left the study prematurely).

Table 14: The characteristics of the study population by serotype.

Variables	N (%) or Median (interquartile range)			
	Primary DF	Secondary DF	Primary DHF	Secondary DHF
	DENV-1 (N=142)			
Number of patients	15 (10.6%)	91 (64.1%)	3 (2.1%)	33 (23.2%)
Age (years)	18 (17-21)	21 (18-26)	18 (17-20)	21 (17-28)
Male sex	8 (53.3%)	65 (71.4%)	0 (0.0%)	20 (60.6%)
NS1 ELISA positive	15 (100%)	84 (92.3%)	3 (100%)	32 (97.0%)
Febrile	15 (100%)	89 (97.8%)	3 (100%)	32 (97.0%)
Time since illness onset (hrs)	51.5 (45.8-56.5)	46.0 (40.0-53.3)	33.0 (23.5-46.5)	51.0 (39.5-55.0)
	DENV-2 (N=51)			
Number of patients	5 (9.8%)	24 (47.1%)	1 (2.0%)	21 (41.2%)
Age (years)	26 (25-28)	20 (17-25)	15	25 (21-31)
Male sex	5 (100%)	14 (58.3%)	1 (100%)	12 (57.1%)
NS1 ELISA positive	3 (60.0%)	14 (58.3%)	1 (100%)	16 (76.2%)
Febrile	5 (100%)	24 (100%)	1 (100%)	21 (100%)
Time since illness onset (hrs)	70.0 (48.0-72.0)	52.5 (47.5-60.4)	72	53.5 (47.5-63.0)
	DENV-3 (N=39)			
Number of patients	6 (15.4%)	23 (59.0%)	-	10 (25.6%)
Age (years)	22 (18-28)	21 (18-27)	-	19 (17-24)
Male sex	4 (66.7%)	14 (60.9%)	-	8 (80.0%)
NS1 ELISA positive	5 (83.3%)	21 (91.3%)	-	10 (100%)
Febrile	5 (83.3%)	22 (95.7%)	-	10 (100%)
Time since illness onset (hrs)	48.8 (34.1-66.4)	50.5 (44.5-53.0)	-	50.5 (46.0-56.6)
	DENV-4 (N=7)			
	-	6 (85.7%)	-	1 (14.3%)
Age (years)	-	26 (23-28)	-	33
Male sex	-	6 (100%)	-	1 (100%)
NS1 ELISA positive	-	6 (100%)	-	1 (100%)
Febrile	-	6 (100%)	-	1 (100%)
Time since illness onset (hrs)	-	48.0 (39.0-53.3)	-	57.0

4.3.2 Viremia kinetics in DF and DHF according to serological status

Median viremia levels by illness day for DENV-1, -2 and -3 are shown in Figure 24. In DF patients with primary infection, DENV-1 viremia levels were significantly higher than DENV-2 or DENV-3 levels at multiple time-points during the acute illness (Figure 1A). In DF patients with secondary infection, the most common serological and clinical grouping, and DHF patients with secondary infection, DENV-1 levels were significantly higher than DENV-2 levels and there was also a non-significant trend towards higher DENV-1 levels than DENV-3 levels (Figure 25B and C). Collectively, and despite small sample sizes for some subgroups, these data suggest that DENV-1 infections were associated with higher viremias (as measured by qRT-PCR) than DENV-2, irrespective of disease severity and immune status. DENV-1 was the commonest serotype detected in this patient population and therefore there was sufficient data to enable direct comparisons of viremia kinetics across serological states and clinical severity whilst controlling for the infecting serotype. These data show that in the early acute phase (illness day 3) patients with DENV-1 infection and DHF had significantly higher viremia levels than DENV-1 patients with DF, irrespective of the patient immune status (Figure 26). These data show also that later in the acute phase (from day 4 of illness) patients with primary DENV-1 infections had significantly higher viremia levels than patients with secondary DENV-1 infections, irrespective of the disease severity (Figure 26).

A limitation of these analyses is that in the majority of patients with secondary infections the viremia was already declining at the time of enrolment i.e. we did not observe an obvious peak viremia (Table 15). Overall, a peak viremia was significantly less often observed in secondary infections than in primary infections for all disease severity grades ($P=0.001$, $OR=3.64$, 95% CI 1.55 - 8.74). However, there were no significant differences in the duration of illness prior to enrolment between patients in different categories of serological status or disease severity (P between 0.11 and 0.96 if all the serotypes are considered, and 0.16 and 0.39 if and only DENV-1), suggesting this snapshot of viremia levels is unbiased by differences in duration of illness at study enrolment.

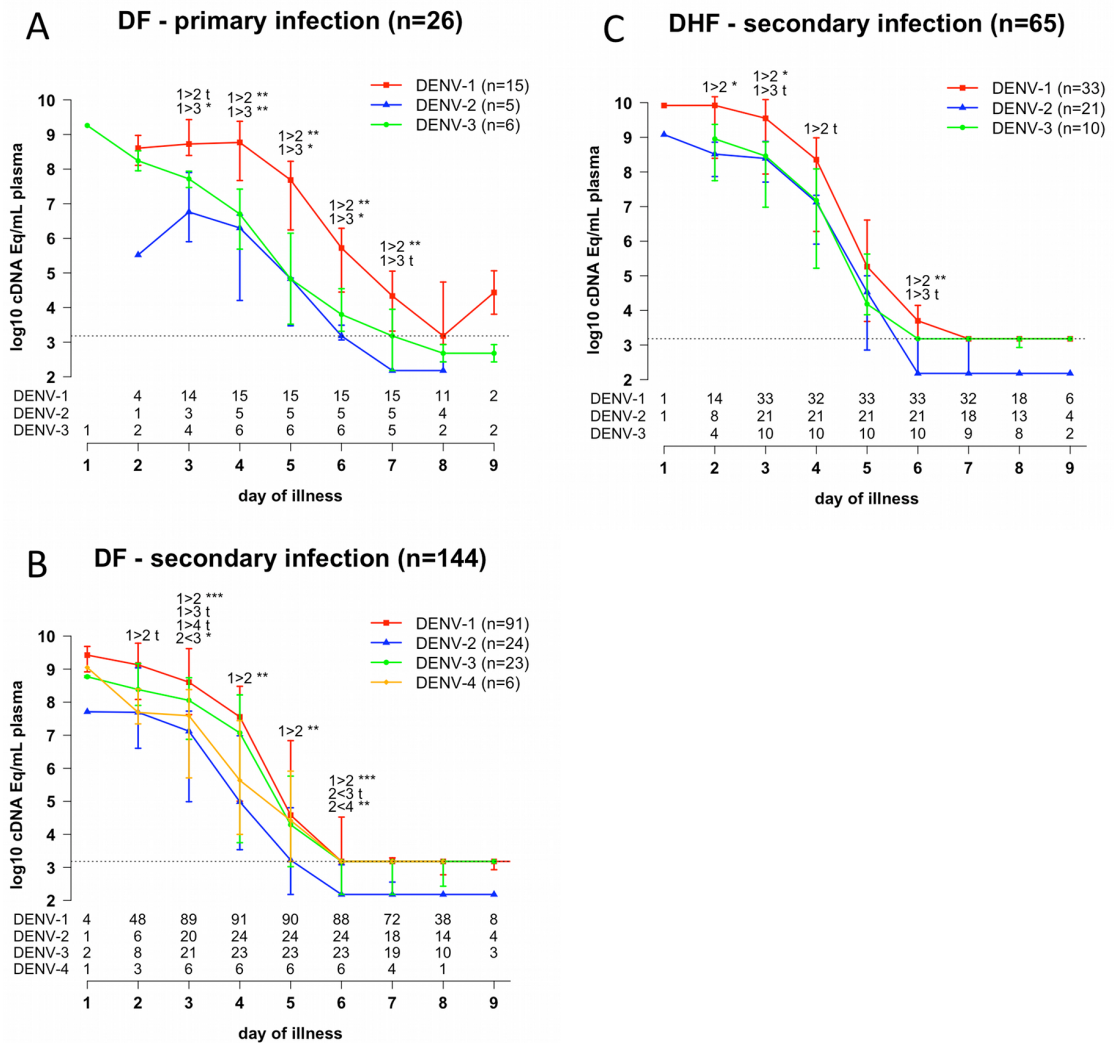


Figure 25: Magnitude of DENV viremia by illness day. Levels of DENV-1, -2, -3 and -4 genome equivalent cDNA copies per millilitre were determined in serial plasma samples from patients with **A)** primary DF (n=26), **B)** secondary DF (n=144) and **C)** secondary DHF (n=64). Data are median and IQR. *** P<0.001 ** 0.001<P<0.01 * 0.01<P<0.05 † 0.05<P<0.1. Data from patients with DENV-1 and -2 primary DHF (n=3 and n=1 respectively) and DENV-4 secondary DHF (n=1) are not displayed because the number of these patients was too small for statistical analysis. The numbers below the graph indicate the numbers of patients at each time point.

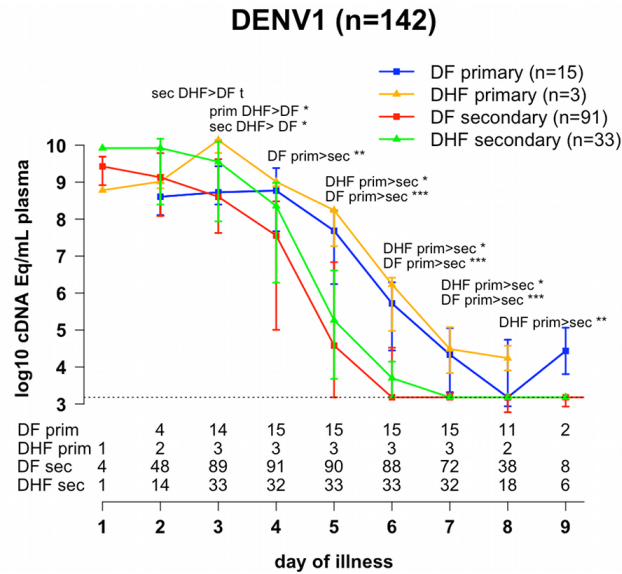


Figure 26: Magnitude of DENV-1 viremia by illness day. Levels of DENV-1 genome equivalent cDNA copies per millilitre were determined in serial plasma samples from DENV-1 infected patients with primary DF (n=15), primary DHF (n=3), secondary DF (n=91) and secondary DHF (n=33). Data are median and IQR. *** P<0.001 ** 0.001<P<0.01 * 0.01<P<0.05 † 0.05<P<0.1. The numbers below the graph indicate the numbers of patients at each time point.

4.3.3 Timing and amplitude of peak viremia and associations with severity and immune status

Peak viremia levels were identified in 72 patients. Peak viremia occurred significantly earlier in secondary DF than in primary DF (P=0.008) and in secondary DHF than in primary DHF (P=0.04) but there were no significant differences between primary DF and primary DHF (P=0.73) and between secondary DF and secondary DHF (P=0.13) for the peak viremia time (Table 15). Amongst DENV-1 patients, peak viremia, when observed (in 51 of 142 patients), happened significantly earlier in secondary DF than in primary DF (P=0.0006) but not in secondary DHF compared to primary DHF (P=0.31), possibly because of small sample size. There was no significant difference between secondary DF and secondary DHF (P=0.48) but only a non-significant trend towards later viremia peaks in primary DF than in primary DHF (P=0.052).

Table 15: Peak viremia analysis.

	N (%) or Median (interquartile range)			
Variables	DF primary	DF secondary	DHF primary	DHF secondary
	All serotypes (N=239)			
Number of patients	26	144	4	65
Time since illness onset (days)	2.12 (1.91-2.74)	2.00 (1.71-2.29)	1.94 (1.18-2.63)	2.08 (1.73-2.29)
Number of patients with peak	13 (50%)	37 (25.7%)	4 (100%)	18 (27.7%)
Time of peak (days)	3.00 (2.42-3.19)	2.46 (2.13-2.78)	2.65 (2.53-3.03)	2.21 (1.99-2.53)
	DENV-1 (N=142)			
Number of patients	15	91	3	33
Highest viremia (log ₁₀ copies / mL of plasma)	9.58 (8.91-9.76)	9.14 (8.11-9.81)	10.26 (9.88-10.27)	9.82 (8.33-10.26)
Time since illness onset (days)	2.15 (1.91 – 2.36)	1.92 (1.67-2.22)	1.38 (0.98-1.94)	2.13 (1.65-2.29)
Number of patients with peak	9 (60%)	29 (23.1%)	3 (100%)	10 (30.3%)
Time of peak (days)	3.08 (2.98-3.25)	2.50 (2.25 -2.77)	2.58 (2.48-2.65)	2.38 (2.01-2.57)
Peak viremia (log ₁₀ copies / mL of plasma)	9.67 (9.02-9.69)	10.11 (9.41-10.38)	10.26 (9.88-10.27)	10.33 (9.99-10.40)

There were sufficient observations of the magnitude of peak viremia in DENV-1 infections to look for associations with clinical outcome in this subgroup (Table 13). Peaks of viremia were observed in 9 primary DF, 29 secondary DF, 3 primary DHF and 10 secondary DHF DENV-1 infected patients. Peak viremia levels were not significantly different between DF and DHF patients (DF vs DHF log₁₀ median peak levels 9.89 vs 10.27, P=0.28) but there was a non-significant trend towards higher peak viremia during secondary infections than primary infections (primary vs secondary P=0.096 and primary DF vs secondary DF P=0.086). If considering the highest viremia levels (as distinct from peak viremia levels) in DENV-1 patients, these were significantly higher in DHF than in DF (log₁₀ median levels 9.84 vs 9.19, P=0.03). Because most DHF cases were associated with secondary infections, for which peak viremia had already past by the time of enrolment, this difference is

probably underestimated. Taken together, these results suggest secondary infections are generally associated with earlier and likely higher peak viremia levels.

4.3.4 Maximum daily rate of virus clearance

In the 239 patients with detectable viremia, the median of maximum daily rates of DENV clearance (estimated as the slope of the steepest descending daily portion of the viremia curve) was 2.2 log₁₀ per day in primary DF, 2.8 log₁₀ per day in secondary DF, 2.1 log₁₀ per day in primary DHF and 3.0 log₁₀ per day in secondary DHF. The maximum daily rate of clearance was significantly higher in patients with secondary infections (median of the maximum daily loss 2.9 logs per day) versus those who experienced primary infections (median of maximum daily losses = 2.1 logs per day, $P < 0.00001$) (primary DF vs secondary DF $P = 0.00004$ and primary DHF vs secondary DHF $P = 0.025$). The results were very similar when considering only DENV-1 patients for analysis (data not shown). These data suggest secondary infection is associated with steeper declines in viremia.

4.3.5 Time to resolution of viremia in DF and DHF according to serological status

Amongst all viremic patients ($n = 239$) time to resolution of viremia was significantly longer in primary infections than in secondary infections (HR=2.88, 95% CI 1.79-4.63, log rank test $P = 0.000005$), in primary DF than in secondary DF (HR=2.60, 95% CI 1.57-4.32, log rank test $P = 0.0001$) and in primary DHF than in secondary DHF (HR=4.92, 95% CI 1.19-20.32, log rank test $P = 0.015$) (Figure 27A). Median times to resolution of dengue viremia were 148hrs (IQR 140 - 173hrs) in primary DF, 162hrs (134 - >171hrs) in primary DHF, 120hrs (97 - 141.5hrs) hrs in secondary DF and 123hrs (113 - 138hrs) in secondary DHF.

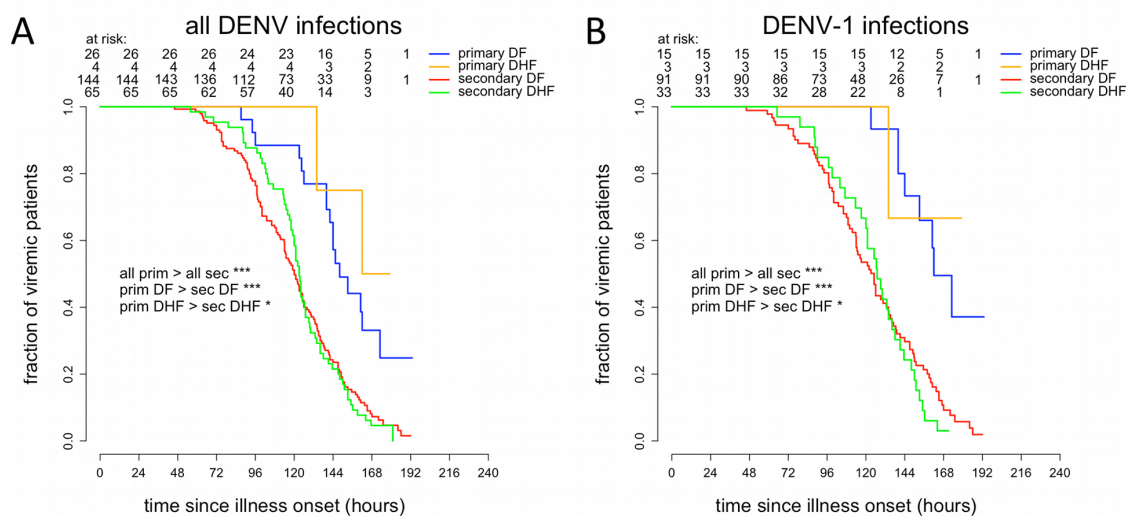


Figure 27: Time to resolution of DENV viremia. Kaplan-Meier survival analysis of time to resolution of plasma viremia in all viremic **A)** or DENV-1 only **B)** patients.

Amongst DENV-1 infected patients only (n=142), times to resolution of viremia were also significantly longer in primary infections than in secondary infections (HR=4.21, 95% CI 2.12-8.35, log rank test P=0.000009), in primary DF than in secondary DF (HR=3.67, 95% CI 1.76-7.62, log rank test P=0.0002) and in primary DHF than in secondary DHF (HR=7.00, 95% CI 0.94-52.17, log rank test P=0.03) (Figure 27B). Median times to resolution of DENV-1 viremia were 162hrs (IQR 144 - >176hrs) in primary DF, >171.1hrs (134 - >179hrs) in primary DHF (since less than 50% of primary DHF had cleared viremia before discharge), 125hrs (99 - 150hrs) in secondary DF and 127hrs (107 - 143.5) in secondary DHF.

4.3.6 Time to resolution of NS1 antigenemia in DF and DHF according to serological status

Of the 248 patients with defined serological and clinical classifications, there were 214 patients NS1 positive at the time of study enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42hrs later). Consistent with the viremia findings,

times to resolution of NS1 antigenemia were significantly longer in primary infections than in secondary infections (HR=4.57, 95% CI 2.01-10.40, log rank test P=0.00007), in primary DF than in secondary DF (HR=3.66, 95% CI 1.47-9.07, log rank test P=0.003), in primary DHF than in secondary DHF (HR=7.04, 95% CI 0.97-51.15, log rank test P=0.02) but also in secondary DF than in secondary DHF (HR=1.86, 95% CI 1.29-2.67, log rank test P=0.0007) (Figure 28A). Interestingly, only 5 of 25 patients (i.e. 20%) with primary DF and 1 of 4 patients (i.e. 25%) with primary DHF had cleared NS1 when discharged from hospital. In patients with secondary dengue, 69 of 127 with secondary DF (i.e. 54.3%) and 51 of 60 with secondary DHF (i.e. 85%) had cleared NS1 when discharged from hospital. Median times to resolution of NS1 antigenemia since illness onset were >166hrs (>146 - >178hrs) in primary DF and >158hrs (138 - >171hrs) in primary DHF (since less than 50% of primary DF and primary DHF had cleared NS1 before discharge), and 137hrs (105 - >174hrs) in secondary DF and 121hrs (100 - 153hrs) in secondary DHF.

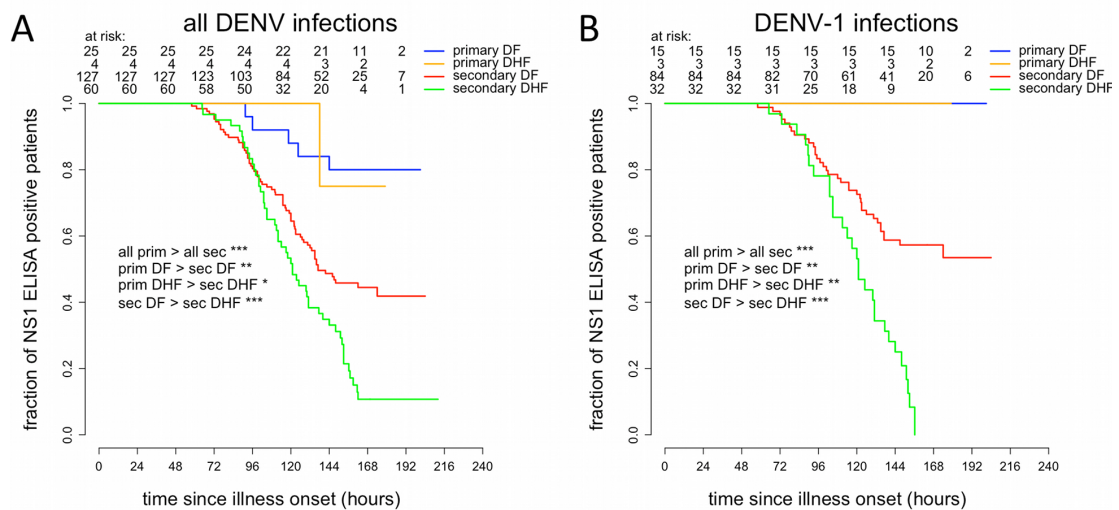


Figure 27. Time to resolution of NS1 antigenemia. Kaplan-Meier survival analysis of time to resolution of NS1 antigenemia in all viremic **A)** or DENV-1 only **B)** patients. Two patients NS1 negative at enrolment were later positive and for the purposes of analysis were considered positive at the time of enrolment.

Amongst DENV-1 infected patients (n=142), 134 were NS1 positive at the time of study enrolment. Times to resolution of NS1 antigenemia were significantly longer in DENV-1 primary infections than in DENV-1 secondary infections (HR=not applicable, log rank test P=0.00008), in DENV-1 primary DF than in DENV-1 secondary DF (HR=3.66, 95% CI 1.47-9.07, log rank test P=0.003), in DENV-1 primary DHF than in DENV-1 secondary DHF (HR=7.04, 95% CI 0.97-51.15, log rank test P=0.004) and in DENV-1 secondary DF than in DENV-1 secondary DHF (HR=1.86, 95% CI 1.29-2.67, log rank test P=0.00001) (Figure 28B). Strikingly, none of the DENV-1 infected patients with primary DF (n=15) or primary DHF (n=3) had cleared NS1 when they were discharged from hospital. In contrast, 36 of 84 (42.9%) secondary DF and 29 of 31 (93.5%) secondary DHF patients had cleared NS1 when they were discharged. Median times to resolution of NS1 antigenemia since illness onset were >172.5hrs in primary DF, >171hrs in primary DHF, >174hrs in secondary DF (since less than 50% of primary and secondary DF, and primary DHF had cleared NS1 before discharge) and 121hrs (103 - 144hrs) in secondary DHF.

Collectively, these results suggest that DENV infection is cleared earlier and faster in secondary infections than in primary infections.

4.3.7 Fever clearance time in DF and DHF according to serological status

There were 240 patients febrile at enrolment (plus 2 afebrile patients who developed fever soon after). Overall, FCT were significantly longer in primary infections than in secondary infections (log rank test P=0.037) but there was no significant difference between primary DF and secondary DF (HR=1.44, 95% CI 0.93-2.23, log rank test P=0.096), and between primary DHF and secondary DHF (HR=1.85, 95% CI 0.67-5.14, log rank test P=0.23) (Figure 29). Median FCT since illness onset was 131hrs (IQR 95.5 - 151.4hrs) in primary DF, 141hrs (135.5 - 160.5hrs) in primary DHF, 118hrs (93.1 - 140.2hrs) in secondary DF and 120hrs (105 - 142hrs) in secondary DHF. Consistent with the viremia and NS1 findings, these data indicate primary infection was associated with a longer-lived febrile period.

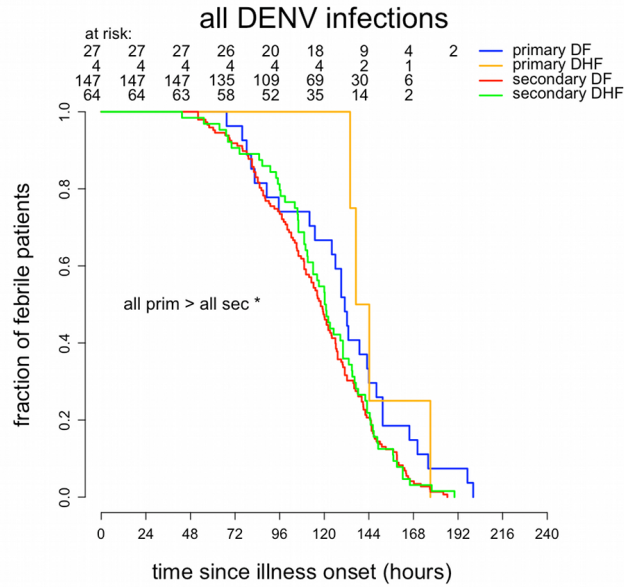


Figure 29: Fever clearance time. Kaplan-Meier survival analysis of fever clearance time in all viremic patients. Two patients afebrile at enrolment developed fever soon after and for the purposes of analysis were considered febrile at the time of enrolment.

4.3.8 DENV-1 viremia kinetics and associations with haematological parameters

For descriptive purposes, the evolution over time of DENV-1 viremia in the context of white blood cell and platelet counts and percentage hemoconcentration was plotted (Figure 30) and summarised in Table 16. The time to platelet nadir and maximum hemoconcentration was shorter in secondary DENV-1 infections ($P < 0.01$). The data also suggest that leucopenia lasts longer in primary infections than in secondary infections.

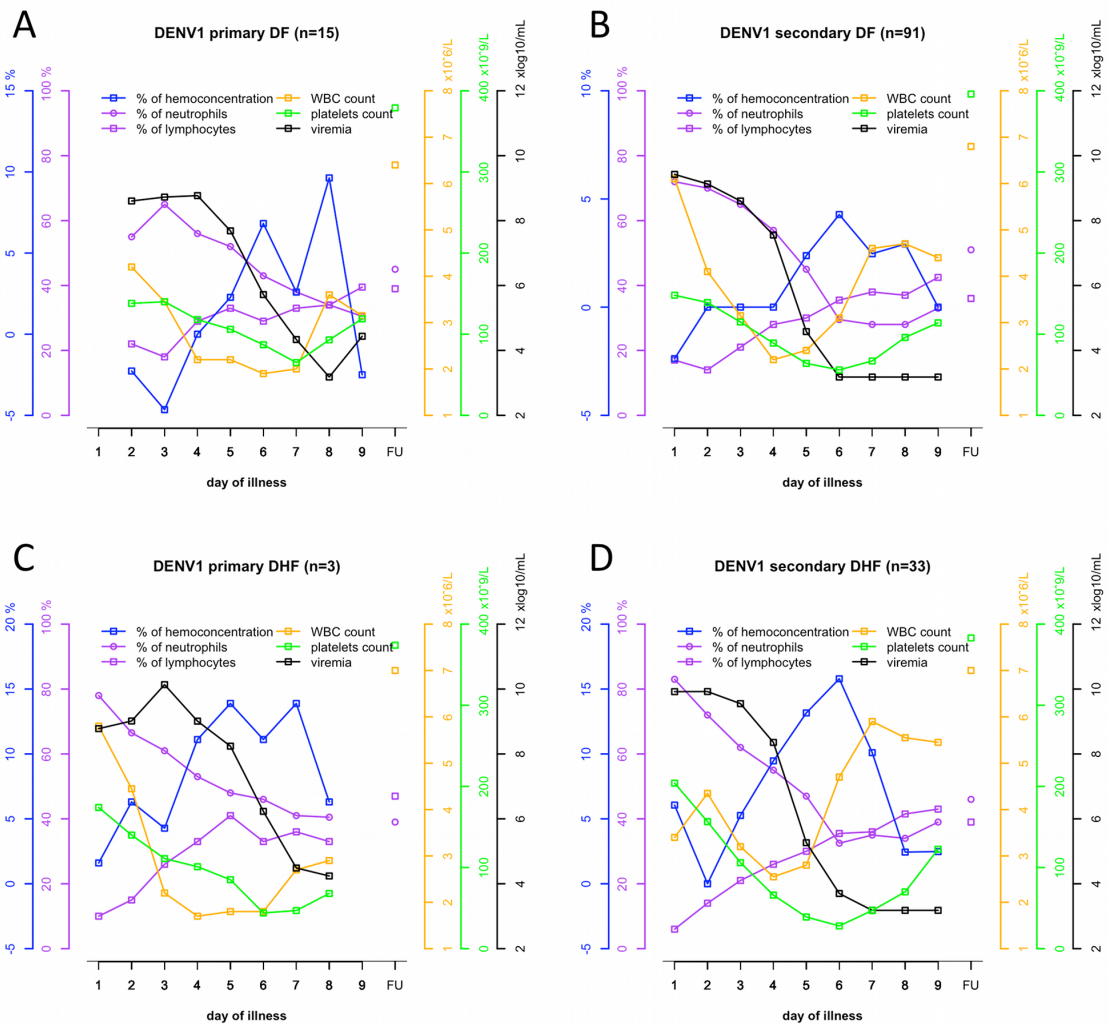


Figure 30: Magnitude of DENV-1 viremia, WBC and platelet counts, hemoconcentration, percentages of lymphocytes and neutrophils by illness day. Medians of viremia levels and medians of haematological parameters determined in serial plasma samples from DENV-1 infected patients with A) primary DF, B) secondary DF, C) primary DHF and D) secondary DHF. The numbers below the graph indicate the numbers of patients at each time point.

Table 16: Haematological markers of the disease severity. All these markers were determined in serial plasma samples from DENV-1 infected patients with primary DF (n=15), primary DHF (n=3), secondary DF (n=91) and secondary DHF (n=33).

Variables	Median (interquartile range)			
	DF primary	DF secondary	DHF primary	DHF secondary
Maximum of hemoconcentration (%)	11,6 (6,9-14,2)	7,7 (4,3-13,4)	23,0 (21,7-26,8)	21,1 (14,6-26,5)
Illness day at maximum of hemoconcentration	7 (6-7)	6 (4-7)	4 (4-5)	5 (4-6)
Platelet nadir (x1000/mm3)	61 (49-81)	46 (33-60)	44 (42-45)	25 (17-33)
Illness day at platelet nadir	7 (7-7)	6 (5-6)	6 (6-7)	6 (5-7)
WBC nadir (x1000/mm3)	1,9 (1,2-2,0)	1,9 (1,5-2,5)	1,7 (1,6-1,8)	2,2 (1,8-2,8)
Illness day at WBC nadir	5 (4-6)	4 (4-5)	4 (4-5)	4 (3-4)

4.4 Discussion

The interplay between DEN virus infection and host immune status is postulated to play a central role in the pathophysiology of severe dengue. In this current study, we observed important features of this dynamic. First, early viremia levels were higher in patients with DHF, even if the peak viremia level was often not observed because it occurred prior to enrolment. Second, DENV-1 infections manifested as higher and longer-lived viremias, suggesting serotype dependent differences in infection kinetics. Third, the clearance of DENV viremia and NS1 antigenemia occurs earlier and faster in patients with secondary dengue and is also consistent with a faster time to defervescence.

Our findings are in agreement with previous studies that found higher viremias associated with more severe disease [237-239]. Our data also suggests that quantitative differences exist between DENV serotypes with respect to the kinetics of viremia and NS1 antigenemia. In particular, DENV-1 infections were associated with

higher and frequently longer-lived viremia levels than infections with either DENV-2 or DENV-3. This is in agreement with recent observations in Vietnamese children and adults [510, 511]. DENV-2 was associated with secondary infection and severe disease in our study; this is also in accordance with previous studies [203, 233, 511]. The mechanisms that facilitate relatively higher viremia in DENV-1 infections relative to DENV-2 infections in our study population, and also recently in Vietnamese children [510], are unknown. Plausibly, DENV-1 has an intrinsically faster rate of replication in this patient population and thereby attains a higher virus biomass *in vivo* than DENV-2. Clearly, further studies will be needed to explore this.

The duration of NS1 antigenemia was shorter in patients with secondary infections and this is consistent with previous studies that have suggested reduced sensitivity of NS1-based diagnostic tests in patients with secondary infections [318-320, 324, 512]. One explanation is that NS1 is less likely to be available for detection when a sufficient level of DENV-reactive IgG (including anti-NS1 IgG) develops during secondary infections. This may serve to mask the antigen from detection in the immunoassay, and/or result in rapid clearance of NS1 in the form of immune-complexes.

Secondary dengue is associated with faster resolution of viremia infection and shorter duration of fever. Interestingly, the daily rates of virus clearance observed in our study were very compatible with those found by Vaughn et al [238] in Thai children. The early adaptive immune response during secondary infection is dominated by populations of memory B and T cells (and possibly memory-like NK cells [513]) and at least some component of this response mediates a strong anti-viral action, as evidenced by faster clearance rates of viral biomarkers. Clearly however, aspects of this rapid host immune response are clinically deleterious given the epidemiological association between secondary dengue and more severe outcomes, and also the timing of when clinical manifestations of capillary permeability occur. This poses the intriguing question of whether modulating the host immune response (e.g. through early corticosteroid therapy) could achieve both a more gradual clearance of the virus biomass and a host immune response that elicits less pathology.

Concerning the significance of our findings with respect to possible immunopathogenetic mechanisms, our data are consistent with theories in which sequential infections can lead to more severe disease through immune enhancement. Indeed, DHF was associated with secondary infection (especially if DENV-2 infection) and higher viremia levels in the early days of symptoms, and secondary infection was associated with steeper decline in viremia and, earlier and likely higher peak viremia level. In the early phase of secondary infection, the antibody-dependent enhancement may transiently boost infection, prompting a robust anamnestic immune response and possibly contribute to more severe disease in some cases. In addition, activation of T-cells may also possibly contribute to more severe disease in secondary infection through activation of memory T-cells with lower avidity for the infecting DENV (“original antigenic sin”) resulting in altered T-cell functional responses leading to plasma leakage in DHF. In several studies, levels of T-cell activation were strongly associated with disease severity and T-cell activation was temporally related to the rapid decline in viremia and was just preceding changes in vascular permeability [293]. Libraty et al. have shown that viremia and subsequent immune activation were of greater magnitude in more severe clinical disease suggesting that a greater virus burden in severe dengue illness reflects a greater burden of virus-infected cells leading to an augmented cascade of antigen-driven innate and adaptive immune responses [239]. However differences between serotypes imply that high virus burden alone is not sufficient to cause DHF and ADE is not necessary as some patients with DHF are primary infected, emphasizing that the clinical illness is the end result of a complex interaction between DENV and the host antigenic sin. In our data, DENV-1 seems to behave differently from other serotypes, suggesting an intrinsic fitness advantage that allows development of higher viremia, even in the absence of pre-existing antibody, but DENV-1 is not associated with more DHF unlike DENV-2. It has been suggested that DENV-2 infections are more likely to be enhanced than DENV-1 infections. This is also supported by other studies in which DHF in children with secondary infection was more common for DENV-2 compared to DENV-1 [203, 205, 233].

Assuming blood viremia is a reasonable surrogate of the whole-body virus burden, then the rapid decline of viremia 48-72hrs after illness onset, especially in secondary

infections that carry higher risk for severe outcomes, has implications for therapeutic pharmacological interventions. It highlights the importance of early diagnosis, since early diagnosis will provide the greatest opportunity for an intervention (e.g. with an anti-viral), to have an impact. Point of care NS1 diagnostics are available but more can be done to improve their sensitivity [514]. More clinical research is also needed to understand if the sensitivity and specificity of early clinical diagnoses (and prognosis) can be improved, particularly in primary health care settings. Recent literature suggests this is feasible [515, 516]. The rapid decline in viremia in secondary infections also indicates an efficacious anti-viral will need favourable pharmacokinetic properties and potency if it is to impact on the viral burden in a rapid and clinically significant manner. Pharmacological targeting (e.g. with corticosteroids) of the host immune response, which accounts for the rapid decline of viremia but which also likely contributes to the capillary permeability syndrome, may equally need to be administered early on in illness e.g. in a prophylactic way, to prevent clinical complications such as DSS.

Current animal models of DENV infection are able to provide for in vivo measurements of anti-viral activity [275, 380]. However these models do not reproduce the temporal changes in virological biomarkers and clinical manifestations seen in naturally infected dengue patients. The lack of concordance between virological and clinical events in small animal models, and what occurs in patients, needs to be carefully considered when evaluating candidate anti-viral drugs for dengue.

There are several limitations to our study. Our results are derived from hospitalized patients who are not necessarily representative of patients being seen at the primary health care level. Interestingly however, the same themes identified in this study in hospitalized Vietnamese adults were also observed in Vietnamese children presenting to primary health care level clinics in Ho Chi Minh City [510]. The majority of the patients in this study were infected by DENV-1. Very few had primary DHF (4 of 248). RT-PCR measurements of viremia in plasma may not be an entirely accurate surrogate of the viral biomass in vivo, although it is certainly a better surrogate of NS1 antigenemia, which persists well after the febrile period and is heavily influenced

by the immune status of the host (i.e. primary versus secondary). A limitation of RT-PCR measurement of viremia is that viruses might be sequestered in other tissues but inaccessible to measurement while still playing a role in disease pathogenesis.

Our study emphasizes the importance of the period before and just after the onset of fever. This and other studies have established that early viremia levels are associated with disease severity, although they are very clearly not the only determinant of outcome. Very little is known of the virological events in the hours preceding and shortly after fever onset, mainly because this is very difficult to investigate without a good experimental model. An interesting insight was provided by clinical trials of DENV-1, -3 and -4 monovalent live attenuated vaccines in the 1980s [350, 517, 518]. These vaccines were not sufficiently attenuated and some volunteers developed dengue fever. These studies suggest that viremic period starts several days before the onset of symptoms. This presymptomatic viremic period should not be underestimated because of its possible contribution to DENV transmission to uninfected mosquitoes. In our study, we also observed prolonged times of virus clearance in primary dengue, and long-lived higher viremia levels in DENV-1 infections. These might lead to a higher possibility of human to mosquito virus transmission by maintaining viremia above the infectious level over a longer period of time.

Collectively, our findings reveal important patterns in the relationship between viremia kinetics, patient immune status and disease severity that have implications for understanding physiopathology, epidemiology and for rational design of therapeutic interventions.

General Conclusion

This work in this thesis has provided new insights into diagnostic methods and therapeutic interventions in dengue, data that is critical for rational design of interventions and for understanding disease pathophysiology.

The search for an antiviral to treat dengue is a new endeavour that is gaining momentum due to both increased awareness of dengue as a public health problem and substantial progress in the structural biology of DENV [1]. Commercial and non-commercial research groups are currently performing preclinical testing of molecules *in vitro* and *in vivo* with potential activity against dengue viruses [519]. However, translation of these preclinical findings to a clinically efficacious drug remains to be achieved. In this thesis, we tested a safe and cheap molecule with broad antimicrobial activity for which we had a reasonable body of preclinical data to expect clinical benefits in dengue. By this first evaluation of a drug to treat acute dengue, we have set out a strategy that is instructive for others to follow in terms of patient enrolment strategies, safety assessments and clinical and virological endpoints. In addition, our study provides a good example of clinical research in resource-limited settings as advocated by Lang et al. [520]. Moreover this is in agreement with the shift of infectious disease research from the developed world to the countries that are most affected by those illnesses [521]. Unfortunately, no effect of CQ was noted on duration of circulation of DENV or NS1 protein or on days of fever. This could be due to various reasons including an absence of CQ activity on DENV at the concentrations attained *in vivo* or an inadequate sample size. Interpretation of results from “negative” RCTs is difficult. These have usually been designed to show an effect and it is important to keep in mind that the absence of evidence does not imply evidence of absence [522]. But the potential “antiviral career” of CQ is not yet over as suggested by the interest shown by recent publications [523, 524]. Another interventional study is ongoing at the Oxford University Clinical Research Unit - Hospital for Tropical Diseases: a study of balapiravir (a drug from Roche initially

designed for HCV infections) in patients with DENV infection (ClinicalTrials.gov NCT01096576).

One of the biggest challenges in testing (and operationally using) an antiviral drug in acute infection is that to be effective the drug should presumably be delivered early after onset of symptoms. This makes the patient recruitment a difficult task: it is necessary to enrol the right persons (ideally patients with a confirmed diagnosis of acute dengue) at the right time (i.e. early in the disease course). One lesson of our study is that a successful recruitment of patients with less than 72 hrs of symptoms only based on suspicion of dengue (i.e. clinical criteria) is achievable but it needs experienced nurses and doctors and adequate laboratory infrastructure to help support the diagnosis. NS1 detection tests and particularly RDTs can improve such recruitment and enrich for the patients with the greatest viremia levels [318, 525]. A point highlighted in this thesis and by other publications is that these NS1 tests can have high specificity during early infection [320, 322, 525, 526]. Our NS1 RDTs evaluation and many other studies have shown that, in the first 3 days of illness, most patients who are NS1 positive are also PCR positive [317, 322, 334, 527, 528]. In addition, patients with detectable plasma NS1 levels have higher viremia levels than patients who are NS1 negative at the same timepoint [318, 320]. Indeed at the time of enrolment into our chloroquine trial, viremia levels were significantly higher in patients who were NS1 positive versus those who were NS1 negative. Accordingly, NS1 detection should allow recruitment of patients with higher viremia, who are of greater interest in an antiviral trial. In such trials, specificity is a priority compared to sensitivity and the fact that not every acute dengue case has detectable NS1 antigenemia is not a problem for recruitment of patients in endemic countries during epidemics when the number of cases is very high. Nevertheless, there is obviously room to improve NS1 tests such that they are sensitive enough to detect all patients with dengue. This might require efforts to dissociate NS1 immune complexes in plasma/serum that could mask the detection of NS1 [318, 320].

In order to deliver the drug early after onset of symptoms, we have enrolled patients with no more than 72hrs of symptoms but this deadline is probably not restrictive enough as our study of viremia kinetics has shown. In the Balapiravir study currently

being conducted at the OUCRU, the recruitment targets patients with confirmed dengue virus infection whose symptoms began within the 48 hours preceding the first drug administration. A major problem is the vast majority of dengue patients present to hospital settings later in the course of their illness. Future trials would then have to capture patients presenting to primary care facilities, where it would be possible to enrol patients at earlier stage of the disease. Also because dengue is mainly a benign disease, the clinical development of antiviral dengue drugs would anyway necessitate large-scale trials focused on ambulatory patients. Because of some advantages compared to ELISA NS1 tests: cheapness, easiness and rapidness, NS1 RDTs seem to us the best tool for the necessary early point-of-care diagnosis. If during an “ideal world” trial (i.e. ideal conditions) it is possible to recruit patients at early stage, is the timeframe for dosing within 48 or 72 hrs realistic for a disease that frequently manifests with nonspecific symptoms in the early phase? What would the deadline be to have a minimal clinical efficacy?

Another challenge is the choice of a correct primary endpoint and particularly the dilemma between clinical or virological endpoints. Efficacy and effectiveness studies should not be confused and it is critical to find an endpoint that reflects the most relevant clinical benefits while “keeping an eye on” the potential antiviral effect. As Thomas Fleming said, “a correlate does not a surrogate make” [529], and the overall priority and aim for an antiviral is to reduce clinical severity with the expectation that this would also be accompanied by evidence of *in vivo* antiviral activity. It is possible that an antiviral effect could be demonstrated in dengue patient who start the treatment several days after illness onset without any clinical benefit if the cascade of events that drives the patient towards severe dengue has already commenced earlier in the disease course. With an antiviral, the clinical benefits we would hope for are a shortening of the duration of signs and symptoms and a reduction in the frequency of patients developing severe complications such as DSS.

However at this stage of exploratory testing of a molecule, it was not realistic to test for clinical efficacy, as sample size would be too large. The primary endpoints of our study were chosen even if the relationship between duration of viremia and antigenemia and duration of illness has not been validated. Clinically, shortening the

disease is probably not a convincing endpoint and reduction of symptoms seems to be more adequate as vascular leakage leading to DSS are the major concern [1]. Nevertheless, it is very likely that aiming to shorten the disease duration would also lead to reduction of the severity of symptoms as observed with oseltamivir in treating acute influenza [530, 531]. Concerning the virological endpoints, if viremia is not necessarily the best reflection of the viral burden, measuring viremia is probably the easiest way to demonstrate an antiviral activity in patients. A major finding of our work is that only viremia levels during the early acute phase (until illness day 3) are associated with disease severity (see chapter 5). This makes the therapeutic window when antivirals could be used to treat dengue very narrow. Another finding is that complex relationships between viremia kinetics, disease severity, patient immune status and dengue serotype make RCTs only based on virological endpoints haphazard. To us, it is clear that both clinical endpoint (as DF vs DHF or reduction of hemoconcentration) and virological endpoint (as reduction of viremia levels measured by daily monitoring) need to be assessed.

A limitation of our trial is the breadth and depth of data concerning the immune response. The way we have monitored the immune response has not been sufficient and cytokines levels should be more often measured over the disease. It is very likely that an antiviral that brings a clinical benefit would also affect certain features of the immune response. Another option is to study gene expression through microarray as described previously in Vietnamese dengue patients [263, 264].

As a Sanofi-Pasteur dengue vaccine candidate has entered in phase III of its clinical development with an expected licensing in 2015-2016, one more challenge will be to find the right place for dengue antivirals when available. Dengue is the most important arboviral disease of humans [532]. The global air transport network is not only aiding the spread of both dengue vectors and virus serotypes within tropical regions suitable for transmission, but also facilitating a substantial increase of imported cases elsewhere [533-535]. The vaccine would be the ideal for countries where dengue is endemic, but the use of an oral antiviral when there is a new outbreak could prove to be more efficient and cheaper than mass vaccination. An interesting parallel can be made with the situation of yellow fever or influenza. In the Mid-20th

Century, an intensive *A. aegypti* eradication campaign was initiated in the Americas, which succeeded in reducing vector populations to undetectable levels throughout most of its range. The production of an effective vaccine in the 1930s led to a change of emphasis from vector control to vaccination for the control of yellow fever. Vaccination campaigns almost eliminated urban yellow fever but incomplete coverage, poor anti-vectorial measures and existence of a sylvatic cycle meant the disease persisted, and outbreaks in remote areas continued [536, 537]. Vaccination alone cannot eradicate yellow fever. To prevent seasonal flu, a vaccine is made every year based on international surveillance and estimations about which types and strains of viruses will circulate in a given year. The CDC considers that influenza vaccination is the most effective method for preventing influenza virus infection and its potentially severe complications. However in 2009, oseltamivir tablets, the first orally active neuraminidase inhibitor commercially developed to treat seasonal flu, were a major component of the public health response to pandemic H1N1 outbreaks in 2009. Thus, the licensing of a dengue vaccine is unlikely to negate the use of a dengue antiviral were it available.

The recent dengue history has seen a marked global emergence and re-emergence with more frequent and larger epidemics and consequently more cases with severe disease. We believe there is a strong argument that an antiviral could be a useful weapon in the fight against dengue. We hope that our work in this thesis will contribute to making a licensed dengue antiviral become reality.

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Annexes

Investigator's Brochure

A research study of the role of chloroquine in treating patients with dengue.

ISRCTN38002730 (International Standard Randomized Controlled Trial Number)

Product: chloroquine

International Nonproprietary Name (INN): chloroquine

Systematic (IUPAC) name: N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine

CAS number: 54-05-7

Sponsor's name: University of Oxford (UK)

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Disease: Dengue fever

Study design: Double blind, randomized, placebo-controlled trial

Location of trial: Hospital for Tropical Diseases

Address: 190 Ben Ham Tu, Dist. 5, Ho Chi Minh City, Viet Nam

Sources of funding: Wellcome Trust (ref: 061330) (UK)

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1. Introduction

1.1. Dengue

Dengue is an acute febrile disease caused by one of four closely related serotypes of DENV, genus *Flavivirus* and family *Flaviviridae*. Each serotype is sufficiently different that there is no cross-protection. Recovery from infection provides thus lifelong immunity against one serotype but not against the three others and epidemics caused by multiple serotypes (hyperendemicity) can occur. DENV is transmitted to humans by the mosquito *Aedes aegypti* (and rarely *Aedes albopictus*).

DENV has re-emerged in recent years as an increasingly important public health threat affecting more than 100 countries worldwide (mostly in tropical and sub-tropical areas), with nearly 50 million infections each year and over 2.5 billion people at risk. An estimated 500,000 cases require hospitalization each year; a large proportion of which are children [1]. Infection with DENV produces a wide spectrum of clinical illness ranging from silent infection to either a mild febrile, self-limited acute syndrome known as dengue fever (DF) or the severe and possibly fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There is neither specific chemotherapy nor vaccine for treatment and prevention of DENV infection. The development of vaccines for DF and DHF is difficult in particular because that may protect against all four serotypes of DENV, rather than just one. Supportive medical care and symptomatic treatment through hydration are the most important aids to patients and to improve survival in the severe forms of disease. Consequently, new approaches for the control of DENV infection are urgently needed.

1.2. Chloroquine

CQ is a synthetic drug used for the treatment and prevention of malaria. It is a member of an important series of chemically related antimalarial agents: the 4-aminoquinoline derivatives and it has been introduced into medicine in the 1940s. CQ is generally administered orally or intramuscularly. CQ is effective against susceptible strains of the malarial parasites as well as certain parasitic worms and amoebas. CQ has long been the mainstay first-line therapy for uncomplicated *P. falciparum* infection. However, resistance to it now exists in most parts of the world and its use as a single first-line drug treatment in *falciparum* malaria is now increasingly limited. But CQ remains the most commonly used treatment for infections with *P. vivax*, *P. ovale*, and *P. malariae* although several studies have reported resistance to CQ in *P. vivax* in Oceania and one study has reported resistance to CQ in *P. malariae* in Indonesia.

A recent study suggests that CQ might once again be effective against *falciparum* malaria after the withdrawal from use. The authors have shown that in Malawi *P. falciparum* parasites have become susceptible to CQ again, 12 years after it was withdrawn from use due to parasite resistance to the drug. Nevertheless they warn that CQ should not be re-introduced as a front-line treatment, but could be used as an intermittent therapy for pregnant women or in combination with other drugs [2].

As CQ has also immuno-modulatory properties, it is used in some inflammatory and autoimmune disorders, such as arthritis in children, high levels of calcium in the blood associated with sarcoidosis, rheumatoid arthritis, systemic lupus erythematosus (lupus; SLE) and various skin disorders.

From few decades, new potential utilizations of this cheap and widely available drug have been investigated. For example, chloroquine is in clinical trials as an investigational antiretroviral in humans with HIV-1/AIDS (see below) and as a potential antiviral agent against chikungunya fever (CuraChik: double blind placebo-controlled randomized trial: efficacy and safety of chloroquine as therapeutic treatment of chikungunya disease. ClinicalTrials.gov identifier: NCT00391313). Moreover, the radio-sensitizing and chemo-sensitizing properties of chloroquine are beginning to be exploited in anticancer strategies in humans [3].

2. Physical, Chemical, and Pharmaceutical Properties and Formulation

2.1. Description of investigational medicinal compound

International Nonproprietary Name (INN): chloroquine

Formula: C₁₈H₂₆N₃Cl

Mol. weight: 319.872 g/mol

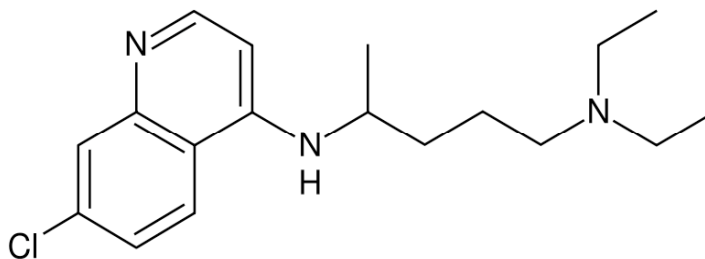
Systematic (IUPAC) name: N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine

Identifiers:

- CAS number: 54-05-7

- ATC code: P01BA01

Structural diagram of CQ:



CQ (diphosphate or sulfate) is a white, odorless, bitter tasting, crystalline powder, freely soluble in water, very little soluble in alcohol and not soluble in organic solvents. CQ should be stored at 25° C (77° F); excursions permitted to 15° - 30° C.

2.2. Description of the formulation

Each drug tablet contains 150mg of chloroquine base. The list of excipients and the description of the placebo are not yet available.

3. Non-clinical Studies

3.1. In vitro anti-viral activity of CQ

3.1.1. General mechanisms of viral inhibition by CQ

CQ/hydroxychloroquine (HCQ is chemically closely related to chloroquine) can impair the replication of several viruses by interacting with the endosome-mediated viral entry or the late stages of replication of enveloped viruses (reviewed in [4]).

3.1.1.1. Endosome-mediated viral entry interaction

Some viruses enter their target cells by endocytosis. This process targets the virus to the lysosomal compartment where the low pH, along with the action of enzymes, disrupts the viral particle, thus liberating the infectious nucleic acid and, in several cases, enzymes necessary for viral replication. CQ has been shown to inhibit different viruses requiring a pH-dependent step for entry, such as the Borna disease virus [5], the minute virus of mice MVMp [6], and the avian leucosis virus [7]. Of particular interest for human pathology is the report that CQ inhibits uncoating of the hepatitis A virus, thus blocking its entire replication cycle [8]. But for Sindbis virus, exposure to low pH is not required for penetration of mosquito cells and chloroquine does not block it [9].

3.1.1.2. Replication of enveloped viruses interaction

For some enveloped viruses, post-translational modifications of envelope glycoproteins occur within the endoplasmic and trans-Golgi network (TGN) vesicles. This process involves proteases and glycosyl-transferases, some of which require a low pH. In line with the pH-dependence of these events, CQ was seen to inhibit budding of Mayaro virus particles [10], and to induce accumulation of non-infectious herpes simplex virus 1 particles in the TGN [11]. CQ also inhibits the replication of members of the Flaviviridae family by affecting the normal proteolytic processing of the flavivirus prM protein to M [12]. As a result, viral infectivity is impaired. Finally CQ induces the production of non-infectious retrovirus particles as shown with the avian reticulo-endotheliosis virus REV-A and with HIV-1 [13]. The mechanism of inhibition seems to be inhibition of glycosylation of the envelope glycoproteins (see below).

3.1.1.3. Glycosylation inhibition

CQ inhibits the glycosylation of the viral envelope glycoprotein gp120, which occurs within the Golgi apparatus. These effects appeared to be specific, since the CQ concentrations effective in vitro neither affected any other step in HIV-1 replication nor were cytotoxic [14]. Glycosylation inhibition might represent a major mechanism for the antiviral effects of CQ, suggesting that specific interactions of CQ with sugar-modifying enzymes or glycosyltransferases may occur within human cells [15].

CQ was recently shown to inhibit quinone reductase 2 [16], a structural neighbour of UDP-N-acetylglucosamine 2-epimerases, which are involved in sialic acid biosynthesis. If CQ should indeed inhibit the biosynthesis of sialic acid, this effect could explain not only the effects of CQ on HIV and SARS coronavirus (sialic acid moieties are present in HIV-1 glycoproteins and SARS coronavirus receptor ACE2), but also the in-vitro effects on orthomyxoviruses (which use sialic acid moieties as receptors [17]).

3.1.2. Anti-SARS coronavirus activity

It has been found that CQ inhibited SARS coronavirus replication with a 50% effective concentration of $8.8\mu\text{mol/L}$ (i.e. within the range of blood concentrations achievable during antimalarial treatment). The dose inducing 50% cytostatic activity was much higher ($261.3\mu\text{mol/L}$) [18]. Time-of-addition experiments indicated that CQ affected an early stage of SARS coronavirus replication. It has been reported potent anti-SARS coronavirus effects of CQ in vitro, attributable to a deficit in the glycosylation of the SARS coronavirus receptor ACE2. Again, the antiviral drug concentrations were not cytotoxic [19].

3.1.3. Anti-influenza virus activity

The effect of CQ against replication of Orthomyxoviridae has long been known. Inhibitory effects of CQ on both type A and B influenza viruses have been described [20, 21]. Savarino et al. investigated the inhibitory effect of CQ on an avian influenza virus strain. Depending on the viral challenging doses and the methods adopted to detect the antiviral effects, the inhibitory concentrations fell within a clinically achievable in plasma during malaria treatment concentrations range [15]. However, they found that the effects of chloroquine depend a lot on the Multiplicity of Infection used and, when they tried on different avian influenza viruses, vary from strain to strain (these data will be published soon).

3.1.4. Anti-DENV activity

The in vitro inhibitory concentration (IC_{50}) for chloroquine against NGC DENV2 strain is $\sim 5\mu\text{M}$ i.e. about 1.5 mg/mL (from Novartis Institute for Tropical Diseases, Singapore). But IC_{50} might depend on DENV strains and the cell culture types.

3.2. In vitro immunomodulatory activity (reviewed in [4])

The accumulation of CQ/HCQ in lymphocytes and macrophages results in anti-inflammatory properties, and has led to its clinical use in inflammatory and autoimmune disorders, for example sarcoidosis, a disease characterized by an overproduction of tumor necrosis factor α ($\text{TNF}\alpha$) by the alveolar macrophages [22]. CQ/HCQ reduces the secretion of these pro-inflammatory cytokines and in particular $\text{TNF}\alpha$, as shown in a murine macrophage cell line [23], and in primary cells such as mouse peritoneal macrophages [24], human peripheral blood mononuclear cells [25], and human whole blood [26].

Several mechanisms have been evoked to explain the CQ/HCQ-induced inhibition of TNF α production by monocyte-macrophages: disruption of cellular iron homeostasis [27], inhibition of TNF α mRNA expression [24], inhibition at a pretranslational stage by a non-lysosomotropic mechanism [28], or at a post-translational stage by blocking the conversion from cell-associated pro-TNF α to a soluble mature form [23]. Apart from inhibiting TNF α production by stimulated monocyte-macrophages, CQ also decreases the surface expression of TNF α receptors in human monocytic cell lines and, hence, the receptor-mediated TNF α signaling [29].

CQ inhibit the antigen processing and presentation (specially a low pH requiring steps: digestion of the protein by acidic hydrolases in the lysosomes, for assembly of the α - β -peptide complex and for its transport to the cell surface) [30].

CQ is known also to block the nuclear factor-kappaB (NF- κ B) induction by interleukin-1 β in epithelial cells and to increase human CD8+ T cell responses against soluble antigens in vivo and the cross-presentation efficiency in vitro [31, 32].

4. Toxicology Studies

See Marketing Experience

5. Effects in Humans

5.1. Pharmacokinetics and Products Metabolism in Humans (reviewed in [33, 34])

CQ is administrated orally (solution or tablet), subcutaneously, intramuscularly and even rectally. If given by intravenous injection, CQ may cause potentially lethal cardiovascular disturbances. The data below concern only the oral administration.

5.1.1. Pharmacokinetics in healthy individuals (and patients with arthritis)

5.1.1.1. Absorption and bioavailability

When administrated orally, CQ is rapidly and almost completely absorbed from the gastrointestinal tract. Its bioavailability is about 80%.

5.1.1.2. Distribution

5.1.1.2.1. Blood and tissue concentrations

The times to reach the maximum concentration in blood circulation following CQ administration (T_{max}) range from approximately 2 to 6 hours. Following single oral dose (600mg base), the peak CQ concentrations (C_{max}) in plasma approximate 1 μ M (i.e. about 0.3 μ g/mL). Following a 3-day course of therapeutical oral doses of CQ (600mg base on days 1 and 2, then 300mg base on day), the peak drug concentrations (C_{max}) range from 0.1 to 0.3 μ g/mL in plasma and 1 to 1.2 μ g/mL in whole blood [35, 36].

In patients chronically treated with CQ (150mg base daily), steady-state CQ plasma concentrations are in the micromolar range with a very high interpatient variability.

After the peak concentration, whole blood or plasma concentrations decline multiexponentially as the drug is distributed to produce an apparent volume of distribution (V_d) several orders of magnitude larger than that of the central compartment (200L/kg when calculated from whole blood concentrations to 200 to 800L/kg from plasma data). This extensive V_d illustrates the ability of the drug to bind to various body tissues, particularly the cellular components of blood.

Following oral administration, whole blood concentrations are 5 to 10 times higher than those observed in plasma, with high proportions of the drug and its main metabolite bound to platelets and granulocytes (CQ concentration versus time course in whole blood was parallel to that in plasma illustrating distribution differences and excluding any metabolism within the blood cells). N.B.: Serum concentrations are higher than plasma concentrations because of the release of platelet-bound chloroquine during clotting.

CQ is extensively sequestered in the tissues; the highest concentrations of approximately 100 μ M were measured in the marrow, liver, spleen and leukocytes.

5.1.1.2.2. Lysosomal trapping

CQ is a weak base with a 4-amino-quinoline nucleus at one end of the planar molecule and a lipophilic side chain at the other end. At physiological pH, a significant concentration of lipophilic free base is in equilibrium with its protonated form. The drug passively diffuses into the cell in a lipophilic unprotonated state, where it becomes protonated and concentrated in acidic, low-pH organelles, such as endosomes, Golgi vesicles, and lysosomes and it increases the pH (entry into the cell is by passive diffusion whereas the trapping mechanism involves a proton pump, which requires energy). The extensive tissue distribution of chloroquine is likely to result from its amphiphilic nature.

It is uncertain whether the weak base effect plays a role pharmacologically. Platelets and lymphocytes are able to concentrate chloroquine by ion trapping within the acidic lysosomes. While red blood cells do not contain these organelles, malaria infected erythrocytes can accumulate CQ inside the acidic digestive food vacuole(s) of the parasite, according to the pH gradient.

5.1.1.2.3. Protein binding

Between 50% and 60% of CQ is bound to non-diffusible plasma constituents (mainly albumin and α 1-acid glycoprotein). CQ binding to plasma is concentration independent. There is a modest stereo-selective difference because around 65% of the (+)-CQ and 45% of (-)-CQ is bound to plasma proteins (the drug is the racemate).

5.1.1.3. Elimination and metabolism

Both the kidney and the liver contribute to CQ elimination. In urine, approximately 50% of a given dose is recovered as unchanged CQ and 10% as desethylchloroquine (DCQ), its primary metabolite. Excretion of CQ is quite slow, but is increased by acidification of the urine. Despite its long CQ terminal elimination half-life, ranging from 20 to 60 days, CQ has a relatively high total clearance approximating 0.10 L/h/kg from whole blood data and 0.7 to 1 L/h/kg from plasma data. Distribution and redistribution processes (from various body compartments back to the intravascular space) rather than slow drug elimination determine the blood concentration profile of CQ. The dosage may have to be modified in patients with renal or/and hepatic insufficiency.

Approximately 30 to 50% of an administered dose of CQ is transformed by the liver. Following oral administration, CQ is rapidly dealkylated into the pharmacologically active DCQ and bidesethylchloroquine (BDCQ). Some minor metabolites are formed by oxidative deamination, elimination of the side chain and N-oxidative pathways. DCQ is rapidly detected in blood or plasma, its concentrations amounting to 20 to 50% of those parents. For BDCQ, plasma or blood concentrations never reach more than 10 to 15% of CQ levels.

An in vitro study suggested that CYP2C8 and CYP3A4 are the most active isoforms of P450 contributing collectively to more than 80% of total CQ N-desethylation and that CYP2D6 play also a significant role at low CQ concentrations [37]. In addition, there may be a non-microsomal metabolism.

5.1.2. Pharmacokinetics in malaria

5.1.2.1. Absorption and Distribution

In Nigerian children with acute uncomplicated malaria, no difference between healthy individuals and patients with malaria was observed. In Thai patients suffering from *P. vivax* malaria, significantly higher blood concentrations and AUCs of both CQ and DCQ were observed. Since CQ pharmacokinetics showed no difference between Thai individuals and patients with malaria following intravenous administration. The authors concluded that malaria-induced absorption changes contributed to the increased systemic exposure to CQ. Indeed higher plasma and blood concentrations during the initial phase of treatment have been observed for other malarial agents. Since acute malaria can induce elevations in α 1-acid glycoprotein level, bound rather than unbound plasma drug concentrations might be increased. Since CQ binding to plasma proteins is moderate, it may not be clinical significance.

Others authors correlated the CQ red cell/plasma ratio with the parasitaemia. Drug-sensitive parasites seem to concentrate quinoline derivatives, differences in red cell concentrations being a reflection of varying degrees of parasitaemia and different stages of parasite development.

5.1.2.2. Elimination

Following a single intravenous dose of CQ the elimination half-time and clearance there were significantly different between healthy individuals and patients with malaria. Liver dysfunction often been reported with malaria and variations in hepatic metabolism could explain some of the variability in CQ pharmacokinetics since, in patients with malaria, there was considerable variation in the ratio of metabolite concentrations.

5.1.3. Pharmacokinetics In malnutrition

5.1.3.1. Absorption and distribution

Following a single dose of CQ (600mg base), there was no difference in the plasma or whole blood concentrations between healthy and malnourished individuals up to 72 hours post-dose. Since malnourished individuals received a higher CQ dose on a mg/kg of bodyweight basis, they appeared to be handling chloroquine more efficiently than healthy individuals. Similarly, after the oral administration of a single dose of 10 mg/kg to Nigerian children, peak plasma CQ concentrations were lower in children with Kwashiorkor than in healthy children. Despite a wide interindividual variability, plasma concentrations remained lower throughout the sampling period leading to smaller AUCs in malnourished children. A Kwashiorkor-induced reduction in chloroquine absorption is a probable explanation for the decreased plasma concentrations of both the parent and metabolite.

5.1.3.2. Elimination

Protein caloric malnourishment can affect drug metabolism, but malnutrition-induced variations in CQ metabolism are not ruled out yet. In malnourished mice the rate of metabolism of CQ was reduced.

5.1.4. Pharmacokinetics In different ethnic groups

The assessment of the relative importance of the 3 variables, disease, nutrition and ethnic origin is a difficult task. Genetic and environmental factors are often indistinguishable as diseases have geographical-dependant incidences, and different ethnic group have typical diets and beverages.

5.1.4.1. Absorption and distribution

In healthy Sudanese individuals, when CQ 600mg base was administered with local beverages, its bioavailability was considerably reduced, mean CQ peak plasma concentrations and AUC 0->24h were decreased by 62 to 73%. Since the 3 selected beverages had a very acidic pH (2.2 to 2.6), reduced CQ could have resulted from decreased intestinal absorption. Increased renal excretion of ionized molecules would be unlikely to occur following a single dose of acidic beverage.

Food was found to enhance CQ bioavailability in healthy Indian volunteers. But CQ bioavailability showed considerable interindividual variability: with or without food, CQ peak plasma concentrations and AUC 0-12h varied 3-fold between individuals.

5.1.4.2. Elimination

Hepatic-oxidative metabolism, which is dependent upon genetic and environmental factors, is a major determinant of drug pharmacokinetics and pharmacodynamics. Since CYP2D6 is implicated in CQ metabolism and frequencies of poor metabolisers in Asians, Blacks and Caucasian individuals and the CYP2D6 activity are different, this might have important clinical significance.

5.2. Anti-Plasmodium activity

Chloroquine has marked and rapid schizonticidal activity against all infections of *P. malariae* and *P. ovale* and against chloroquine-sensitive infections of *P. falciparum* and *P. vivax*. It is also gametocytocidal against *P. vivax*, *P. malariae* and *P. ovale* as well as immature gametocytes (stages 1-3) of *P. falciparum*. It is not active against intrahepatic forms, and should therefore be used with primaquine to effect radical cure of *P. vivax* and *P. ovale*.

Chloroquine interferes with parasite haem detoxification. Resistance is related to genetic changes in transporters (PfCRT, PfMDR), which reduce the concentrations of chloroquine at its site of action, the parasite food vacuole [38].

5.3. Anti-HIV activity

CQ/HCQ has been adopted to treat HIV-1-infected patients in clinical trials. CQ (250mg twice daily) has been administered to HIV-1-infected patients with baseline viral loads over 50000 copies per mL, in combination with lamivudine (150mg twice daily) and hydroxyurea (HU) (500mg twice daily) in an ongoing clinical trial in India. Ten out of 18 volunteers had an undetectable viral load at week 24. The median drop in viral load was more than 2.0 log, more than the median 1.5 log drop seen with a nucleoside reverse transcriptase inhibitor (NRTI) and HU alone. These results are different from those of another trial in Singapore using didanosine (125–250mg twice daily), HU (500mg twice daily), and HCQ (200mg twice daily, corresponding to 125mg of CQ). The median drop in viral load was 1.3 log, similar to that induced by a NRTI plus HU. Follow-up of these patients at week 144 suggests that the value of HCQ may lie in the maintenance of the effects of didanosine/HU.

The discrepancy between the two studies, besides differences in the design and patients enrolled, probably reflects the different dosages of CQ/HCQ. Drops in viral load are reported to occur using daily doses of 800 mg of HCQ, 1 corresponding to 500 mg of CQ (as used in the Indian study), but not using 250 mg of CQ daily, 5 corresponding to 400 mg of HCQ (as adopted in the Singapore study).

5.4. Safety and efficacy

See Marketing Experience

6. Marketing Experience (from the last FDA label for Aralen®)

Chloroquine has a well-documented adverse effects profile due to an about 50 years attendance on market.

6.1. Contraindications

Use of CQ is contraindicated in the presence of retinal or visual field changes either attributable to 4-amino-quinoline compounds or to any other etiology, and in patients with known hypersensitivity to 4-amino-quinoline compounds. However, in the treatment of acute attacks of malaria caused by susceptible strains of plasmodia, the physician may elect to use this drug after carefully weighing the possible benefits and risks to the patient.

6.2. Warnings

Irreversible retinal damage has been observed in some patients who had received long-term or high-dosage 4-amino-quinoline therapy. Retinopathy has been reported to be dose related. When prolonged therapy with any antimalarial compound is contemplated, initial (base line) and periodic ophthalmologic examinations (including visual acuity, expert slit-lamp, funduscopy, and visual field tests) should be performed. If there is any indication (past or present) of abnormality in the visual acuity, visual field, or retinal macular areas (such as pigmentary changes, loss of foveal reflex), or any visual symptoms (such as light flashes and streaks) which are not fully explainable by difficulties of accommodation or corneal opacities, the drug should be discontinued immediately and the patient closely observed for possible progression. Retinal changes (and visual disturbances) may progress even after cessation of therapy.

All patients on long-term therapy with this preparation should be questioned and examined periodically, including testing knee and ankle reflexes, to detect any evidence of muscular weakness. If weakness occurs, discontinue the drug.

A number of fatalities have been reported following the accidental ingestion of chloroquine, sometimes in relatively small doses (0.75 g or 1 g chloroquine phosphate in one 3-year-old child). Patients should be strongly warned to keep this drug out of the reach of children because they are especially sensitive to the 4-amino-quinoline compounds.

Use of CQ in patients with psoriasis may precipitate a severe attack of psoriasis. When used in patients with porphyria the condition may be exacerbated. CQ should not be used in these conditions unless the benefit to the patient outweighs the potential risks.

Usage in Pregnancy: Radioactively tagged CQ administered intravenously to pregnant pigmented CBA mice passed rapidly across the placenta and accumulated selectively in the melanin structures of the fetal eyes. It was retained in the ocular tissues for five months after the drug had been eliminated from the rest of the body. There are no adequate and well-controlled studies evaluating the safety and efficacy of CQ in pregnant women. Usage of CQ during pregnancy should be avoided except in the suppression or treatment of malaria when in the judgment of the physician the benefit outweighs the potential risk to the fetus.

6.3. Precautions

6.3.1. Hematological effects

Complete blood cell counts should be made periodically if patients are given prolonged therapy. If any severe blood disorder appears which is not attributable to the disease under treatment, discontinuance of the drug should be considered.

CQ should be administered with caution to patients having G-6-PD (glucose-6 phosphate dehydrogenase) deficiency because of the risk of hemolytic anemia.

6.3.2. Auditory effects

In patients with preexisting auditory damage, CQ should be administered with caution. In case of any defects in hearing, CQ should be immediately discontinued, and the patient closely observed (see Adverse reactions).

6.3.3. Hepatic effects

Since this drug is known to concentrate in the liver, it should be used with caution in patients with hepatic disease or alcoholism or in conjunction with known hepatotoxic drugs.

6.3.4. Central Nervous System Effects

Patients with history of epilepsy should be advised about the risk of CQ provoking seizures.

6.4. Drug Interactions

Antacids and kaolin: Antacids and kaolin can reduce absorption of CQ; an interval of at least 4 hours between intake of these agents and CQ should be observed.

Cimetidine: Cimetidine can inhibit the metabolism of CQ, increasing its plasma level. Concomitant use of cimetidine should be avoided.

Ampicillin: In a study of healthy volunteers, CQ significantly reduced the bioavailability of ampicillin. An interval of at least two hours between intake of this agent and CQ should be observed.

Cyclosporine: After introduction of CQ (oral form), a sudden increase in serum cyclosporine level has been reported. Therefore, close monitoring of serum cyclosporine level is recommended and, if necessary, CQ should be discontinued.

6.5. Nursing Mothers

Because of the potential for serious adverse reactions in nursing infants from CQ, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the potential clinical benefit of the drug to the mother.

6.6. Geriatric Use

Clinical studies of CQ did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. However, this drug is known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection and it may be useful to monitor renal function.

6.7. Adverse reactions

Special Senses:

Ocular: Irreversible retinal damage in patients receiving long-term or high-dosage 4-amino-quinoline therapy; visual disturbances (blurring of vision and difficulty of focusing or accommodation); nyctalopia; scotomatous vision with field defects of paracentral, pericentral ring types, and typically temporal scotomas, e.g., difficulty in reading with words tending to disappear, seeing half an object, misty vision, and fog before the eyes.

Auditory: Nerve type deafness; tinnitus, reduced hearing in patients with preexisting auditory damage.

Musculoskeletal system: Skeletal muscle myopathy or neuromyopathy leading to progressive weakness and atrophy of proximal muscle groups, which may be associated with mild sensory changes, depression of tendon reflexes and abnormal nerve conduction, have been noted.

Gastrointestinal system: Anorexia, nausea, vomiting, diarrhea, abdominal cramps.

Skin and appendages: Pleomorphic skin eruptions, skin and mucosal pigmentary changes; lichen planus-like eruptions, pruritus, photosensitivity and hair loss and bleaching of hair pigment.

Hematologic system: Rarely, aplastic anemia, reversible agranulocytosis, thrombocytopenia and neutropenia.

Central Nervous system: Convulsive seizures, mild and transient headache, neuropsychiatric changes including psychosis, delirium, personality changes and depression.

Cardiovascular system: Rarely, hypotension, electrocardiographic changes (particularly, inversion or depression of the T-wave with widening of the QRS complex), and cardiomyopathy.

6.8. Overdosage

6.8.1. Symptoms

CQ is very rapidly and completely absorbed after ingestion. Toxic doses of CQ can be fatal. As little as 1 g may be fatal in children. Toxic symptoms can occur within minutes. These consist of headache, drowsiness, visual disturbances, nausea and vomiting, cardiovascular collapse, shock and convulsions followed by sudden and early respiratory and cardiac arrest. The electrocardiogram may reveal atrial standstill, nodal rhythm, prolonged intraventricular conduction time, and progressive bradycardia leading to ventricular fibrillation and/or arrest.

6.8.2. Treatment

Treatment is symptomatic and must be prompt with immediate evacuation of the stomach by emesis (at home, before transportation to the hospital) or gastric lavage until the stomach is completely emptied. If finely powdered, activated charcoal is introduced by stomach tube, after lavage, and within 30 minutes after ingestion of the antimalarial, it may inhibit further intestinal absorption of the drug. To be effective, the dose of activated charcoal should be at least five times the estimated dose of CQ ingested.

6.8.3. Convulsions

If present, should be controlled before attempting gastric lavage. If due to cerebral stimulation, cautious administration of an ultra short-acting barbiturate may be tried but, if due to anoxia, it should be corrected by oxygen administration and artificial respiration. Monitor ECG. In shock with hypotension, a potent vasopressor should be administered. Replace fluids and electrolytes as needed. Cardiac compressing or pacing may be indicated to sustain the circulation. Because of the importance of supporting respiration, tracheal intubation or tracheostomy, followed by gastric lavage, may also be necessary. Peritoneal dialysis and exchange transfusions have also been suggested to reduce the level of the drug in the blood.

NB: A patient who survives the acute phase and is asymptomatic should be closely observed for at least six hours. Fluids may be forced, and sufficient ammonium chloride (8 g daily in divided doses for adults) may be administered for a few days to acidify the urine to help promote urinary excretion in cases of both overdosage or sensitivity.

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Chloroquine for dengue.

Study Code: FR

OXTREC 005-06

1. Principal investigators

Dr Tran Tinh Hien, Dr Cameron Simmons, Dr Jeremy Farrar

2. Institution and Department

Oxford University Clinical Research Unit Ho Chi Minh City Viet Nam

3. Project Title

A double-blind, randomized, placebo-controlled, trial designed to assess the antiviral efficacy of Chloroquine in adult patients hospitalized with dengue.

Proposed start date: May 2007.

4. Data and Safety Monitoring Committee

Dr Phuong Quoc Tuan (OUCRU), Professor Nick White (Wellcome Trust/Bangkok),
Dr Guy Thwaites (Imperial College, UK), Dr Paul Young (University of Queensland,
Australia)

Introduction and rationale

Dengue fever (DF) is the most common vector-borne viral disease of humans, with more than 100 million cases recorded each year in tropical and subtropical countries and an increasing incidence in more temperate regions. Over half of the world's population live in areas at risk of infection. In its most serious forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), it is also a major cause of morbidity and mortality, particularly in Southeast Asia where it is the principle reason for paediatric admission to hospital during the rainy season. In VietNam over the past three years we have noted an increasing incidence in adults. Data from dengue protein crystallographic studies have revealed opportunities to intervene in the replication cycle of dengue viruses (DENV). The pH-dependent structural changes involving the fusion loop in the envelope protein are central to successful virion fusion with the endosomal membrane and release of infectious viral nucleic acid into the cytoplasm (1). Chloroquine, a safe and pharmacologically well-characterized drug, inhibits several viruses requiring a pH dependent step for cell entry (2)(3). The in vitro inhibitory concentration (IC_{50}) for chloroquine against dengue viruses is $\sim 5 \mu\text{M}$ (Fig. 1 from Novartis Institute for Tropical Diseases, Singapore). Furthermore, chloroquine inhibits the normal proteolytic processing of certain dengue virus proteins thereby reducing the infectivity of the mature virions (4). Following a 3-day course of therapeutic oral doses of chloroquine (600mg base on days 1 and 2, then 300mg base on day), the peak drug concentration (C_{max}) ranges from 0.1 to $0.3 \mu\text{g/mL}$ ($\sim 0.3\text{-}1 \mu\text{M}$) in plasma and 1 to $1.2 \mu\text{g/mL}$ ($3.2\text{-}3.8 \mu\text{M}$) in whole blood. Chloroquine is extensively sequestered in the tissues. In animals, from 200 to 600 times the plasma concentration may be found in the liver, spleen, kidney, and lung (5, 6). In humans, the highest concentrations of approximately $100 \mu\text{M}$ were measured in the marrow, liver, spleen and leukocytes (7). These data suggest a therapeutic potential for chloroquine in dengue-infected patients since the C_{max} in relevant organs is likely to exceed the IC_{50} value. This trial asks the question whether chloroquine, by its action on the acidification of the intracellular vacuole, reduces viral load in patients with dengue.

Study Aims

To measure the antiviral efficacy of chloroquine in the treatment of dengue.

Primary endpoint

The primary endpoint is;

1. time to negative DENV NS1 antigenaemia in plasma
2. time to negative reverse transcriptase (RT)-PCR detection of viral RNA in plasma

For the purposes of the study, time to a negative NS1 or PCR will be the interval between starting treatment and the first time NS1 or PCR is negative and remains negative for two consecutive measurements.

Sample Size

The following assumptions were made in calculating the sample size for EF study:

1. The median time to NS1 and PCR clearance in dengue population with normal standard of care is 72 hours
2. Most (90%) of dengue patients admitted to the trial will be day 3 of illness
3. The average proportion of cases that are NS1 and/or PCR positive on day 3 is 80%
4. At the end of follow-up (5 days after study entry), 20% of cases will still be positive for NS1
5. 10% of patients lost to follow-up

Under these assumptions, the minimum number of NS1 or PCR clearance cases required to be able to detect a 36 hours reduction of median time in Chloroquine arm as compared to standard care arm with a power of 0.8 and type I error 0.05-- equivalently a hazard ratio relating to NS1 clearance of 2—is 72. Therefore total number will be $138 / \{0.8 * (1-0.1) * (1-0.1)\} = 220$ or 110 patients in each arm. The effect size in this sample size calculation study is large, but can be justified on the basis that a smaller effect size is unlikely to be clinically significant.

Patient selection:

Patients will be eligible to be admitted to the study if they have suspected uncomplicated dengue and:

1. Are >14 years of age
2. They have a history of symptoms of 3 days or less.
3. They give fully informed consent.
4. No previous history of hypersensitivity to Chloroquine
5. They are not pregnant.
6. They are not G6PD deficient
7. They are not receiving therapy for other disorders

Randomisation and Treatment

This will be a double-blind randomised comparison. The randomization code will be computer generated and will be made for blocks of 40 cases. The study drugs will be presented in sealed plastic bottles; each bottle will contain sufficient tablets to complete one course of treatment (10 tablets, 150mg chloroquine base per tablet). Once the bottle is opened the patient will be considered to have entered the trial whatever happens subsequently. Patients will be randomized to group A or group B.

Group A: Chloroquine: 600mg base (4 tablets) on admission to the study, then 600mg 24hrs later (day 2), then 300mg on day 3.

GroupB: Placebo. Same regime of tablets (identically sized) as per chloroquine arm

Observations and Investigations

A full history and clinical examination will be performed at study entry and every day during the inpatient period. All clinical data will be documented in the case record form. The following investigations will be performed;

1. Chest X-ray or ultrasound on day of defervescence.
2. Hct, white cell count/differential, platelet count, SGOT, SGPT daily
3. Twice daily viral culture from plasma for minimum 5 days
4. Twice daily quantitative viral PCR from plasma for minimum 5 days
5. Twice daily assessment of Dengue NS1 levels for minimum 5 days

6. Twice daily measurement of plasma concentrations of the pro-inflammatory cytokines TNF, IL-6, IL-1B, IL12, IL-8 and the anti-inflammatory, IL-10 for minimum 5 days
7. ELISPOT assays to quantitate DENV-specific T cell responses
8. Microarray and PCR investigations to measure the effect of chloroquine on host gene-expression responses during acute disease

Trial monitoring and Adverse events

There will not be an interim analysis of the trial because of the relatively small sample size. All serious adverse events will be reported to the independent data safety and monitoring committee (DSMC). All adverse events will be recorded in the adverse events form found in the case record forms.

Blood sample collection

Venous blood (2ml) will be collected from all cases twice daily for a minimum of 5 days after starting treatment. This 2ml sample is sufficient to evaluate viral and haematological parameters. At the time of discharge, a 5ml blood sample will be collected to measure dengue-specific T cell responses.

Vomiting protocol

In the event the patient vomits within 5 minutes of receiving the study drug, then the patient will be re-dosed.

Analysis plan- primary endpoints

Baseline variables at randomization will be compared between the 2 treatment groups and analysed using the Kruskal-Wallis test for continuous variables and the chi-square test (or Fisher's exact test) for categorical variables. Only study subjects who have detectable NS1 antigenaemia or viraemia (detected by PCR) at the time of study entry will be analysed for the primary endpoint measures. The primary endpoints are time to negative DENV NS1 antigenaemia in plasma and time to negative reverse transcriptase (RT)-PCR detection of viral RNA in plasma. Comparison of Kaplan-Meier survival curves (representing proportion of cases NS1 or PCR positive) between treatment groups using log-rank test will be performed. Adjusted treatment

effect will be estimated and independent predictors will be identified using Cox regression analysis with stepwise forward variable selection procedure.

Analysis plan- secondary endpoints

Analysis of secondary outcome measures will be performed at the end of the trial.

Secondary endpoints include;

1. Fever clearance time (Time to first fall $\leq 37.5^{\circ}\text{C}$, axillary, and to remain $\leq 37.5^{\circ}\text{C}$ for 48 hours)
2. Time to plasma concentrations of the pro-inflammatory cytokines TNF, IL-6, IL-1B, IL12, IL-8 and the anti-inflammatory, IL-10 to return to normal range (defined a priori)
3. Time to SGOT, SGPT to return to normal range (defined a priori)
4. Time to platelet count to exceed $>100,000$ per ul

These secondary outcome measures will be analysed by Kaplan-Meier estimates with comparison of curves between treatment groups using log-rank test. Adjusted treatment effect will be estimated and independent predictors will be identified using Cox regression analysis with stepwise forward variable selection procedure.

Other secondary endpoints include;

5. Frequency of grade 3 and 4 adverse events as defined in Appendix 1 that are probably or definitely related to the intervention will be compared between treatment groups.
6. Frequency of new, clinically significant complications after commencement of treatment, including;
 - a. Dengue shock syndrome as defined by WHO criteria
 - b. Gastrointestinal bleeding requiring transfusion with blood or blood products
7. ELISPOT assays to measure DENV-specific T cell responses. The magnitude and breadth of DENV-specific T cell responses will be compared between treatment groups.
8. The magnitude of gene expression changes in peripheral blood measured by microarray or RT-PCR will be compared between treatment groups.

Frequencies of these secondary endpoints will be compared between treatment groups. Statistical analysis will be by parametric (student t-test) or non-parametric (Mann-Whitney U test) approaches. Comparison of proportions will be performed using chi-square (or Fishers exact) test.

Subgroup analysis

The following sub-group analyses will be performed:

1. Comparison of primary and secondary outcomes for those with primary or secondary dengue
2. Comparison of primary and secondary outcomes for each DENV serotype
3. Comparison of primary outcome for cases with DENV viraemias $>10,000$ cDNA equivalents per ml at the commencement of treatment
4. Comparison of primary outcome for cases with NS1 antigenaemias $>500\text{ng/ml}$ at the commencement of treatment
5. In subjects with at least 4 consecutive positive RT-PCR, we will determine the slope of the line representing changes in viral load over time.

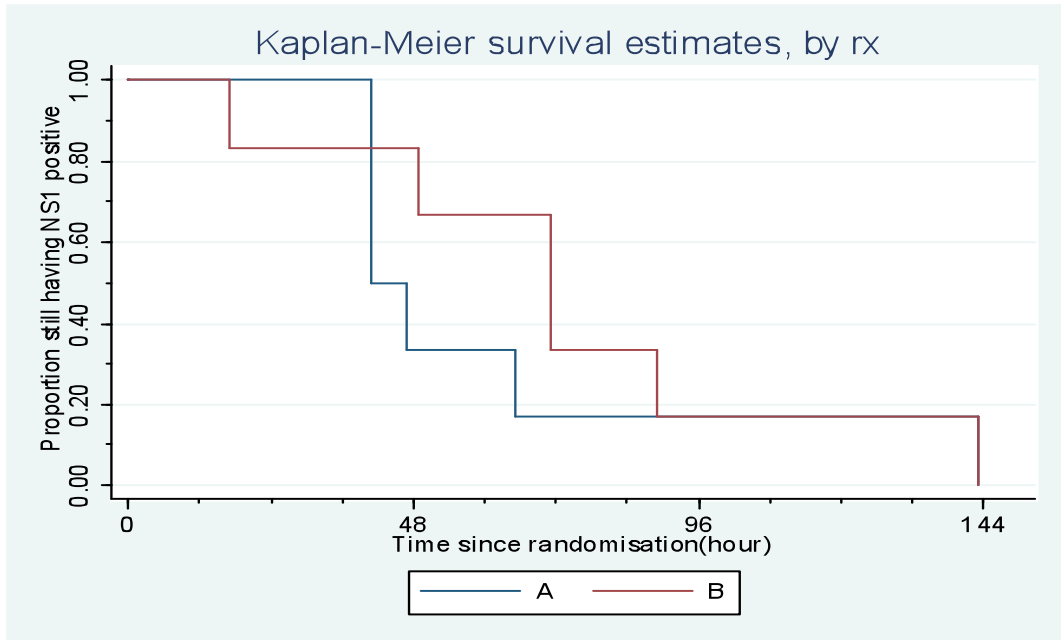
Hospital costs

For patients in the study, the OUCRU will pay hospital costs that are consistent with routine care of uncomplicated dengue.

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Figure 2. KPM survival analysis of a pilot study of chloroquine use in dengue patients. Shown is the proportion of adult patients (n=20) with NS1 antigenaemia since beginning randomised treatment (chloroquine or placebo).



A Randomized Controlled Trial of Chloroquine for the Treatment of Dengue in Vietnamese Adults

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Abstract

Background: There is currently no licensed antiviral drug for treatment of dengue. Chloroquine (CQ) inhibits the replication of dengue virus (DENV) *in vitro*.

Methods and Findings: A double-blind, randomized, placebo-controlled trial of CQ in 307 adults hospitalized for suspected DENV infection was conducted at the Hospital for Tropical Diseases (Ho Chi Minh City, Vietnam) between May 2007 and July 2008. Patients with illness histories of 72 hours or less were randomized to a 3-day course of CQ (n = 153) or placebo (n = 154). Laboratory-confirmation of DENV infection was made in 257 (84%) patients. The primary endpoints were time to resolution of DENV viraemia and time to resolution of DENV NS1 antigenaemia. In patients treated with CQ there was a trend toward a longer duration of DENV viraemia (hazard ratio (HR) = 0.80, 95% CI 0.62–1.05), but we did not find any difference for the time to resolution of NS1 antigenaemia (HR = 1.07, 95% CI 0.76–1.51). Interestingly, CQ was associated with a significant reduction in fever clearance time in the intention-to-treat population (HR = 1.37, 95% CI 1.08–1.74) but not in the per-protocol population. There was also a trend towards a lower incidence of dengue hemorrhagic fever (odds ratio = 0.60, PP 95% CI 0.34–1.04) in patients treated with CQ. Differences in levels of T cell activation or pro- or anti-inflammatory plasma cytokine concentrations between CQ- and placebo-treated patients did not explain the trend towards less dengue hemorrhagic fever in the CQ arm. CQ was associated with significantly more adverse events, primarily vomiting.

Conclusions: CQ does not reduce the durations of viraemia and NS1 antigenaemia in dengue patients. Further trials, with appropriate endpoints, would be required to determine if CQ treatment has any clinical benefit in dengue.

Trial Registration: Current Controlled Trials number ISRCTN38002730.

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Introduction

Dengue is a globally important public health problem. This mosquito-borne viral infection results in an estimated 50 million cases of symptomatic illness each year in over 100 affected countries [1]. There are no licensed vaccines to prevent dengue and no specific therapies to stop or limit viral replication or modulate the severity of symptoms in patients.

Infection with any of the four dengue virus serotypes can cause clinically apparent disease. A measurable viraemia is typically present for the duration of the febrile period, with the first 48–72hrs characterized by relatively high viraemia levels that then rapidly decline as acquired humoral and cellular immune responses resolve infection [2]. NS1, a non-structural protein secreted by virus-infected cells, can be detected in the peripheral blood in some, but not all, symptomatic individuals [3,4]. Both viraemia and NS1 levels are higher in patients with more severe clinical patterns of disease [5]. The majority of symptomatic infections manifest as an acute systemic febrile illness that is

clinically uncomplicated and lasts for 3–7 days. For reasons not fully elucidated, some DENV infections result in severe dengue, a syndrome usually characterized by transiently increased capillary permeability and a hemorrhagic diathesis. Parenteral fluids are used to replenish the intravascular volume and maintain cardiovascular stability during the period of maximum capillary permeability. Mortality in severe dengue can be reduced to less than 1% in experienced settings.

Previous randomized controlled trials in dengue have focused on supportive management and to our knowledge, there has never been a trial directed towards reducing the virus burden. Chloroquine (CQ) is a cheap, widely available and well-tolerated lysosomotropic 4-amino-quinoline derivative. *In vitro*, CQ has modest anti-viral effects on replication of viruses from diverse taxonomic families (reviewed in [6]). This has led to speculation that CQ could have a therapeutic role in the treatment of viral diseases where there are limited or no other therapies [6,7]. In the context of DENV, the lysosomotropic and weak base properties of CQ could exert anti-viral activity by interfering with endosomal

Author Summary

There is no available drug or vaccine against dengue, an acute viral disease that affects ~50 million people annually in tropical and sub-tropical countries. Chloroquine (CQ), a cheap and well-tolerated drug, inhibits the growth of dengue viruses in the laboratory with concentrations achievable in the body. To measure the antiviral efficacy of CQ in dengue, we conducted a study involving 307 adults with suspected dengue. Patients received a 3-day oral dosage of placebo or CQ early in their illness. Unfortunately, we did not see an effect of CQ on the duration of viral infection. We did, however, observe that CQ had a modest anti-fever effect. In patients treated with CQ, we observed a trend towards a lower incidence of dengue hemorrhagic fever, a severe form of dengue. We did not find any differences in the immune response that can explain this trend. We also found more adverse events, primarily vomiting, with CQ. This trial provides valuable new information on how to perform trials of antiviral drugs for dengue.

trans-Golgi network respectively [8,9]. Indeed, treatment of mammalian-expressing cells with chloroquine inhibits DENV infection [10]. Furthermore, treatment of DENV-2 infected mammalian cells with chloroquine reduces the infectivity of the produced virus by six- to eightfold, possibly by reducing the efficiency of the virus maturation process [11]. The 50% inhibitory concentration of chloroquine for DENV [10] is achievable inside human cells following ingestion of standard doses of CQ [12,13]. CQ could also modulate the host response to virus infection. Recognition of viral products by plasmacytoid dendritic cells (pDCs) occurs through a TLR-dependent pathway that requires endosomes acidification [14,15]; chloroquine-mediated blocking of this process partially inhibited West Nile virus-induced IFN- α production by pDC cultures [16]. CQ could also modulate antigen processing via an increased export of soluble antigens into the cytosol of DCs [17]. CQ also attenuates inflammatory cytokine responses [18,19] and this may in part explain why CQ is used as a 2nd line therapy in the treatment of inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus [20,21,22,23]. Possibly related to its anti-inflammatory properties, CQ exerts an antipyretic effect equal to paracetamol during treatment of uncomplicated *P. falciparum* malaria [24].

fusion and furin-dependent virus maturation, which both require low pH environments in late endosomes and the lumen of the

Against a backdrop of interest in CQ as a therapeutic for acute viral infections [6,7], the purpose of this study was to evaluate CQ

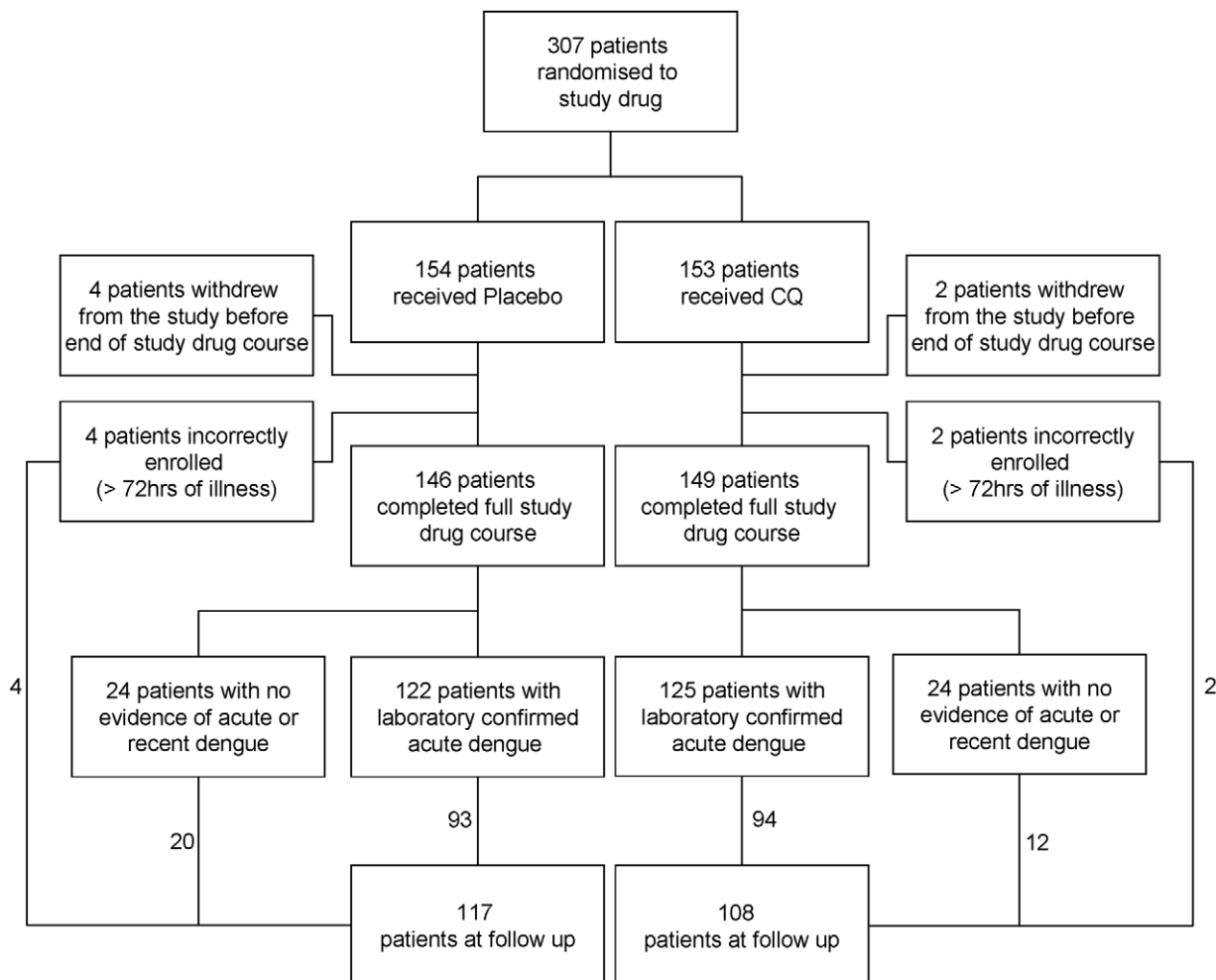


Figure 1. Participant flow in the randomized controlled trial of CQ vs. Placebo.

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as potential anti-viral therapy in a randomized, double-blind placebo-controlled trial of adolescents and adults with dengue.

Materials and Methods

Study setting, participants and treatment allocation

We performed a randomized (allocation ratio 1:1), double blind, placebo-controlled parallel-group study in 307 adults hospitalized for suspected DENV infection. Study participants were recruited from the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam. Patients were eligible if they were ≥ 15 yrs, had a self-reported illness history of 72 hrs or less and were suspected of having dengue. Patients were excluded if they were pregnant or receiving therapy for other chronic disorders, had a history of hypersensitivity to CQ, or written consent from either the patient or a parent was not obtained. Physicians in the Hospital for Tropical Diseases were responsible for enrolment. Patients were randomly assigned to receive CQ (Mekophar Chemical-Pharmaceutical Joint-Stock Company, Ho Chi Minh City, Viet Nam) or placebo. The regimen for CQ was 600mg base (4×150 mg tablets) on enrolment to the study, then 600mg on day 2 and 300mg on day 3 (following the World Health Organization recommended treatment regimen for CQ susceptible *P. vivax*) [25]. Patients in the placebo arm received the same regimen of tablets (identical color

and size). All treatment courses were contained in identical pre-packed bottles that were randomly assigned to patients via a computer-generated sequence of random numbers in blocks of 20 patients. A pharmacist generated the random sequence and was the only person who knew the content of each bottle. All patients, care providers and study investigators were blinded to treatment assignments. Physicians in the Hospital for Tropical Diseases were responsible for ensuring that the correct sequence of study codes, and therefore the treatment allocation, was followed. The study medication was given within 1 hr of a baseline blood sample being collected. Clinical care, including other treatments such as parenteral fluid therapy was at the discretion of the attending physician and following hospital guidelines. Case classification was according to 1997 WHO classification criteria and was applied to each case after review of study notes [26]. The Scientific and Ethical committee of the HTD and the Oxford Tropical Research Ethical Committee approved the study protocol and all patients gave written informed consent. The trial was registered with the ISRCTN Register (ISRCTN38002730).

Investigations

Clinical and laboratory investigations. Clinical history and examination findings were recorded daily into case record forms. An ultrasound was performed in all patients within 24hrs of

Table 1. Baseline characteristics in the intention-to-treat population.

Variables	CQ group (N = 153)	Placebo group (N = 154)
	N (%) or Median (interquartile range)	
Age (years)	22 (18–27)	22 (19–28)
Male sex	104 (68.0%)	106 (68.8%)
Dengue confirmed	128 (83.7%)	129 (84.3%)
Viremic	124 (81.0%)	124 (80.6%)
Infecting serotype:		
DENV-1	80 (64.5%)	67 (54.0%)
DENV-2	26 (21.0%)	27 (21.8%)
DENV-3	14 (11.3%)	27 (21.8%)
DENV-4	4 (3.2%)	3 (2.4%)
viraemia (log₁₀ copies/mL of plasma):		
All serotypes	9.0 (8.0–9.6) ^a	8.5 (7.6–9.3)
DENV-1	9.2 (8.3–9.8)	8.9 (7.9–9.7)
DENV-2	8.3 (7.0–9.4) ^a	7.7 (6.3–8.6)
DENV-3	8.3 (7.6–9.1)	8.2 (7.4–9.3)
DENV-4	7.7 (6.9–8.7)	7.7 (6.0–8.5)
Serological status:		
Primary	20 (15.7%)	12 (9.3%)
Secondary	105 (82.7%)	110 (85.3%)
Ambiguous	2 (1.6%)	7 (5.4%)
NS1 ELISA positive	113 (73.9%) ^b	110 (71.4%)
Febrile	150 (98.0%) ^c	147 (95.5%) ^c
Temperature (°C)	39.3 (38.7–40)	39.5 (38.8–40)
Time since illness onset (hrs)	49 (40–56)	48 (40–57)
Platelet count at enrolment	129,000 (95,000–168,000)	125,000 (91,000–167,000)

^aBaseline viraemia value missing for 1 patient in the CQ arm.

^bTwo patients NS1 negative at enrolment became positive later (both in the CQ arm).

^cThree patients afebrile at enrolment later developed fever (1 in the CQ arm and 2 in the Placebo arm).

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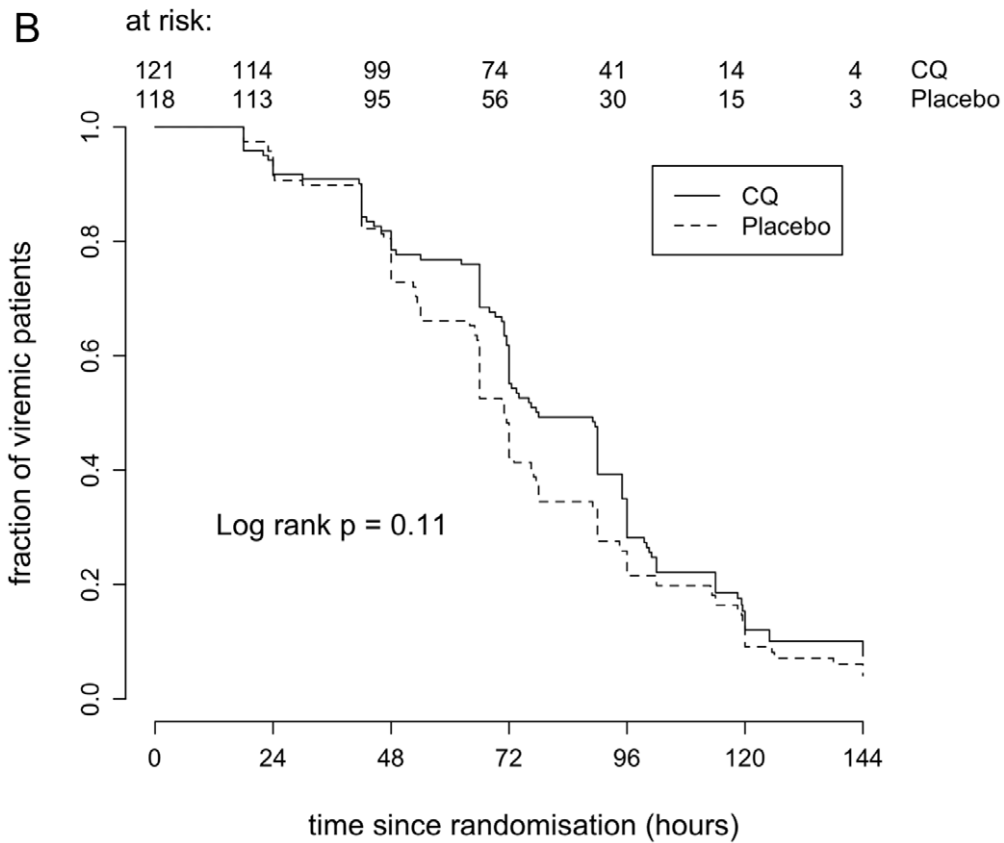
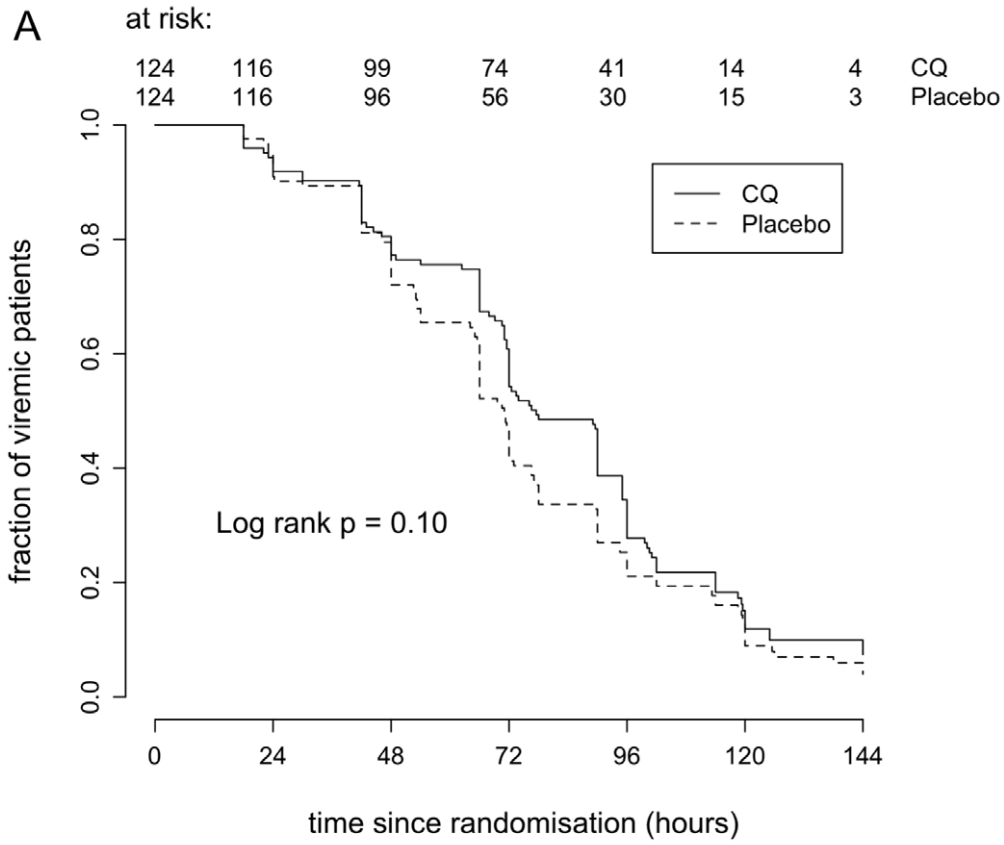


Figure 2. Time to fever clearance. Kaplan – Meier survival analysis of time to fever clearance by treatment group (CQ or placebo) and population; **A)** Intention to treat population and **B)** Per Protocol population. Three patients afebrile at enrollment developed fever later (1 in the CQ arm and 2 in the Placebo arm) and for the purposes of analysis were considered positive at the time of enrolment. doi:10.1371/journal.pntd.0000785.g002

defervescence. Venous blood samples were collected at hospital admission (prior to study drug administration), then twice daily (around 9am and 3pm) for a minimum of 5 days after starting treatment (defined as study day 1) and again 10–14 days after discharge from the hospital. Plasma, for use in diagnostic investigations, was stored frozen in multiple aliquots at -80°C until use. A complete blood count, including hematocrit (Hct) and platelet measurements, was performed daily for all patients. Hct measurements were performed more frequently if clinically indicated. The extent of hemoconcentration during symptomatic illness was determined by comparing the maximum Hct recorded during hospitalization with either the value recorded at follow-up when available or against a sex-matched population value.

Adverse events. Adverse events (AE) were defined as any unfavorable and unintended abnormal laboratory finding, symptom or disease that occurred during the course of the study, regardless of whether it was considered to be related to the intervention. AEs were classified as mild (grade 1), moderate (grade 2), severe (Grade 3) and life-threatening (grade 4) according to the Common Terminology Criteria for Adverse Events from National Cancer Institute. The relatedness of the AEs to study drug was investigated and graded as definitely, probably, possibly, unlikely to be, or not related.

Dengue diagnostics. A diagnosis of “confirmed acute dengue” was reached using previously described serological methods and a diagnostic algorithm [27]. DENV viraemia in plasma was measured using an internally controlled, serotype-specific, real-time RT-PCR TaqMan assay that has been described elsewhere [28]. RNA extraction was automated (NucliSens easyMAG, BioMerieux, Marcy l’Etoile, France). Results were expressed as cDNA equivalents per ml of plasma. Sample measurements were only valid when there was a detectable signal from the internal control amplicon and were considered as positive if above the assay limit of detection defined as the last dilution of standard that gave a specific signal. NS1 was detected by using the NS1 Platelia assay from BioRad (Hercules, CA) according to the manufacturer’s instructions.

Outcome assessment

Primary outcomes. The primary endpoints were the time to resolution of viraemia and the time to resolution of NS1 antigenaemia. Time to resolution of viraemia was defined as the time from the start of treatment until the first of two consecutive plasma samples were RT-PCR negative. Time to resolution of NS1 antigenaemia was defined as the time from the start of treatment until the first of two consecutive plasma samples were NS1 ELISA negative. Patients who did not reach viraemia or NS1 antigenaemia clearance were treated as censored at their last date of viraemia or NS1 antigenaemia measurement.

Secondary endpoints. The fever clearance time (FCT) was defined as the time from the start of treatment to the start of the first 48 hour period during which axillary temperature remained below 37.5°C . Other pre-defined secondary endpoints were- a) the median nadir platelet count, b) the mean maximum % hemoconcentration (calculated as (maximum hematocrit recorded during the inpatient period)–(hematocrit at follow-up when available or a sex-matched population value)/(hematocrit at follow-up when available or a sex-matched population value) $\times 100$), c) the proportion of patients who were treated with intravenous fluid (the

decision was based in clinical signs and conducted according to Hospital for Tropical Diseases protocols ; briefly, intravenous fluids were given if the attending physician believed treatment was necessary because of persistent vomiting, gastrointestinal bleeding, hemoconcentration or hypotension), d) the proportion of patients in each arm classified as having dengue hemorrhagic fever (DHF), e) the proportion of patients in each arm with grade 3 or 4 adverse events that were probably or definitely related to the intervention, f) the proportion of patients in each arm with one or more episodes of vomiting and g) the proportion of patients in each arm with bleeding that required blood transfusion.

T cell and cytokine investigations

To determine the optimal time point for cytokine measurement, levels of IL-1 β , IL-6, IL-8, IL-10, IL-12p70, and TNF- α were measured on serial plasma samples from 39 patients by using a CBA Human Inflammatory Cytokines kit (Becton Dickinson, San Jose, CA) according to the manufacturer’s instructions (except that all samples were fixed in 4% paraformaldehyde before being analyzed). Subsequently, a luminex-based Bio-Plex system (Bio-Rad Laboratories, Hercules, CA) was used according to the manufacturer’s instructions to measure simultaneous plasma levels of IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF), INF- γ , and TNF- α in 1 plasma sample from each patient. Flow-cytometric analysis of whole-blood samples stained with fluorochrome-conjugated monoclonal antibodies (CD3-Cy, CD4-PE-Cy7, CD8-PE, CD38-FITC, HLA-DR-PerCP and Ki67FITC) was performed by use of a FACScalibur flow cytometer (Becton Dickinson (BD)). Cell-surface staining was routinely performed on 150 μL of fresh whole blood. All antibodies were purchased from BD. Whole-blood samples from healthy volunteer subjects were used as group control.

Sample size calculation

Assuming a median time from enrolment to resolution of viraemia or NS1 antigenaemia in the placebo group of 72 hours and a reduction of this time by 24 hours due to CQ treatment (corresponding to a hazard ratio of 0.67 assuming an exponential distribution of the resolution times), we would need to observe viraemia or NS1 antigenaemia resolution in 191 patients to show such an effect with 80% power at the two-sided 5% significance level. Assuming sufficient follow-up to observe viraemia or NS1 antigenaemia resolution in 90% of patients, we would need to include at least 213 patients with confirmed dengue.

Statistical methods

The statistician was unblinded for the data analysis. Data stayed blinded until the database was cleaned and locked ready for data analysis. All statistical analyses were performed using Intercooled STATA version 9.2 (StataCorp, TX). A two-sided p-value ≤ 0.05 was considered significant for all parameters. The intention-to-treat (ITT) population was defined as all subjects who were randomized regardless of whether or not they began the treatment regimen. All laboratory confirmed dengue patients completing the expected number of days of treatment who fulfilled the inclusion/exclusion criteria of the protocol and who did not leave before the end of the study drug course formed the per-protocol (PP) population. Secondary endpoints (except the FCT) were compared between the 2 groups and analyzed using the Kruskal-Wallis test

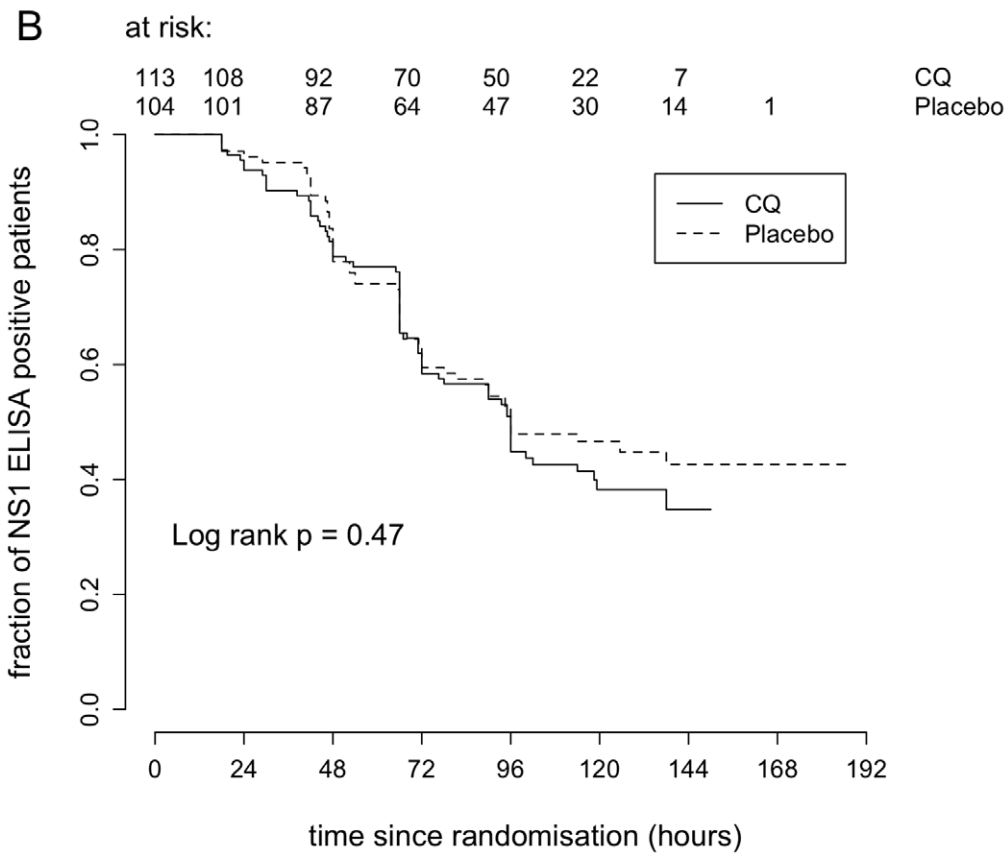
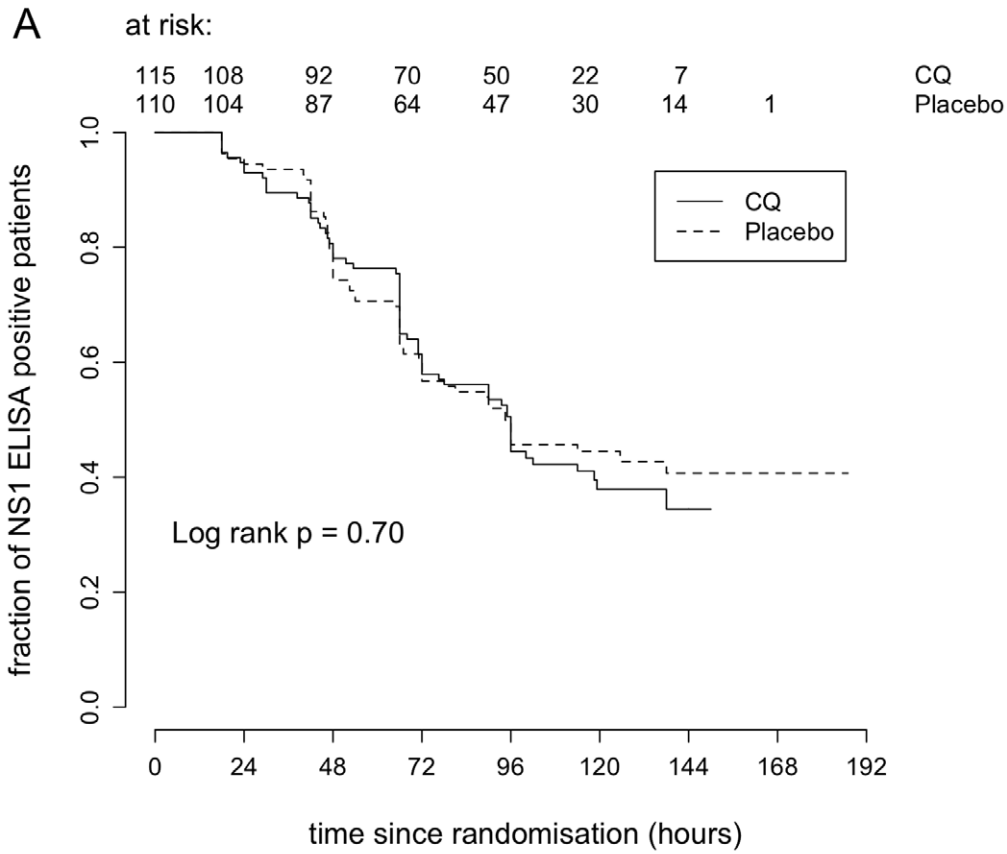


Figure 3. Time to resolution of viraemia. Kaplan – Meier survival analysis of time to resolution of plasma viraemia by treatment group (CQ or placebo) and population; **A)** Intention to treat population and **B)** Per Protocol population.
doi:10.1371/journal.pntd.0000785.g003

for continuous variables and the Fisher's exact test for categorical variables. For the primary endpoints and the FCT, the null hypothesis is that CQ has no effect on duration of DENV viraemia, NS1 antigenaemia and fever. Survival analysis using the Kaplan-Meier (KM) method and log-rank test was used for all time-to-event outcomes. Cox regression was used to quantify the difference in risk between treatment groups and to adjust for all the following baseline variables: time since illness onset at enrolment, serological status, serotype (DENV-1 vs other), viraemia and temperature. Because these covariates were thought to influence the time to resolution of viraemia, the time to resolution of NS1 antigenaemia and the FCT, all were retained in the final adjusted models. The proportional hazards assumptions were checked using a test based on Schoenfeld residuals.

Results

Baseline characteristics of enrolled patients

Between May 2007 and July 2008, 307 adults with suspected dengue were randomized to CQ or placebo (Fig. 1). Of these 307 patients, 257 had laboratory confirmed dengue and 50 had no evidence of recent or acute dengue. All patients recovered fully. The baseline characteristics of the study population are summarized in Table 1. Baseline characteristics were generally well-balanced between the two groups except for baseline viraemia which tended to be higher in the CQ group (median 9.04 vs 8.52 Log₁₀ copies/mL) and the proportion of DENV3 infected patients, which was lower in the CQ arm (11.3% CQ vs 21.8% placebo).

Primary endpoints

DENV viraemia clearance times. There were 248 patients viraemic at enrolment in the ITT population and 239 in the PP population (Table 1). Viraemia clearance times were not significantly different in the CQ arm compared to the placebo arm for either the ITT or PP analysis (Fig. 2A and B) (ITT hazard ratio (HR) 0.80, 95% CI 0.62–1.05, log rank test $P=0.10$ and PP HR = 0.80, 95% CI 0.61–1.05, log rank test $P=0.11$). Median times to resolution of DENV viraemia were similar in the ITT and PP population: ITT 77.5hrs and PP 78hrs (ITT inter-quartile range (IQR) 53–100hrs and PP IQR 66–100.5hrs) for CQ arm and both ITT and PP 71hrs (IQR ITT 48–94.5hrs and PP 48–95.5hrs) for Placebo arm. Adjusting for baseline covariates did not alter these findings (ITT HR = 0.95, 95% CI 0.72–1.26 and PP HR = 0.94, 95% CI 0.71–1.25). Of the 24 patients still qRT-PCR positive at discharge (median discharge time for those patients = 5 days since enrolment) only 14 presented at follow-up and none were qRT-PCR positive (median follow up time for those patients = 13 days after enrolment).

Time to negative NS1 antigenaemia. In the ITT population, there were 223 (72.6%) patients NS1 positive at the time of enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42hrs later) (Table 1). Time to resolution of NS1 antigenaemia was not significantly different between CQ and placebo arms (Fig. 3A) (HR = 1.07, 95% CI 0.76–1.51, log rank test $P=0.70$). Adjusting for baseline covariates did not alter these findings (HR = 1.18, 95% CI 0.82–1.68). Median times to resolution of NS1 antigenaemia were 96hrs (IQR 65.5–115hrs) in the CQ arm and 94.5hrs (48–120hrs) in the placebo arm. There were 96 patients still NS1 ELISA positive at discharge. This

suggests that NS1 antigenaemia is relatively long lived. Moreover 17 patients (~27% of patients NS1 positive at discharge and with a follow-up sample) were still NS1 positive at follow-up (time range: 10.7–14 days after enrolment).

In the PP population, there were 215 (87.0%) patients NS1 positive at the time of enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42hrs later) (Table 1). Time to resolution of NS1 antigenaemia was not significantly different between CQ and placebo arms (Fig. 3B) (HR = 1.14, 95% CI 0.80–1.63, log rank test $P=0.47$). Adjusting for baseline covariates did not alter these findings (HR = 1.19, 95% CI 0.83–1.71). Median times to resolution of NS1 antigenaemia were 96hrs (IQR 66–116hrs) and 96hrs (54–120hrs) respectively for CQ and placebo arms.

Secondary endpoints

Fever clearance times. In the ITT population, there were 297 patients febrile at enrolment (plus 3 afebrile who developed fever soon after) (Table 1). FCTs were significantly shorter in the CQ arm compared to the placebo arm (HR = 1.37, 95% CI 1.08–1.74, log rank test $P=0.01$ but there was a trend that the hazards were non-proportional $p=0.07$) (Fig. 4A). However, when adjusted for baseline covariates, the rate of fever clearance among patients who received CQ was not different from patients who received placebo (HR = 1.16, 95% CI 0.89–1.51, $P=0.28$). Median FCTs were 69hrs (IQR 45–93hrs) and 75hrs (IQR 36.5–99hrs) respectively for CQ and placebo arms.

There were 240 patients febrile at enrolment (plus 2 afebrile who developed fever soon after) in the PP population (Table 1). FCTs were not different between the 2 groups (HR = 1.24, 95% CI 0.96–1.60, log rank test $P=0.10$) (Fig. 4B). Adjusting for baseline covariates did not alter these findings (HR = 1.28, 95% CI 0.98–1.68, $P=0.07$). Median FCTs were 69hrs (IQR 45–93hrs) and 76hrs (IQR 46–99hrs) respectively for CQ and placebo arms.

Platelet nadir and maximum hemoconcentration. The median nadir platelet count was the same in the CQ and placebo arms 45,000 (IQR 25,000–60,000) (Mann-Whitney $p=0.61$, PP population). There were also no significant differences between the two arms in the mean level of hemoconcentration detected (10.8% (8.9–12.7) and 12.2% (10.3–14.1) for the CQ and placebo respectively (Mann-Whitney $p=0.27$, per protocol).

DF vs. DHF in each arm. There was a trend, though not significant ($P=0.09$), towards fewer patients with DHF in the CQ arm (Table 2). There were 29 patients (23.2%) with DHF in the CQ arm compared to 41 (33.6%) in the Placebo arm (odds ratio 0.60, 95% CI 0.34–1.04, $P=0.07$). As the infecting DENV serotype might influence clinical severity, and at baseline the two arms differed in the prevalence of each serotype, the analysis of the effect of CQ on disease severity was also adjusted for serotype by logistic regression, but this did not alter these findings.

Adverse events. Two patients in the CQ arm developed severe adverse events (both grade 3) that were possibly related to CQ. One patient with hematemesis was admitted to the ICU for 3 days, with stable vital signs. The 2nd patient was anorexic and vomiting with a narrow pulse pressure 100/80 mmHg) and was admitted to the ICU for 3 days. There were no severe AEs in the placebo arm. Significantly more adverse events occurred in the CQ arm: 18 patients reported a total of 33 AEs versus 6 patients with 8 AEs in the placebo arm (Fisher's exact test $P=0.01$)

Figure 4. Time to negative NS1 antigenaemia. Kaplan – Meier survival analysis of time to resolution of NS1 antigenaemia by treatment group (CQ or placebo) and population; **A)** Intention to treat population and **B)** Per Protocol population. Two patients NS1 negative at enrollment were later positive (both in the CQ arm) and for the purposes of analysis were considered positive at the time of enrolment. doi:10.1371/journal.pntd.0000785.g004

(Table 2). The most common adverse event was vomiting (~51% of all grade adverse events) (Table 2).

Number of patients requiring fluid and blood transfusion

Thirty two patients (21 in CQ arm and 11 in placebo arm) required parenteral crystalloid fluid therapy during their hospitalization (for rehydration, and/or maintenance) but none required blood transfusion (Table 2). There was no significant difference between the 2 groups in the need for fluid therapy (p-value = 0.11 in the PP population and 0.06 in the ITT population).

T cell activation in peripheral blood of study participants

Given the clinical experience of using CQ therapy in inflammatory autoimmune disorders, we investigated whether CQ was associated with a measurable attenuation of the T cell response. To this end, the activation state of peripheral blood CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was assessed in fresh whole-blood at the time of enrolment, on illness day 6, and again at follow-up in 172 consecutive patients enrolled in the study between September 07 and June 08 (85 in CQ arm, 87 in placebo arm), amongst whom there were 147 laboratory-confirmed dengue patients. The activation markers used were CD38, HLA-DR and Ki-67. As a reference, we also phenotyped T cells in fresh whole blood from 9 healthy adult volunteers. Strikingly, in dengue patients we observed a large population of surface-activated (CD38⁺ or HLA-DR⁺) and proliferating (Ki-67⁺) CD8⁺ T cells at early convalescence that were mostly absent at the time of enrolment and follow-up (Fig. 5). There was no evidence however of a significant difference in the proportion of activated T cells in patients treated with CQ or placebo.

Plasma concentrations of cytokines/chemokines

To understand if CQ modulated the cytokine response to DENV infection, plasma concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF- γ , and TNF- α were measured in plasma from 234 laboratory-confirmed dengue patients (121 in CQ arm, 113 in placebo arm) 2 or 3 days after randomization (Fig. 6). However, there was no significant difference in plasma concentrations of any of these cytokines between CQ or placebo treated patients (Mann-Whitney P>0.1).

Table 2. Summary of secondary endpoints.

	CQ N (%)	Placebo N (%)	P value ^a
Intention to treat population	153	154	
Patient with AE	18 (11.8%)	6 (3.9%)	0.01
Patient with vomiting	15 (9.8%)	6 (3.9%)	0.04
Patient requiring IV fluid	21 (13.7%)	11 (7.1%)	0.06
Per protocol population	125	122	
Patient with AE	17 (13.6%)	5 (4.1%)	0.01
Patient with vomiting	14 (11.2%)	5 (4.1%)	0.05
Patient requiring IV fluid	19 (15.2%)	10 (8.2%)	0.11
DHF patient	29 (23.2%)	41 (33.6%)	0.09

^aFisher's exact test.

doi:10.1371/journal.pntd.0000785.t002

Discussion

There are no specific therapies for treating dengue. This controlled trial was conducted to determine if CQ could reduce the viral burden in dengue patients. We found no evidence that CQ reduced the duration of viraemia or NS1 antigenaemia in adult dengue patients, but did observe a modest anti-pyretic activity of CQ in the intention to treat population, but not in dengue laboratory-confirmed cases. CQ was associated with a higher frequency of adverse events compared to placebo, but these were generally mild. There was no evidence that CQ reduced the magnitude of cytokine or T cell responses to DENV infection.

To our knowledge the only previous therapeutic trial of CQ for an acute viral infection has been in a small number of patients with Chikungunya virus infection [29], in which CQ had no impact on either duration of febrile arthralgia or viraemia. Several possible reasons could explain the lack of measurable activity in this study of CQ against virological markers of DENV infection in vivo. Although the C_{max} of CQ inside cells approximates the IC₅₀ value of CQ against DENV in vitro, it is possible that CQ does not achieve inhibitory concentrations inside the reticuloendothelial cells where DENV replication is believed to occur [30]. Furthermore, it may not achieve the same pH modulation in vivo that is postulated to explain its activity on cultured virus in vitro.

Alternative trial designs and protocols, such as increasing the therapeutic dose, dosing patients earlier in their illness or increasing the sample size substantially might increase the chances of observing an in vivo effect by CQ on the duration of DENV viraemia and NS1 antigenaemia. The importance of treating early is highlighted by the fact that in this trial the median duration of illness prior to treatment was relatively short (~48 hrs) and the median viraemia clearance times after treatment were ~3.75 days in the CQ arm and ~3 days in the placebo arm. Strikingly however, the duration of NS1 antigenaemia was relatively long, with as many as 92/243 (38%) of dengue patients still NS1 positive at the time of discharge from hospital, although most of this antigen is probably generated in the first few days of illness and its prolonged clearance simply reflects its large, oligomeric structure [31,32]. The time to resolution of NS1 antigenaemia may therefore not be an optimal endpoint and an alternative approach could have been to compare the proportion of patients that were positive at a single post-therapy timepoint (e.g. study day 5). Collectively, these data underscore that there is only a brief therapeutic window of opportunity to improve upon the host's virus-eliminating immune response. Encouragingly however, strategies to diagnose patients very early in their illness are available in the form of NS1 rapid diagnostic tests [27,33,34,35] and these could in principal guide rational treatment with an antiviral or other intervention as early as 24–48hrs into the illness course. Of additional value, but not yet identified, would be early prognostic markers of severe outcome, so that interventions can be delivered to those patients at higher risk.

A CQ-mediated anti-pyretic effect equal to paracetamol has been shown during treatment of uncomplicated *P. falciparum* malaria [24,36,37]. This effect may be explained by CQ's anti-inflammatory properties, including CQ effects on TLR signaling [38,39]. Fever during an infection is thought to be initiated by virtually immediate cyclooxygenase-2, prostaglandin E2 (PGE2) production, activation of hypothalamic PGE2 receptors and then

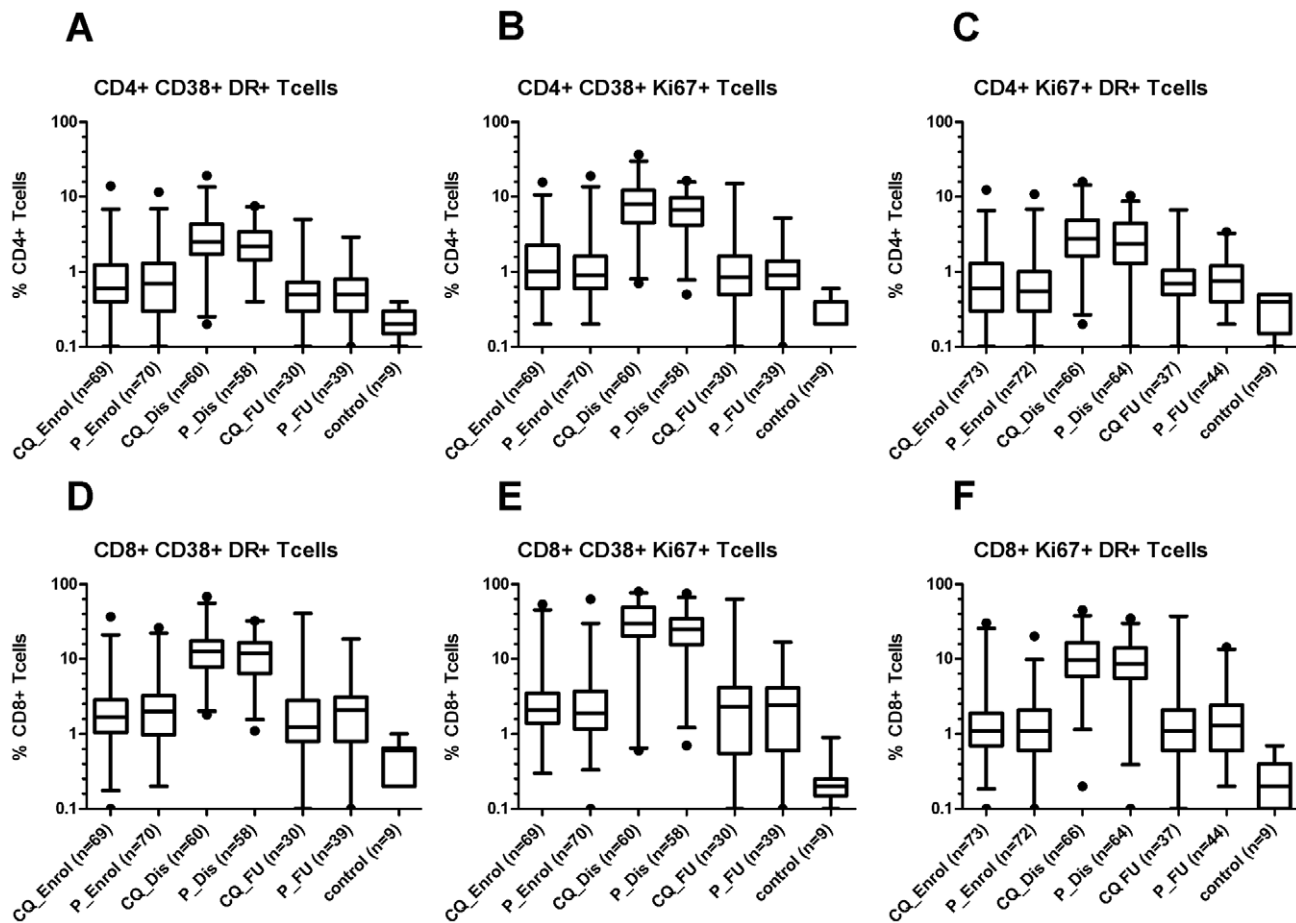


Figure 5. Surface phenotypes of CD4⁺ and CD8⁺ T cells in laboratory-confirmed dengue patients randomized to placebo or CQ. The Box and Whisker plots show the median number and range (2.5–97.5 percentile) of percentages of surface-activated T cells in peripheral blood from CQ (n = 74) and Placebo (n = 73) treated laboratory-confirmed dengue patients at different time points. The median illness day (range) for enrolment samples was 2 (0–3) days, for hospital discharge was 6 (4–8) days and for follow-up was 15.5 (13–30) days. Shown are percentages of peripheral blood CD4⁺ T cells that were **A)** CD38⁺HLA-DR⁺, **B)** CD38⁺Ki67⁺, and **C)** Ki67⁺ HLA-DR⁺. Also shown are percentages of CD8⁺ T cells that were, **D)** CD38⁺ HLA-DR⁺, **E)** CD38⁺Ki67⁺, and **F)** Ki67⁺ HLA-DR⁺. The labels below the graphs indicate the time at which sample collection occurred. doi:10.1371/journal.pntd.0000785.g005

cytokines and TLR ligand activity [40]. It is reasonable to believe that CQ mediates an anti-pyretic effect by altering the levels and balance of these pyretic mediators during infection. Accordingly, we found a small reduction in fever clearance median times (~6 hrs) amongst CQ patients in the intention-to-treat patient population, and whilst a similar trend was observed amongst the dengue confirmed patients, it was not statistically significant. CQ might be a better anti-pyretic in non-dengue patients in this study because these patients had milder infections, albeit of unknown origin.

Fewer patients receiving CQ developed DHF. The intriguing possibility that CQ mediated an anti-disease effect, but not a measurable anti-viral effect in this trial is plausible given the literature on CQ as a pleiotropic immune-modulatory drug. To find support for this possibility we measured pro- and anti-inflammatory plasma cytokine concentrations and T cell activation markers in dengue patients. Of particular interest were vasodilatory and pyretic cytokines such as TNF- α that have been identified as susceptible to CQ modulation [18,41] and important in the pathogenesis of the dengue capillary leak syndrome [42]. Similarly, the magnitude of T cell activation has been postulated to be associated with dengue severity [43]. Whilst robust T cell activation and cellular proliferation was indeed present around the

time of defervescence, there was no evidence of a difference between CQ and placebo arms for the cellular markers we investigated nor in the cytokines that were measured. The absence of a measurable impact by CQ on these elements of the host response might suggest any trend towards less DHF in the CQ arm is simply chance or reflects our inability to identify and measure true immunological correlates of disease. Only further large trials, with clinical endpoints, will determine if CQ has a disease modulating effect.

Our study had several limitations. The study was hospital-based and therefore the patient population, although presenting early in their illness, may not reflect that seen in primary health care settings where milder infections might be expected. The study was performed in adults, who generally compensate well for capillary permeability, and it's plausible that different findings might be observed in children, who in most endemic settings carry much of the disease burden. We measured viraemia by quantitative RT-PCR as a surrogate and well characterised marker of infection though we recognise this is not that same as a quantitative biological assay of infectious virus. Finally, we did not formally conduct pharmacokinetic analysis of CQ in treated patients and this could have aided the interpretation of the final outcomes.

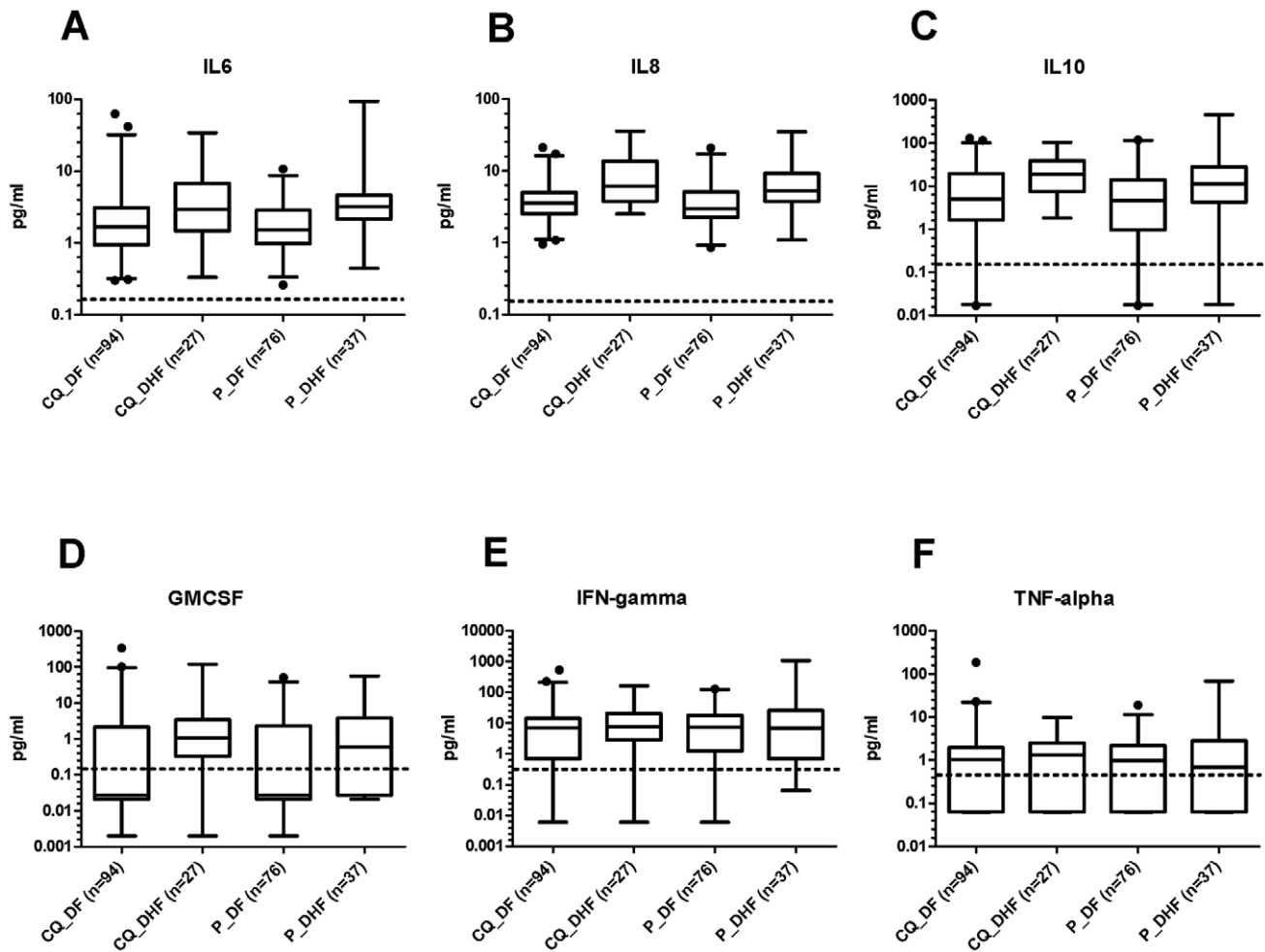


Figure 6. Plasma concentrations of pro- and anti-inflammatory cytokines in laboratory-confirmed dengue patients randomized to placebo or CQ. The Box and Whisker plots show the median and range (2.5th–97.5th percentile) of plasma concentrations of **A)** IL-6, **B)** IL-8, **C)** IL-10, **D)** GM-CSF, **E)** IFN- γ , and **F)** TNF- α from DF and DHF patients treated with CQ or Placebo. The dashed line represents the assay limit of detection. Concentrations of IL-2 and IL-4 are not shown because they were below the limit of detection. doi:10.1371/journal.pntd.0000785.g006

There is growing interest in the potential for anti-viral therapies for dengue [44,45]. This study illuminated several important issues in the design of anti-viral interventions trials. Most striking is the rapid decline in the DENV viraemia beginning ~72hrs into the illness, highlighting the fact that anti-viral interventions will likely need to be delivered very early and aggressively, preferably guided by cheap, sensitive and specific diagnostics. NS1 is a useful and easily assayed biomarker of DENV infection and in the context of a trial it conceivably provides a slightly different insight into virus infection than is given by measurement of viral RNA in plasma. In early phase trials, measurement of virological and clinical markers at multiple time-points per day is strongly recommended given the speed of viral clearance and evolution of disease. In later phase trials, the choice of clinical endpoints will depend on the target patient population and the setting. In children, single or combination endpoints around dengue shock syndrome, the most common life-threatening complication in children, should be considered. In adults, other complications such as severe bleeding may also be relevant.

In summary, this study suggests CQ has no measurable impact on virological or immunological parameters of DENV infection in

young adults. We also found no convincing evidence that CQ reduces the time to fever resolution in adults with dengue. Interventions with either more potent anti-viral molecules and/or immunomodulatory drugs are needed to improve clinical outcomes for patients in endemic settings.

Supporting Information

Checklist S1 CONSORT Checklist

Found at: doi:10.1371/journal.pntd.0000785.s001 (0.18 MB DOC)

Protocol S1 Trial Protocol

Found at: doi:10.1371/journal.pntd.0000785.s002 (0.14 MB DOC)

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Author Contributions

Conceived and designed the experiments: VT NNM JF BW HTT CPS.
Performed the experiments: VT NNM. Analyzed the data: VT NNM SJL

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RESEARCH ARTICLE

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Comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses

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Abstract

Background: Dengue is a major public health problem in tropical and subtropical countries. Rapid and easy diagnosis of dengue can assist patient triage and care-management. The detection of DENV NS1 on rapid lateral flow tests offers a fast route to a presumptive dengue diagnosis but careful evaluations are urgently needed as more and more people use them.

Methods: The sensitivity and specificity of the Bio-Rad NS1 Ag Strip and SD Dengue Duo (NS1/IgM/IgG) lateral flow rapid tests were evaluated in a panel of plasma samples from 245 Vietnamese patients with RT-PCR confirmed dengue and 47 with other febrile illnesses.

Results: The NS1 rapid tests had similar diagnostic sensitivities (respectively 61.6% and 62.4%) in confirmed dengue cases but were 100% specific. When IgM/IgG results from the SD Dengue Duo were included in the test interpretation, the sensitivity improved significantly from 62.4% with NS1 alone to 75.5% when NS1 and/or IgM was positive and 83.7% when NS1 and/or IgM and/or IgG was positive. Both NS1 assays were significantly more sensitive for primary than secondary dengue. NS1 positivity was associated with the underlying viraemia as NS1-positive samples had a significantly higher viraemia than NS1-negative samples.

Conclusions: These data suggest that the NS1 test component of these assays are highly specific and have similar levels of sensitivity. The IgM parameter in the SD Duo test improved overall test sensitivity without compromising specificity. The SD Dengue Duo lateral flow rapid test deserves further prospective evaluation in dengue endemic settings.

Background

Dengue is a major public health problem in tropical and subtropical countries [1,2]. Each of the four serotypes of dengue virus (DENV-1 to DENV-4) is able to cause disease. Clinically apparent disease varies in severity from mild undifferentiated fever through to more severe syndromes, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF is a vasculopathy characterized by capillary leakage and haematological deregulation, which in severe cases can result in life-threatening hypovolemic shock (DSS). There are no

licensed vaccines or specific therapies for dengue, and patient management relies on good supportive care.

There are several reasons why early and accurate diagnosis of dengue is important. First, an early and accurate diagnosis can assist in patient management by directing clinical attention to the appearance of major warning signs of severe or even life threatening complications, e.g. rapidly rising hematocrit, poor peripheral perfusion. Second, an accurate dengue diagnosis prevents unnecessary and possibly expensive antibiotic usage. Third, prompt diagnosis of index cases can facilitate vector control activities in the community so as to mitigate further transmission. Lastly, the expanded use of accurate dengue diagnostics provides important data on the epidemiology and health burden of dengue and in doing so can inform

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and guide public health policy, particularly as dengue vaccines and anti-virals make their way through development pipelines.

Commercial ELISA tests that detect the DENV NS1 protein in plasma/sera have provided a new avenue for diagnosing dengue [3-10]. The detection of NS1 on rapid lateral flow point-of-care tests offers an even faster route to a presumptive dengue diagnosis [11]. As more point-of-care rapid diagnostic tests (RDT) for dengue, particularly those targeting NS1, reach the marketplace their prices will likely drop to the point they are affordable for use in even resource limited health-care settings. The example of RDTs for malaria that target HRP2 provides a useful example of a point-of-care antigen-detection test where a large number of manufacturers in the market have resulted in cheaper tests such that they are being promoted in even the most resource limited settings [12].

The purpose of the current study was to compare the sensitivity and specificity of 2 commercially available, lateral-flow dengue RDTs (Bio-Rad NS1 Ag Strip and SD Dengue Duo) in a panel of plasma samples from dengue patients with different viral serotypes and viraemia levels. The SD Dengue Duo is distinguished from the Bio-Rad NS1 Ag Strip in that it also tests for DENV IgM and IgG.

Methods

Patient samples

The panel of plasma samples used in this study was from patients enrolled in the DENCO study, a multi-centre descriptive study of dengue conducted at the Hospital for Tropical Diseases, Paediatric Hospital #1 and Paediatric Hospital #2 in Ho Chi Minh City, Viet Nam from August 2006 to May 2007. Following written informed consent by the study participant, or a parent/guardian in the case of children, patients above 6 months of age with clinically suspected dengue and fever for less than 7 days were enrolled in the study. Ethical approval was obtained from the Ethics Review Committee of the Hospital for Tropical Diseases, Paediatric Hospital #1 and #2. Two plasma or sera samples were collected from each patient, one at day of enrolment and the second 7-14 days after fever onset.

Dengue diagnostics

All of the plasma samples used in the panel described in this study were RT-PCR positive for dengue virus using an assay described previously [11]. IgM and IgG antibody capture ELISA (MAC and GAC ELISA), using DENV/JEV antigens and mAb reagents provided by Venture Technologies (Sarawak, Malaysia), were performed as previously described [13]. The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA units in early convalescent plasma samples taking into account the day of illness. The cut-off in IgG ELISA units for distinguishing primary

from secondary dengue by day of illness was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed at the Centre for Vaccine Development, Mahidol University, Bangkok, Thailand using a reference IgM and IgG antibody capture ELISA described previously [14]. DENV loads in plasma were measured using an internally-controlled, serotype-specific, real-time RT-PCR assay that has been described previously [15]. Results were expressed as cDNA equivalents per milliliter of plasma.

The Bio-Rad NS1 Ag Strip and SD Dengue Duo rapid tests were provided by Bio-Rad (Hercules, CA) and Standard Diagnostics (Kyonggi-do, Korea) respectively and were performed according to the manufacturer's instructions. Each plasma sample for assessment was tested on both rapid tests in parallel. Each assay strip was independently assessed after the incubation time suggested by the manufacturer by the technician conducting the test and by a 2nd analyst who was blind to the first assessment. Discordant results were referred to a 3rd analyst whose decision was final. The analysts performing and scoring the assays were blind to the reference assay results and to any clinical information on the patients.

Analysis and Statistics

Concerning the SD Dengue Duo rapid test and for comparative purposes only, the IgM and IgG parameters were included in the interpretation of the test in some analyses. The statements "NS1 or IgM" and "NS1 or IgM or IgG" were then used and should be understood as if at least one of these parameters (i.e. NS1, IgM or IgG) is positive the sample is considered as positive. However, the detection of IgM and/or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue.

All statistical analysis was performed using Intercooled STATA version 9.2 (StataCorp, TX). Significance was assigned at $P < 0.05$ for all parameters and were two-sided unless otherwise indicated. Uncertainty was expressed by 95% confidence intervals. Categorical variables between groups were compared by Fisher's exact test. The *t*-test was used for continuous variables.

Results

Characteristics of the study population

The characteristics of the study population ($n = 292$ cases) that contributed acute plasma to the test panel is shown in Table 1. The panel of dengue cases ($n = 245$) were consecutively enrolled, qRT-PCR positive patients in the DENCO study (see M&M). The median duration of illness prior to the test plasma sample being collected was 3 days (range: 1-6). There were 47 patients in whom there was no virological or serological evidence of acute or recent dengue in paired plasma specimens.

Table 1: Baseline table summarizing the characteristics of the patient population that contributed to the sample panel

Variable	Confirmed dengue (n = 245)	Other febrile illness (n = 47)
	N (%) or Median (range)	N (%) or Median (range)
Male sex	141 (57.8%) ^a	25 (55.6%) ^b
Age (years)	12 (1-49) ^a	9 (2-53) ^b
Day of illness	3 (1-6)	2 (1-6) ^b
Infesting serotype and serological status		
DENV-1	138 (56.3%)	
Primary	49 (35.6%)	
Secondary	87 (63.0%)	
Indeterminate	2 (1.4%)	
DENV-2	91 (37.61%)	
Primary	9 (9.9%)	
Secondary	81 (89.0%)	
Indeterminate	1 (1.1%)	
DENV-3	16 (6.6%)	
Primary	8 (50.0%)	
Secondary	8 (50.0%)	

^asex and age was unknown in 1/245 acute dengue patients

^bsex, age and day of illness was unknown in 2/47 patients with other febrile illness

Sensitivity and specificity of NS1 tests versus reference algorithm and qRT-PCR

Bio-Rad and SD Duo NS1 rapid tests were equally sensitive for the diagnosis of acute dengue relative to the reference qRT-PCR test (Table 2; Bio-Rad 61.6% vs SD Duo NS1 62.4%, $P = 0.93$). The specificity of both NS1 tests was 100%, albeit the number of patients who had no evidence of acute or recent dengue was small ($n = 47$). Inclusion of the IgM parameter in the interpretation of the SD Duo test significantly increased its diagnostic sensitivity (Table 2; 75.5% vs 62.4%, $P = 0.0024$). Inclusion of the IgM and the IgG parameters in the interpretation of the SD Duo test further increased the diagnostic sensitivity over NS1 alone (Table 2; 83.7% vs 62.4%, $P < 0.0001$) but came at a cost of reduced specificity (100% for NS1 alone or NS1/IgM versus 98% for NS1/IgM/IgG).

Sensitivity of NS1 tests by day of illness

The sensitivity of NS1 tests alone was not significantly different between test samples collected within 3 days of illness onset versus those collected at a later time (Table 3). However, if the result of SD IgM and IgG parameters

were included in the interpretation of the SD test then sensitivity was significantly improved in test samples collected after 3 days of illness onset (Table 3; $P = 0.05$).

NS1 sensitivity in primary or secondary infection

NS1 detection rates with both RDTs were significantly lower in patients with secondary dengue than primary dengue (Table 4). Interestingly, inclusion of the IgM and IgG parameters in the interpretation of the SD Duo test significantly increased the diagnostic sensitivity over NS1 alone in patients with secondary dengue (Table 4). The overall difference in sensitivity between primary and secondary dengue was not associated with the illness day at the time of testing (primary dengue, mean day of illness: 3.30 days versus secondary dengue: 3.26 days, $P = 0.71$). Reduced sensitivity was also not associated with viraemia levels between primary and secondary dengue cases (log10 mean viraemia for primary: 7.04 versus for secondary 6.78, $P = 0.29$). In a more stratified analysis, NS1 sensitivity was also higher in DENV-1 infected patients (where the sample size was highest) with primary dengue

Table 2: Sensitivity and specificity, positive and negative predictive values of each assay against the gold standard algorithm

Assay parameter	Patients (n =)	Acute Dengue cases (n =)	Number positive in rapid test (n =)	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV (%) (95% CI)
BR NS1	292	245	151	61.6 (55.2 - 67.8)	100 (93.8 - 100) ^a	100 (98.0 - 100) ^a	33.3 (25.6 - 41.8)
SD NS1 alone	292	245	153	62.4 (56.1 - 68.5)	100 (93.8 - 100) ^a	100 (98.1 - 100) ^a	33.8 (26.0 - 42.3)
SD NS1 or IgM ^b	292	245	185	75.5 (69.6 - 80.8)	100 (93.8 - 100) ^a	100 (98.4 - 100) ^a	43.9 (34.3 - 53.9)
SD NS1 or IgM or IgG ^b	292	245	206	83.7 (78.4 - 88.1)	97.9 (88.7 - 99.9)	99.5 (97.3 - 100.0)	53.5 (42.4 - 64.3)

^aone-sided, 95% Confidence Interval

^bthe detection of IgM or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue and results are reported here for comparative purposes only

compared to secondary dengue at all time points of acute illness, (Figure 1A and 1B).

A possible basis for reduced sensitivity in secondary dengue is that NS1, along with other viral antigens, is less likely to be available for detection when a substantial level of DENV-reactive IgG is present. To explore this further, we analyzed NS1 detection sensitivity in the context of DENV-reactive IgG and IgM status as defined by the reference ELISA in the test sample. The presence of measurable DENV-reactive IgG (in the GAC ELISA) in the test sample was associated with a significant reduction in NS1 sensitivity in the SD assay (Table 5). On the contrary, the presence of measurable DENV-reactive IgM (in the MAC ELISA) but not IgG (in the GAC ELISA) in the test sam-

ple was associated with a significant increase in NS1 sensitivity relative to seronegative test samples (Table 5).

NS1 sensitivity in relation to viraemia levels

We hypothesized that viraemia levels would be associated with the detection of plasma NS1. Accordingly, in DENV-1 patients with equivalent illness durations (3 and 4 days of illness) viraemia levels were significantly higher ($P = 0.0005$) in patients who were NS1-positive versus those who NS1 negative in both tests at day 3 (with Bio-Rad and SD rapid test: log₁₀ mean viraemia = 7.71 versus log₁₀ mean viraemia = 6.11 respectively) (Figure 2A and 2B). To place NS1 detection in the context of both viraemia and serological response, individual test results for

Table 3: Sensitivity of the dengue RDTs in samples collected within 3 days of illness onset versus those collected at a later time

Status	Total (n =)	BR-NS1	SD-NS1	SD NS1 or IgMb	SD NS1 or IgM or IgGb
		% Sensitivity (95% CI)			
Test sample ≤ 3 days of illness	156	60.9 (52.8 - 68.6)	62.2 (54.1 - 69.8)	73.1 (65.4 - 79.9)	80.1 (73.0 - 86.1)
Test sample > 3 days of illness	89	62.9 (52.0 - 72.9)	62.9 (52.0 - 72.9)	79.8 (69.9 - 87.6)	89.9 (81.7 - 95.3)
<i>P</i> value ^a		0.7860	1.0000	0.2809	0.0498

^aFisher's exact test

^bthe detection of IgM or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue and results are reported here for comparative purposes only

Table 4: Sensitivity of dengue RDTs in patients with primary and secondary serological profiles

		BR-NS1	SD-NS1	SD NS1 or IgMb	SD NS1 or IgM or IgGb
Status	Total (n =)	% Sensitivity (95% CI)			
Primary dengue	66	80.3 (68.7 - 89.1)	80.3 (68.7 - 89.1)	83.3 (72.1 - 91.4)	83.3 (72.1 - 91.4)
Secondary dengue	176	55.1 (47.4 - 62.6)	56.3 (48.6 - 63.7)	72.7 (65.5 - 79.2)	84.1 (77.8 - 89.2)
<i>P</i> value ^a		0.0003	0.0006	0.0951	0.8472

^aFisher's exact test

^bthe detection of IgM or IgG in the rapid test is not sufficient for a definitive diagnosis of dengue and results are reported here for comparative purposes only

the Bio-Rad NS1 rapid test in DENV-1 infected patients were plotted against corresponding viraemia and serological responses in the same test sample (Figure 3A and 3B). These graphs illustrate the dynamic relationship between the detection of NS1, time since illness onset and in particular, the interfering effect of DENV-reactive IgG in the test sample.

Discussion

In the present study we showed that two different commercially available lateral flow RDTs, the Bio-Rad NS1 Ag Strip and SD Dengue Duo, have similar sensitivities (61.6% vs 62.4% respectively) for the detection of NS1 in plasma from RT-PCR positive patients. The factors negatively influencing the detection of NS1 included the presence of DENV-reactive IgG in the test sample and the

presence of secondary infection. The inclusion of the IgM test in the SD Dengue Duo provides for greater sensitivity (75.5%) without compromising specificity. The reasonable sensitivity and specificity of the SD Dengue Duo suggests it warrants additional prospective evaluations.

There is, as yet, no dengue RDT that could be considered as providing a definitive diagnosis of dengue. At best, current tests provide a presumptive diagnosis for a patient with a dengue like illness. A recent, TDR/WHO sponsored multi-centre evaluation of anti-dengue virus IgM rapid tests highlighted the very modest specificity of several commercially available IgM rapid tests, particularly in samples from patients with other infections, with malaria being a major confounder [16]. As acknowledged by the authors of the evaluation study, specificity issues must be considered in the context of the setting in which

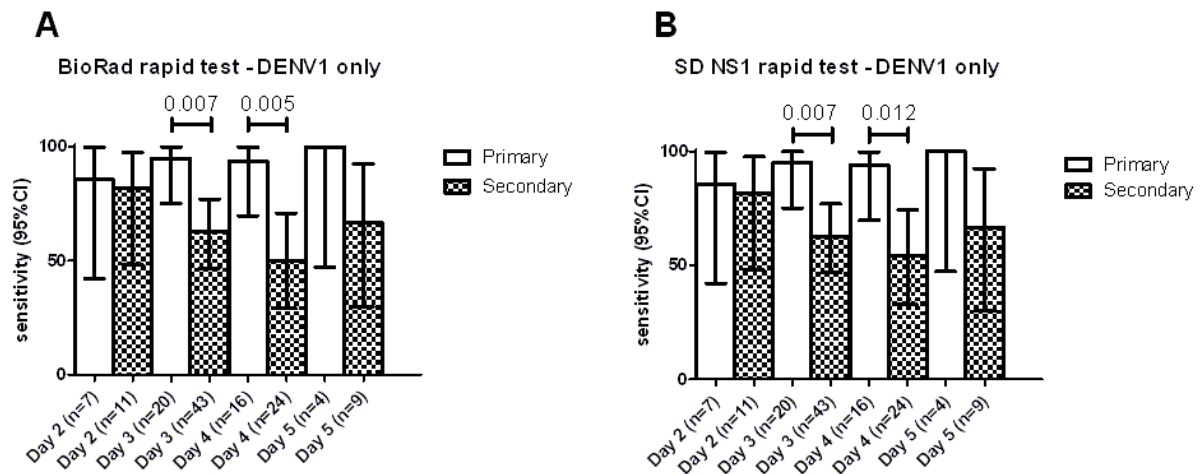


Figure 1 NS1 sensitivity of Bio-Rad and SD rapid tests in relation to primary and secondary serological status and day of illness in DENV-1 infection. Shown is the sensitivity of NS1 detection in the test sample according to primary and secondary serological status and day of illness for **A**) Bio-Rad NS1 test and **B**) SD NS1 test.

Table 5: Sensitivity (95% CI) of SD NS1 RDT assay in the presence or absence of measurable DENV-reactive IgG or IgM detected by ELISA in the test sample

Status ^a	IgG+ & IgM-	IgG+ & IgM+	IgG- & IgM-	IgG- & IgM+
% Sensitivity (95% CI)	14.3 (4.0 - 32.7)	31.7 (18.1 - 48.1)	67.9 (58.2 - 76.7)	91.4 (82.3 - 96.8)
Confirmed acute Dengue cases (n =)	28	41	106	70
P value^b		0.1546	0.0001	0.0002

^aaccording to the MAC and GAC reference ELISAs.

^bFisher's exact test

a particular test is to be used [16]. For example, in SE Asia there is no Yellow Fever virus transmission and in many urban settings there is no malaria transmission that could serve to limit the accuracy of a dengue RDT. The challenge then is to identify the best test for a particular setting and this is best done by accumulating more systematic data across a range of patient populations in different health care settings. Decision-tree based algorithms, as suggested for selecting malaria rapid tests, could be helpful in this regard for dengue RDTs [17].

To the best of our knowledge this is the first side-by-side assessment of different NS1 RDTs. Our finding that they were equally sensitive for NS1 detection is encourag-

ing and suggests other points of difference should be considered, e.g. ease of use, cost and stability at room temperature. The strength of the SD Dengue Duo is that the DENV IgM and IgG test windows provides an additional diagnostic investigation that complements NS1 detection. The inclusion of the DENV-IgM result in this current evaluation improved sensitivity without a reduction in the very high specificity associated with NS1 detection (100%). The inclusion of the IgG test result modestly further improved sensitivity. Caution is needed however as a positive IgM or IgG result alone could also represent infection anytime in the previous few months and should therefore be considered a presumptive diag-

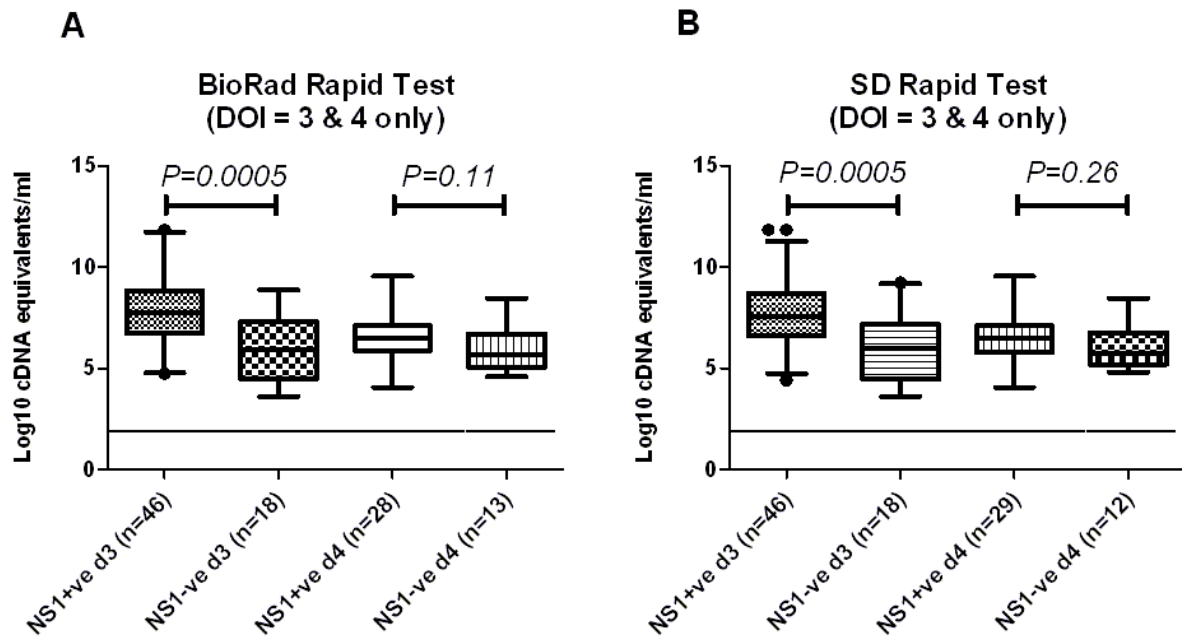


Figure 2 Viraemia by NS1 status in DENV-1 infected patients samples at 3 and 4 days after illness onset. Shown is the median (interquartile range, 95% range of data, and outliers) viraemia level in NS1 positive and NS1 negative patients with the same illness duration (3 and 4 days) tested by **A**) Bio-Rad NS1 test or **B**) SD Duo test. The limit of detection of the assay is shown with a dashed line. Viraemia levels were significantly higher in NS1 positive patients relative to NS1 negative patients (*t*-test) at day 3 of illness.

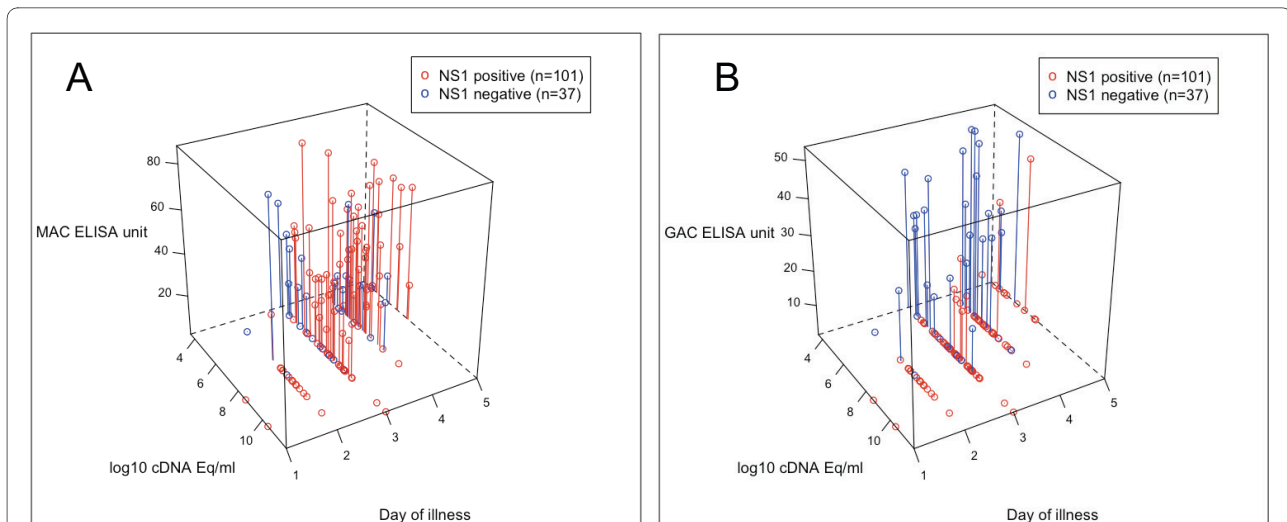


Figure 3 NS1 status (with Bio-Rad rapid test) in relation to MAC and GAC ELISA results, viraemia and day of illness in DENV-1 infection. Shown are **A**) MAC or **B**) GAC ELISA units, viraemia level and day of illness in NS1 positive and NS1 negative patients.

nosis. The potential for reduced specificity was highlighted in that one patient with no laboratory acute evidence of dengue had a positive IgG test result alone. Nevertheless the use of IgM and IgG test parameters in a NS1 RDT is rational as it will likely provide improved presumptive diagnostic coverage towards the end of the acute illness when NS1 levels are declining but the DENV-specific IgM and IgG titres are climbing rapidly. The sensitivity and specificity of the IgM and IgG test components of the SD Dengue Duo have been described previously as part of the TDR/WHO assessment of dengue RDTs [16].

The presence of DENV-reactive IgG in the test sample, a relatively low viraemia and secondary dengue were the major factors associated with a negative NS1 finding on both tests. The bias of these RDTs towards patients with higher viraemia levels is probably a positive feature of these tests in that they are likely biased towards patients at risk for complications during their illness [18]. Somewhat surprisingly we did not replicate our previous observations in a different patient population that NS1 sensitivity by Bio-Rad rapid test was higher in the first 3 days of illness that at later times [11]. Differences exist between the two studies and these include- a) the first study included adults and b) the first study was done prospectively in real time with fresh plasma rather than frozen stored plasma. It is possible these factors might account for the differences between the study findings. It may also reflect chance differences associated with relatively small sample sizes. Similarly, we found greater NS1 sensitivity in samples where there was a measurable DENV-reactive IgM level, a finding inconsistent with our previous observations using ELISA based NS1 detection

where the presence of IgM was not associated with higher or lower sensitivity [11]. The different findings might reflect chance differences in the patient population or differences between ELISA versus RDT.

A weakness of this study was that it was performed using a panel of stored plasma specimens and was heavily biased towards DENV-1, the most common serotype in circulation in Viet Nam since 2006. Similarly, assay performance and interpretation were performed by experienced lab analysts and not by clinicians "at the bedside". Different results may also have been obtained if an outpatient population, rather than a hospitalized set of patients, were used to generate the assessment panel used here. The evaluation panel is also biased in that all samples from dengue patients were RT-PCR positive. Despite these limitations, the current study provides a baseline in terms of sensitivity and specificity of these two RDTs for Vietnamese dengue patients and highlights virological and immunological factors associated with assay performance. Further prospective evaluations of both tests are warranted.

Conclusions

Our findings suggest that the NS1 components of each test are specific tools for diagnosing acute dengue, though the sensitivity of both is influenced by the level of viraemia and host humoral immune response. The addition of an IgM/G component to the SD Dengue Duo significantly improved diagnostic sensitivity above NS1 testing alone.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VT participated in the study design, the experimental work, the analysis and interpretation of the data and drafted the manuscript. HTTPV participated in the experimental work and helped draft the manuscript. NVNQ participated in the study design, the experimental work and the analysis and interpretation of the data. CVVN, HTTP, JF and BW participated in the study design, interpretation of data and revising of the manuscript. CPS conceived and designed the study and participated in the analysis and interpretation of the data and writing of the manuscript. All authors have read and approved the final manuscript.

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Kinetics of Viremia and NS1 Antigenemia Are Shaped by Immune Status and Virus Serotype in Adults with Dengue

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Abstract

Background: Dengue is a major public health problem in tropical and subtropical countries. Exploring the relationships between virological features of infection with patient immune status and outcome may help to identify predictors of disease severity and enable rational therapeutic strategies.

Methods: Clinical features, antibody responses and virological markers were characterized in Vietnamese adults participating in a randomised controlled treatment trial of chloroquine.

Results: Of the 248 patients with laboratory-confirmed dengue and defined serological and clinical classifications 29 (11.7%) had primary DF, 150 (60.5%) had secondary DF, 4 (1.6%) had primary DHF and 65 (26.2%) had secondary DHF. DENV-1 was the commonest serotype (57.3%), then DENV-2 (20.6%), DENV-3 (15.7%) and DENV-4 (2.8%). DHF was associated with secondary infection (Odds ratio = 3.13, 95% CI 1.04–12.75). DENV-1 infections resulted in significantly higher viremia levels than DENV-2 infections. Early viremia levels were higher in DENV-1 patients with DHF than with DF, even if the peak viremia level was often not observed because it occurred prior to enrolment. Peak viremias were significantly less often observed during secondary infections than primary for all disease severity grades ($P=0.001$). The clearance of DENV viremia and NS1 antigenemia occurs earlier and faster in patients with secondary dengue ($P<0.0001$). The maximum daily rate of viremia clearance was significantly higher in patients with secondary infections than primary ($P<0.00001$).

Conclusions: Collectively, our findings suggest that the early magnitude of viremia is positively associated with disease severity. The clearance of DENV is associated with immune status, and there are serotype dependent differences in infection kinetics. These findings are relevant for the rational design of randomized controlled trials of therapeutic interventions, especially antivirals.

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Introduction

Dengue viruses (DENVs) are members of the Flavivirus genus and are the most important arboviral pathogens of humans. The four DENVs are antigenically-related and have single-stranded, positive-sense RNA genomes that share 60–70% sequence identity between each others [1]. There are no licensed vaccines to prevent dengue and vector control remains the cornerstone of public health interventions.

The clinical outcome from DENV infection ranges from the asymptomatic to an acute, often debilitating illness called dengue fever (DF), to the severe and potentially life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The cardinal feature of DHF/DSS is a capillary permeability syndrome characterised by plasma leaking from the vasculature into interstitial spaces. Thrombocytopenia, a coagulopathy and a hemorrhagic diathesis are also common findings. DSS manifests when capillary

permeability is severe enough to result in an inadequate intravascular volume that then leads to poor tissue perfusion. DSS is managed, and possibly prevented, by careful restoration and maintenance of the intravascular volume by use of parenteral fluids. Viral strain and host immune status have been suggested as major risk factors for DHF/DSS. In particular, two sequential infections, with the second infection caused by a DENV serotype different from the first, is a risk factor for severe disease in children and adults [2–5]. A process called antibody dependent enhancement of infection (ADE), coupled with strong anamnestic cellular immune responses is the leading hypothesis to mechanistically explain more severe disease in secondary infections [6–9]. Severe dengue can also occur in primary infection of infants born to dengue-immune mothers with, indicating anamnestic immune responses are not absolutely critical for eliciting the capillary permeability syndrome in all patients. Viral traits may also be important in pathogenesis, with strong evidence that some viral genotypes are fitter than others [10,11].

Author Summary

Dengue is an acute viral disease that affects tens of millions of people annually in tropical and sub-tropical countries. In some cases, this infection happens to be severe and even life threatening. Severe cases have been associated with higher levels of virus in the blood. Several hypotheses have been proposed to explain the occurrence of these cases notably by involving the patient's history of previous DEN virus infection(s). Little is known about the relationships between the evolution over time of virus levels in the blood, the clinical outcome and the previous infection(s) history—a better understanding of these features could help in anti-viral drug development. To analyze these relationships, we studied well characterized patients who participated in a clinical trial. The majority of these patients were infected by DENV-1 serotype and had higher levels of virus than those infected by DENV-2 and sometimes DENV-3 serotypes. We also found that patients with more severe symptoms had higher levels of virus in the first days of their illness. We found as well that the virus was cleared faster and earlier from the blood of patients previously infected. These findings are of major importance for further anti-viral drug testing.

The literature describing the overall relationship between plasma/serum viral burden, disease severity and immune status generally supports the hypothesis that there is a positive correlation between markers of viral burden in the first 2–3 days of fever and the severity of clinical outcomes. For example, during the febrile and early convalescent periods, Taiwanese adults with secondary DENV-3 infections and DHF had higher plasma levels of viral RNA than did patients with DF [12]. An association between higher peak viremia and increased disease severity was observed in Thai children with acute DENV-1 and -2 infections [13]. Similarly, DHF was associated with higher plasma viremia early in illness in Thai children with secondary DENV-3 infections [14]. Duyen et al recently showed that DENV-1 infections were associated with higher viremia and NS1 antigenemia than DENV-2 infections in ambulatory Vietnamese paediatric patients [15]. In the same patients, viremia and NS1 antigenemia persisted for longer in patients with primary infections.

In adults, where the risk of clinically apparent disease occurring in primary infection is possibly greater [16,17], there is less evidence relating virological features of infection to immune status or clinical outcome. Kuberski et al reported in 1977 that the magnitude of viremia in young adult patients was higher in primary than secondary DENV-1 infections [18]. More recently, DENV viremia levels from Taiwanese adult patients were reported to be lower in secondary than in primary DENV-2 infections [19].

The dynamics of virus clearance might also be relevant to clinical outcome. In Thai children, Vaughn and others have shown that the slope of the descending portion of the viremia curve was steeper for patients with secondary infection versus those with primary infection and viremia decreased more quickly for patients with DHF than for patients with DF at defervescence [13]. The accelerated clearance of viremia in secondary infection most likely reflects the contribution of anamnestic humoral and cellular immune responses, which themselves have been implicated in the pathogenesis of capillary leakage. Conversely however, Wang et al suggested clearance of the virus and virus-containing immune complexes was slower in adult DHF patients [20].

A better understanding of the relationship between biomarkers of virus infection, the immune response and disease evolution is

critical for the rational use of intervention therapies in dengue, e.g. anti-viral drugs or immune-modulating therapies. To this end, this study describes the kinetics of viremia and NS1 in an intensively investigated cohort of Vietnamese adults with dengue and less than 72 hrs of fever enrolled in a randomized placebo-controlled trial (RCT).

Methods

Study setting, participants and ethical considerations

A double blind RCT of chloroquine (CQ) in 307 adults hospitalized for suspected DENV infection was conducted at the Hospital for Tropical Diseases (Ho Chi Minh City, Vietnam) between May 2007 and July 2008. Information on recruitment, inclusion criteria, randomization, treatment and investigations have been published previously [21]. The Scientific and Ethical committee of the HTD and the Oxford Tropical Research Ethical Committee approved the study protocol and all patients gave written informed consent. The trial was registered with the ISRCTN Register (ISRCTN38002730). Herein we describe the clinical and virological features of the 257 patients with laboratory confirmed dengue enrolled into this trial, which hitherto have not been described in detail.

Dengue diagnostics and detection of DENV RNA and NS1 in plasma

A diagnosis of laboratory confirmed dengue was reached using serological, antigen detection and molecular methods [22]. In brief, RT-PCR detection of DENV RNA in plasma was performed using an internally controlled, serotype-specific, real-time RT-PCR TaqMan assay that has been described previously [23]. RNA extraction from plasma samples was automated (NucliSens easyMAG, BioMerieux, Lyon, France). Results were expressed as cDNA equivalents per mL of plasma. A capture IgM and IgG ELISA (MAC and GAC ELISA) using DENV/JEV antigens and mAb reagents provided by Venture Technologies (Sarawak, Malaysia), was performed as previously described [24]. NS1 was detected by using the NS1 Platelia ELISA assay from BioRad (Hercules, CA) according to the manufacturer's instructions. Samples defined as equivocal in the NS1 Platelia ELISA assay were repeated and if they were still equivocal they were regarded as being negative.

Host immune status

The interpretation of primary and secondary serological responses was based on the magnitude of IgG ELISA units in early convalescent plasma samples taking into account the illness day. The cut-off in IgG ELISA units for distinguishing primary from secondary dengue by illness day was calibrated using a panel of acute and early convalescent sera from Vietnamese dengue patients that were assayed at the Centre for Vaccine Development, Mahidol University, Bangkok, Thailand using a reference IgM and IgG antibody capture ELISA described previously [25].

Clinical and laboratory investigations

Clinical history and examination findings were recorded daily into case record forms. An ultrasound was performed in all patients within 24 hrs of defervescence. Venous blood samples were collected at hospital admission, then twice daily (around 9am and 3pm) for a minimum of 5 days after hospital admission and again 10–14 days after discharge from the hospital. A complete blood count, including hematocrit (Hct) and platelet measurements, was performed daily for all patients. Hct measurements were performed more frequently if clinically indicated. The extent

of hemoconcentration during symptomatic illness was determined by comparing the maximum Hct recorded during hospitalization with either the value recorded at follow-up when available (191/248 i.e. 77% of the patients) or against a sex- and age-matched population value. Plasma was stored frozen in multiple aliquots at -80°C until use in the real-time RT-PCR and NS1 ELISA. The day of fever onset was self-reported by the patient and was designated illness day 1.

Case definition

DF and DHF were diagnosed according to 1997 World Health Organization (WHO) classification criteria and was applied to each case after review of study notes [26]. The 1997 definitions were used for this study because at the time of clinical assessment the 2009 WHO Guidelines and revised classification scheme was not available. DF was defined as a laboratory confirmed dengue case with no evidence of capillary permeability as defined for a DHF case. DHF was defined as laboratory confirmed dengue case with thrombocytopenia ($<100,000$ platelets/ mm^3), any hemorrhagic manifestation, and evidence of plasma leakage (as denoted by a $>20\%$ increase in the Hct from the baseline value or by the presence of pleural or abdominal effusions).

Analysis and statistics

The data used in this analysis was taken from a randomised controlled treatment trial of dengue. Since the intervention (CQ) had no measurable impact on virological or immunological outcomes, for the purposes of this analysis we did not distinguish between patients in the CQ or placebo arms of the study. All statistical analysis was performed and figures designed using the software R (version 2.10.1). Significance was assigned at $P<0.05$ and were two-sided unless otherwise indicated. Uncertainty was expressed by 95% confidence intervals. The Kruskal-Wallis rank sum test was used for continuous variables and the Fisher's exact test for categorical variables. For the viremia kinetics analysis, when the RT-PCR signal was below the assay limit of detection (defined as the last dilution of standard that gave a specific signal), a value equal to concentration of the last dilution of standard that gave a specific signal divided by 10 was assigned. The maximum viremia level was defined as the highest plasma viremia level measured during illness. The maximum viremia level was considered to be a peak viremia level only in cases in which viremia rose after the enrolment specimen. To compare kinetics of viremia between patients with different serological status, disease severity and serotype, the means of log-transformed viremia measurements made on the same illness day were used as a summary measure of the viremia on that day. To estimate the maximum daily rate of DENV clearance, the slope of the viremia curve was calculated for each illness day as the change in the means of log-transformed viremia measurements made on the same illness day. Only the maximum decreasing daily rate of each patient was used for analysis. Survival analysis using the Kaplan-Meier method and log-rank test was used for all time-to-event outcomes. Time to resolution of viremia or NS1 antigenemia was defined as the time from the start of symptoms until the first of two consecutive plasma samples below the RT-PCR limit of detection or NS1 ELISA negative. The fever clearance time (FCT) was defined as the time from the start of symptoms to the start of the first 48 hours period during which axillary temperature remained below 37.5°C .

Results

Characteristics of the study population

Of the 307 adults with suspected dengue enrolled in the CQ RCT between May 2007 and July 2008, 257 had laboratory-

confirmed dengue including 248 patients with a defined serological and clinical classification and 9 patients with ambiguous or unknown clinical outcomes or serology (mainly because they left the study prematurely). The characteristics of the study population are summarized in Table 1 (and Table S1). DENV-1 (57.3%) was the commonest serotype detected in this population of patients, then DENV-2 (20.6%), DENV-3 (15.7%) and DENV-4 (2.8%). DHF was significantly associated with secondary infection compared with primary infection (65/215 vs 4/33 i.e. 30.2% vs 12.1%, $P=0.04$, Odds ratio (OR) = 3.13, 95% CI 1.04–12.75). DHF resulting from secondary infection was more commonly associated with DENV-2 (21/45 (46.7%)) than for other serotypes (DENV-1: 33/124 (26.6%), DENV-3: 10/33 (30.3%) and DENV-4: 1/7 (14.3%) (DENV-2 vs DENV-1, -3 and -4 $P=0.02$, OR = 2.38, 95% CI 1.14–4.96) (Table S1).

Viremia kinetics in DF and DHF according to serological status

Median viremia levels by illness day for DENV-1, -2 and -3 are shown in Figure 1 (and Table S2). In DF patients with primary infection, DENV-1 viremia levels were significantly higher than DENV-2 or DENV-3 levels at multiple time-points during the acute illness (Figure 1A). In DF patients with secondary infection, the most common serological and clinical grouping, and DHF patients with secondary infection, DENV-1 levels were significantly higher than DENV-2 levels and there was also a non-significant trend towards higher DENV-1 levels than DENV-3 levels (Figure 1B and C). Collectively, and despite small sample sizes for some subgroups, these data suggest that DENV-1 infections were associated with higher viremias (as measured by qRT-PCR) than DENV-2, irrespective of disease severity and immune status.

DENV-1 was the commonest serotype detected in this patient population and therefore there was sufficient data to enable direct comparisons of viremia kinetics across serological states and clinical severity whilst controlling for the infecting serotype (Table S2). These data show that in the early acute phase (illness day 3) patients with DENV-1 infection and DHF had significantly higher viremia levels than DENV-1 patients with DF, irrespective of the patient immune status (Figure 2). These data show also that later in the acute phase (from day 4 of illness) patients with primary DENV-1 infections had significantly higher viremia levels than patients with secondary DENV-1 infections, irrespective of the disease severity (Figure 2).

A limitation of these analyses is that in the majority of patients with secondary infections the viremia was already declining at the time of enrolment i.e. we did not observe an obvious peak viremia (Table S3). Overall, a peak viremia was significantly less often observed in secondary infections than in primary infections for all disease severity grades ($P=0.001$, OR = 3.64, 95% CI 1.55–8.74). However, there were no significant differences in the duration of illness prior to enrolment between patients in different categories of serological status or disease severity (P between 0.11 and 0.96 if all the serotypes are considered, and 0.16 and 0.39 if and only DENV-1), suggesting this snapshot of viremia levels is unbiased by differences in duration of illness at study enrolment.

Timing and amplitude of peak viremia and associations with severity and immune status

Peak viremia levels were identified in 72 patients. Peak viremia occurred significantly earlier in secondary DF than in primary DF ($P=0.008$) and in secondary DHF than in primary DHF ($P=0.04$) but there were no significant differences between primary DF and

Table 1. The characteristics of the study population^a.

Variables	N (%) or Median (interquartile range)			
	Primary (N = 33)		Secondary (N = 215)	
Age (years)	19 (17–25)		22 (18–27)	
Male sex	20 (60.6%)		147 (68.4%)	
Viremic	30 (90.9%)		209 (97.2%)	
Infecting serotype:				
DENV-1	18 (60.0%)		124 (59.3%)	
DENV-2	6 (20.0%)		45 (21.5%)	
DENV-3	6 (20.0%)		33 (15.8%)	
DENV-4	0 (0.0%)		7 (3.4%)	
NS1 ELISA positive	28 (84.8%)		186 (86.5%)	
Febrile	31 (93.9%)		209 (97.2%)	
Time since illness onset (hrs)	51.5 (43.5–68.0)		49.0 (41.3–55.0)	
Disease severity:				
DF	29 (87.9%)		150 (69.8%)	
DHF	4 (12.1%)		65 (30.2%)	
Treatment allocation by disease severity^b:				
	CQ	Placebo	CQ	Placebo
DF	19 (65.5%)	10 (34.5%)	78 (52.0%)	72 (48.0%)
DHF	2 (50.0%)	2 (50.0%)	27 (41.5%)	38 (58.5%)

^aWere omitted from this table 9 patients with ambiguous or unknown clinical outcomes or serology (mainly because they left the study prematurely).

^bPercentages given here are within the same group of immune status and disease severity.

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primary DHF ($P=0.73$) and between secondary DF and secondary DHF ($P=0.13$) for the peak viremia time (Table S3). Amongst DENV-1 infected patients, peak viremia levels, when observed (in 51 of 142 DENV-1 infected patients), happened significantly earlier in secondary DF than in primary DF ($P=0.0006$) but, possibly because of small sample size, not in secondary DHF compared to primary DHF ($P=0.31$). There was no significant difference between secondary DF and secondary DHF ($p=0.48$) but there was a non-significant trend towards later viremia peaks in primary DF than in primary DHF ($P=0.052$).

There were sufficient observations of the magnitude of peak viremia in DENV-1 infections to look for associations with clinical outcome in this subgroup (Table S3). Peaks of viremia were observed in 9 primary DF, 29 secondary DF, 3 primary DHF and 10 secondary DHF DENV-1 infected patients. Peak viremia levels were not significantly different between DF and DHF patients (DF vs DHF log₁₀ median peak levels 9.89 vs 10.27, $P=0.28$) but there was a non-significant trend towards higher peak viremia during secondary infections than primary infections (primary vs secondary $P=0.096$ and primary DF vs secondary DF $P=0.086$). If considering the highest viremia levels (as distinct from peak viremia levels) in DENV-1 patients, these were significantly higher in DHF than in DF (log₁₀ median levels 9.84 vs 9.19, $P=0.03$). Because most DHF cases were associated with secondary infections, for which peak viremia had already past by the time of enrolment, this difference is probably underestimated. These results suggest secondary infections are generally associated with earlier peak viremia but do not provide any conclusive evidence of higher peak viremia levels in DHF and/or secondary infections.

Maximum daily rate of virus clearance

In the 239 patients with detectable viremia, the median of maximum daily rates of DENV clearance (estimated as the slope of

the steepest descending daily portion of the viremia curve) was 2.2 log₁₀ per day in primary DF, 2.8 log₁₀ per day in secondary DF, 2.1 log₁₀ per day in primary DHF and 3.0 log₁₀ per day in secondary DHF. The maximum daily rate of clearance was significantly higher in patients with secondary infections (median of the maximum daily loss 2.9 logs per day) versus those who experienced primary infections (median of maximum daily losses = 2.1 logs per day, $P<0.00001$) (primary DF vs secondary DF $P=0.00004$ and primary DHF vs secondary DHF $P=0.025$). The results were very similar when considering only DENV-1 patients for analysis (data not shown). These data suggest secondary infection is associated with steeper declines in viremia.

Time to resolution of viremia in DF and DHF according to serological status

Amongst all viremic patients ($n=239$) time to resolution of viremia was significantly longer in primary infections than in secondary infections (hazard ratio (HR) = 2.88, 95% CI 1.79–4.63, log rank test $P=0.000005$), in primary DF than in secondary DF (HR = 2.60, 95% CI 1.57–4.32, log rank test $P=0.0001$) and in primary DHF than in secondary DHF (HR = 4.92, 95% CI 1.19–20.32, log rank test $P=0.015$) (Figure 3A). Median times to resolution of dengue viremia were 148 hrs (IQR 140–173 hrs) in primary DF, 162 hrs (134–>171 hrs) in primary DHF, 120 hrs (97–141.5 hrs) in secondary DF and 123 hrs (113–138 hrs) in secondary DHF.

Amongst DENV-1 infected patients only ($n=142$), times to resolution of viremia were also significantly longer in primary infections than in secondary infections (HR = 4.21, 95% CI 2.12–8.35, log rank test $P=0.000009$), in primary DF than in secondary DF (HR = 3.67, 95% CI 1.76–7.62, log rank test $P=0.0002$) and in primary DHF than in secondary DHF (HR = 7.00, 95% CI 0.94–52.17, log rank test $P=0.03$) (Figure 3B). Median times to

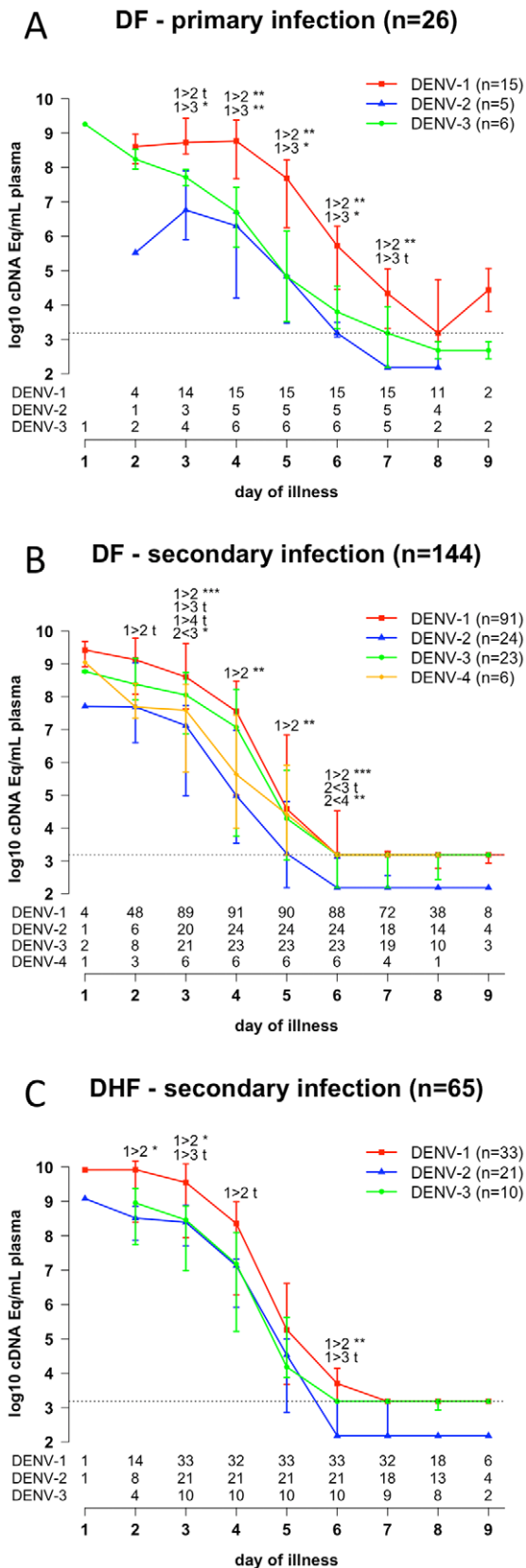


Figure 1. Magnitude of DENV viremia by illness day. Levels of DENV-1, -2, -3 and -4 genome equivalent cDNA copies per millilitre were determined in serial plasma samples from patients with **A**) primary DF (n=26), **B**) secondary DF (n=144) and **C**) secondary DHF (n=64). Data

are median and IQR. *** $P < 0.001$ ** $0.001 < P < 0.01$ * $0.01 < P < 0.05$ † $0.05 < P < 0.1$. Data from patients with DENV-1 and -2 primary DHF (n=3 and n=1 respectively) and DENV-4 secondary DHF (n=1) are not displayed because the number of these patients was too small for statistical analysis. The numbers below the graph indicate the numbers of patients at each time point. The dashed line represents the assay limit of detection. doi:10.1371/journal.pntd.0001309.g001

resolution of DENV-1 viremia were 162 hrs (IQR 144–>176 hrs) in primary DF, >171.1 hrs (134–>179 hrs) in primary DHF (since less than 50% of primary DHF had cleared viremia before discharge), 125 hrs (99–150 hrs) in secondary DF and 127 hrs (107–143.5) in secondary DHF.

Time to resolution of NS1 antigenemia in DF and DHF according to serological status

Of the 248 patients with defined serological and clinical classifications, there were 214 patients NS1 positive at the time of study enrolment (plus 2 patients negative at enrolment but NS1 positive 24 and 42 hrs later). Consistent with the viremia findings, times to resolution of NS1 antigenemia were significantly longer in primary infections than in secondary infections (HR = 4.57, 95% CI 2.01–10.40, log rank test $P = 0.00007$), in primary DF than in secondary DF (HR = 3.66, 95% CI 1.47–9.07, log rank test $P = 0.003$), in primary DHF than in secondary DHF (HR = 7.04, 95% CI 0.97–51.15, log rank test $P = 0.02$) but also in secondary DF than in secondary DHF (HR = 1.86, 95% CI 1.29–2.67, log rank test $P = 0.0007$) (Figure 4A). Interestingly, only 5 of 25 patients (i.e. 20%) with primary DF and 1 of 4 patients (i.e. 25%) with primary DHF had cleared NS1 when discharged from hospital. In patients with secondary dengue, 69 of 127 with secondary DF (i.e. 54.3%) and 51 of 60 with secondary DHF (i.e. 85%) had cleared NS1 when discharged from hospital. Median times to resolution of NS1 antigenemia since illness onset were

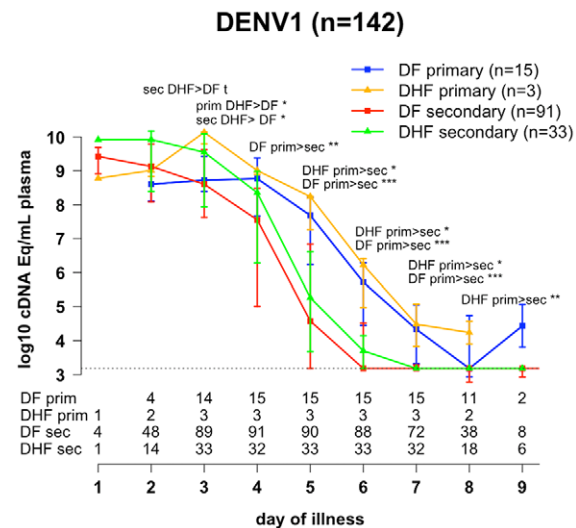


Figure 2. Magnitude of DENV-1 viremia by illness day. Levels of DENV-1 genome equivalent cDNA copies per millilitre were determined in serial plasma samples from DENV-1 infected patients with primary DF (n=15), primary DHF (n=3), secondary DF (n=91) and secondary DHF (n=33). Data are median and IQR. *** $P < 0.001$ ** $0.001 < P < 0.01$ * $0.01 < P < 0.05$ † $0.05 < P < 0.1$. The numbers below the graph indicate the numbers of patients at each time point (2 per patient for most of the time points). The dashed line represents the assay limit of detection. doi:10.1371/journal.pntd.0001309.g002

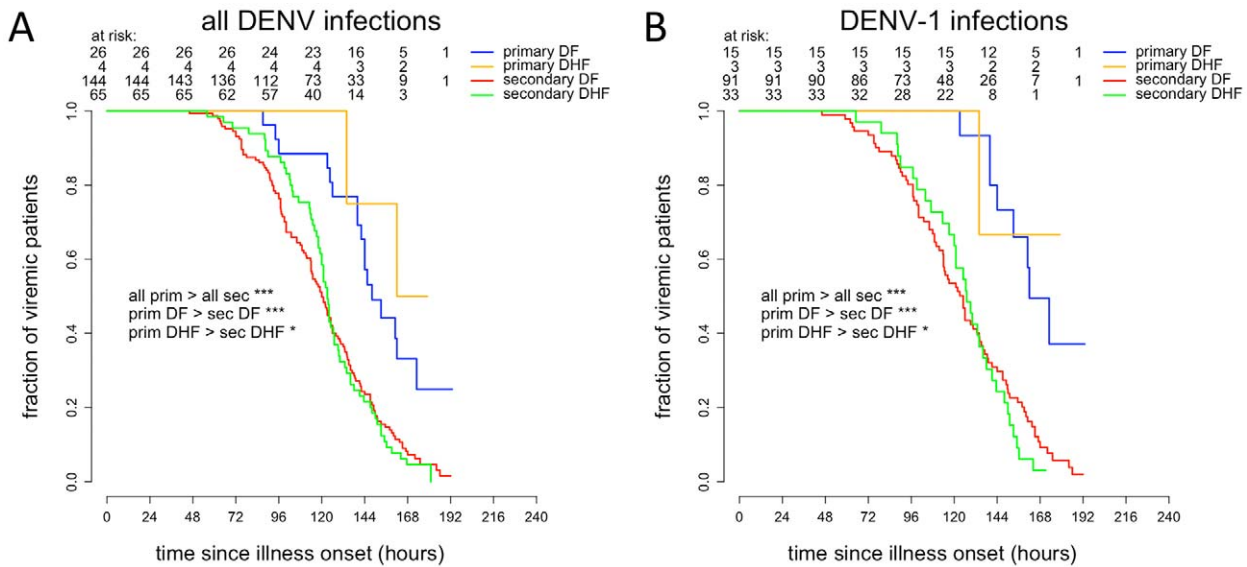


Figure 3. Time to resolution of DENV viremia. Kaplan-Meier survival analysis of time to resolution of plasma viremia in all viremic **A**) or DENV-1 only **B**) patients.
doi:10.1371/journal.pntd.0001309.g003

>166 hrs (>146–>178 hrs) in primary DF and >158 hrs (138–>171 hrs) in primary DHF (since less than 50% of primary DF and primary DHF had cleared NS1 before discharge), and 137 hrs (105–>174 hrs) in secondary DF and 121 hrs (100–153 hrs) in secondary DHF.

Amongst DENV-1 infected patients (n = 142), 134 were NS1 positive at the time of study enrolment. Times to resolution of NS1 antigenemia were significantly longer in DENV-1 primary infections than in DENV-1 secondary infections (HR = not applicable, log rank test $P=0.00008$), in DENV-1 primary DF than in DENV-1 secondary DF (HR = 3.66, 95% CI 1.47–9.07,

log rank test $P=0.003$), in DENV-1 primary DHF than in DENV-1 secondary DHF (HR = 7.04, 95% CI 0.97–51.15, log rank test $P=0.004$) and in DENV-1 secondary DF than in DENV-1 secondary DHF (HR = 1.86, 95% CI 1.29–2.67, log rank test $P=0.00001$) (Figure 4B). Strikingly, none of the DENV-1 infected patients with primary DF (n = 15) or primary DHF (n = 3) had cleared NS1 when they were discharged from hospital. In contrast, 36 of 84 (42.9%) secondary DF and 29 of 31 (93.5%) secondary DHF patients had cleared NS1 when they were discharged. Median times to resolution of NS1 antigenemia since illness onset were >172.5 hrs in primary DF, >171 hrs in primary DHF,

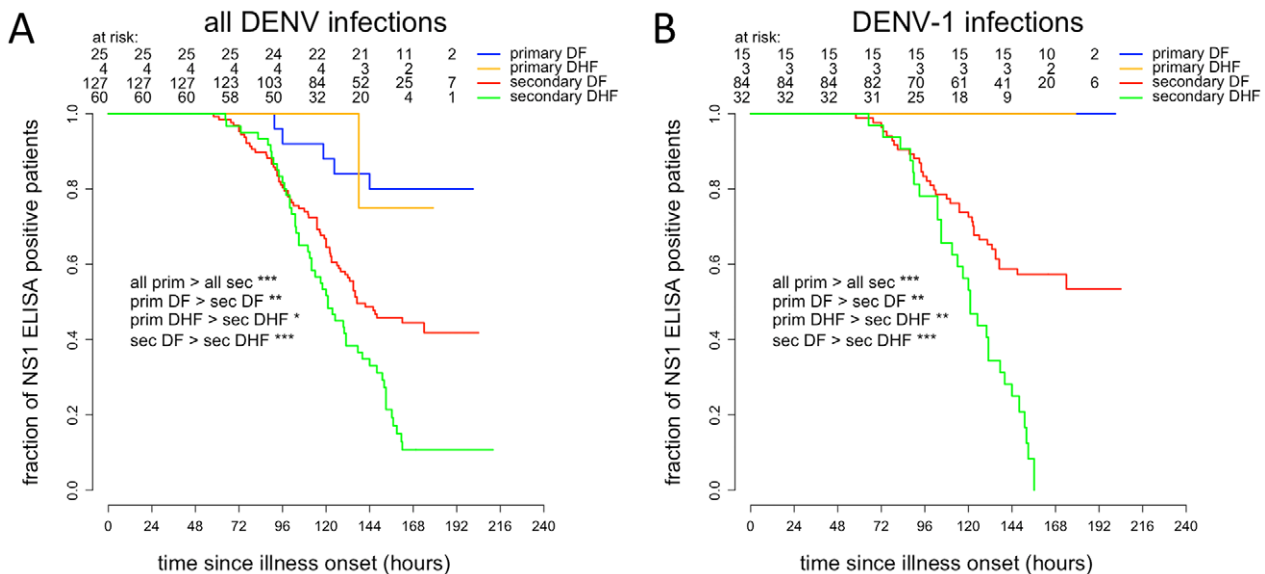


Figure 4. Time to resolution of NS1 antigenemia. Kaplan-Meier survival analysis of time to resolution of NS1 antigenemia in all viremic **A**) or DENV-1 only **B**) patients. Two patients NS1 negative at enrolment were later positive and for the purposes of analysis were considered positive at the time of enrolment.
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>174 hrs in secondary DF (since less than 50% of primary and secondary DF, and primary DHF had cleared NS1 before discharge) and 121 hrs (103–144 hrs) in secondary DHF.

Collectively, these results suggest that DENV infection is cleared earlier and faster in secondary infections than in primary infections.

Fever clearance time in DF and DHF according to serological status

There were 240 patients febrile at enrolment (plus 2 afebrile patients who developed fever soon after). Overall, FCT were significantly longer in primary infections than in secondary infections (log rank test $P=0.037$) but there was no significant difference between primary DF and secondary DF (HR = 1.44, 95% CI 0.93–2.23, log rank test $P=0.096$), and between primary DHF and secondary DHF (HR = 1.85, 95% CI 0.67–5.14, log rank test $P=0.23$) (Figure 5). Median FCT since illness onset was 131 hrs (IQR 95.5–151.4 hrs) in primary DF, 141 hrs (135.5–160.5 hrs) in primary DHF, 118 hrs (93.1–140.2 hrs) in secondary DF and 120 hrs (105–142 hrs) in secondary DHF. Consistent with the viremia and NS1 findings, these data indicate primary infection was associated with a longer-lived febrile period.

DENV-1 viremia kinetics and associations with haematological parameters

For descriptive purposes, the evolution over time of DENV-1 viremia in the context of white blood cell and platelet counts and percentage hemoconcentration was plotted (Figure 6) and summarised in Table S4. The highest levels of hemoconcentration and the lowest platelet counts occur when the viremia is close to resolution and when the patient is already or very nearly afebrile. The time to platelet nadir and maximum hemoconcentration was shorter in secondary DENV-1 infections ($P<0.01$). The data also suggest that leucopenia lasts longer in primary infections than in secondary infections.

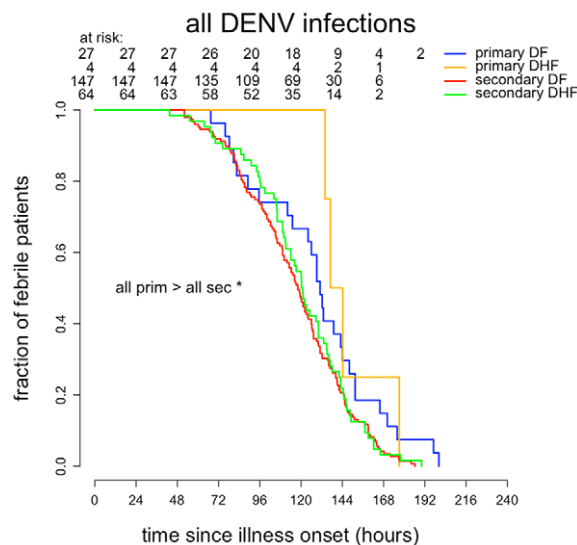


Figure 5. Fever clearance time. Kaplan-Meier survival analysis of fever clearance time in all viremic patients. Two patients afebrile at enrolment developed fever soon after and for the purposes of analysis were considered febrile at the time of enrolment. doi:10.1371/journal.pntd.0001309.g005

Discussion

The interplay between DEN virus infection and host immune status is postulated to play a central role in the pathophysiology of severe dengue. In this current study, we observed important features of this dynamic. First, early viremia levels were higher in patients with DHF, even if the peak viremia level was often not observed because it occurred prior to enrolment. Second, DENV-1 infections manifested as higher and longer-lived viremias, suggesting serotype dependent differences in infection kinetics. Third, the clearance of DENV viremia and NS1 antigenemia occurs earlier and faster in patients with secondary dengue and is also consistent with a faster time to defervescence.

Our findings are in agreement with previous studies that found higher viremias associated with more severe disease [12–14]. Our data also suggests that quantitative differences exist between DENV serotypes with respect to the kinetics of viremia and NS1 antigenemia. In particular, DENV-1 infections were associated with higher and frequently longer-lived viremia levels than infections with either DENV-2 or DENV-3. This is in agreement with recent observations in Vietnamese children and adults [15,27]. DENV-2 was associated with secondary infection and severe disease in our study; this is also in accordance with previous studies [2,27,28]. The mechanisms that facilitate relatively higher viremia in DENV-1 infections relative to DENV-2 infections in our study population, and also recently in Vietnamese children [15], are unknown. Plausibly, DENV-1 has an intrinsically faster rate of replication in this patient population and thereby attains a higher infected cell mass in vivo than DENV-2. Clearly, further studies will be needed to explore this.

The duration of NS1 antigenemia was shorter in patients with secondary infections and this is consistent with previous studies that have suggested reduced sensitivity of NS1-based diagnostic tests in patients with secondary infections [22,29–32]. One explanation is that NS1 is less likely to be available for detection when a sufficient level of DENV-reactive IgG (including anti-NS1 IgG) develops during secondary infections. This may serve to mask the antigen from detection in the immunoassay, and/or result in rapid clearance of NS1 in the form of immune-complexes.

Secondary dengue is associated with faster resolution of viremia infection and shorter duration of fever. Interestingly, the daily rates of virus clearance observed in our study were very compatible with those found by Vaughn et al in Thai children [13]. The early adaptive immune response during secondary infection is dominated by populations of memory B and T cells (and possibly memory-like NK cells [33]) and at least some components of this response mediate a strong anti-viral action, as evidence by faster clearance rates of the viremia and the NS1 antigenemia. Clearly however, aspects of this rapid host immune response are clinically deleterious given the epidemiological association between secondary dengue and more severe outcomes, and also the timing of when clinical manifestations of capillary permeability occur. This poses the intriguing question of whether modulating the host immune response (e.g. through early corticosteroid therapy) could achieve both a more gradual clearance of the virus and a host immune response that elicits less pathology.

Assuming blood viremia is a reasonable surrogate of the whole-body virus burden, then the rapid decline of viremia 48–72 hrs after illness onset, especially in secondary infections that carry higher risk for severe outcomes, has implications for rational design of therapeutic pharmacological interventions. An efficacious anti-viral will need favourable pharmacokinetic properties and potency if it is to impact on the viral burden in a rapid and clinically significant

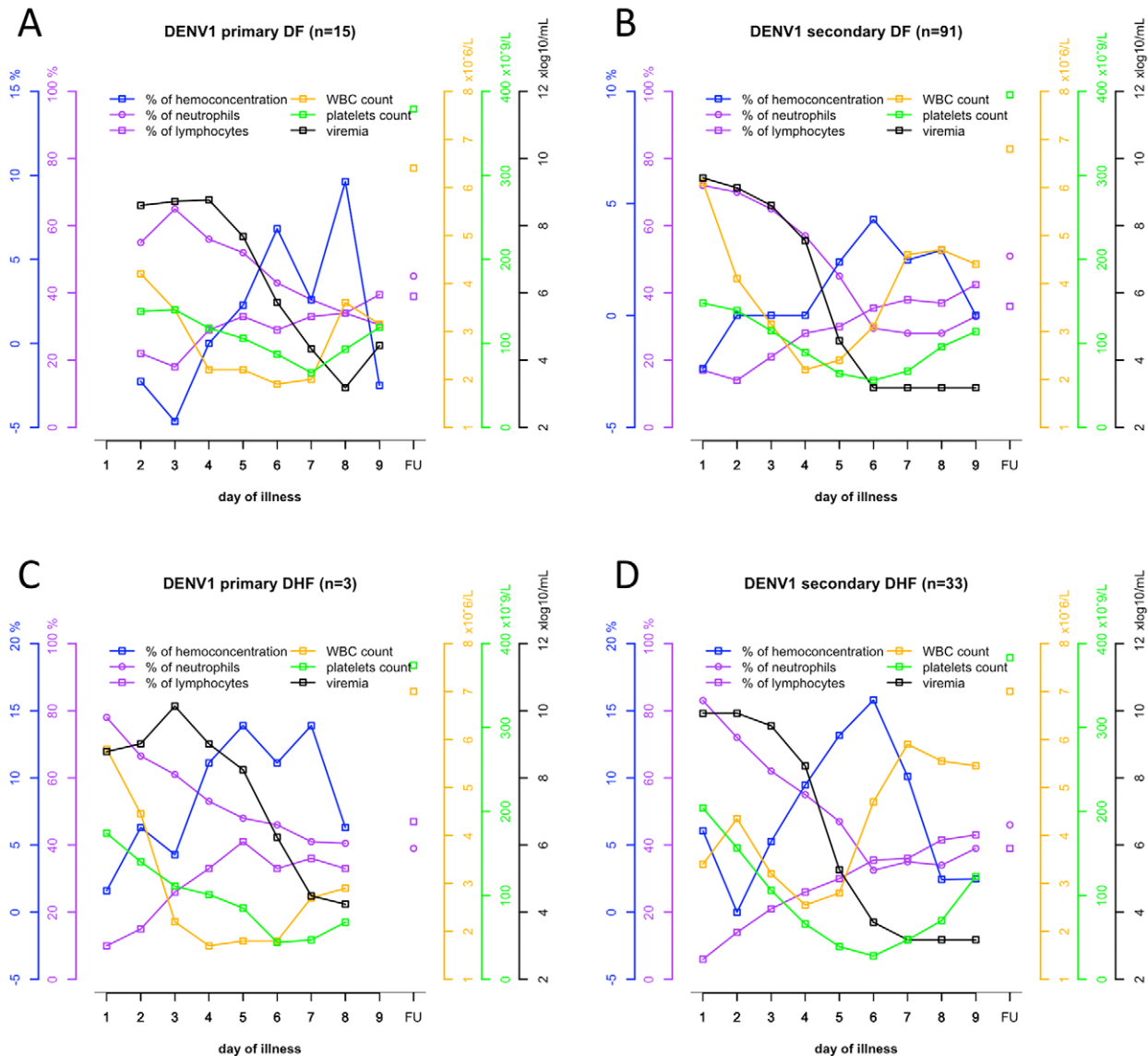


Figure 6. Profile of haematological parameters and viremia in DENV-1 infected patients. Medians of viremia levels, WBC and platelet counts, hemoconcentration, and percentages of lymphocytes and neutrophils were determined in serial plasma samples from DENV-1 infected patients with **A)** primary DF, **B)** secondary DF, **C)** primary DHF and **D)** secondary DHF. The numbers below the graph indicate the numbers of patients for reasons of clarity, error bars are not shown. doi:10.1371/journal.pntd.0001309.g006

manner. Pharmacological targeting (e.g. with corticosteroids) of the host immune response, which accounts for the rapid decline of viremia but which also likely contributes to the capillary permeability syndrome, may equally need to be administered early on in illness e.g. in a prophylactic way, to prevent clinical complications such as DSS. The rapid decline in viremia in secondary infections also highlights the importance of early diagnosis, since early diagnosis will provide the greatest opportunity for an intervention (e.g. an anti-viral), to have an impact. Point of care NS1 diagnostics are available but more can be done to improve their sensitivity [34]. More clinical research is also needed to understand if the sensitivity and specificity of early clinical diagnoses (and prognosis) can be improved, particularly in primary health care settings. Recent literature suggests this is feasible [35,36].

Current animal models of DENV infection are able to provide for in vivo measurements of anti-viral activity [37,38]. However

these models do not reproduce the temporal changes in virological biomarkers and clinical manifestations seen in naturally infected dengue patients. The lack of concordance between virological and clinical events in small animal models, and what occurs in patients, needs to be carefully considered when evaluating candidate anti-viral drugs for dengue. For instance, the onset of vascular permeability does not follow the disappearance of virus during enhanced DENV infection in mouse models [38,39].

There are several limitations to our study. Our results are derived from hospitalized patients who are not necessarily representative of patients being seen at the primary health care level. Interestingly however, the same themes identified in this study in hospitalized Vietnamese adults were also observed in Vietnamese children presenting to primary health care level clinics in Ho Chi Minh City [15]. Of the 248 patients in this study with a defined serological and clinical classification, 126 received a 3 day

course of CQ and, whilst no virological or immunological effects were detected, an effect of CQ on the clinical phenotype cannot be excluded because vomiting was more frequent in this treatment arm, possibly leading to more dehydration [21]. The majority of the patients in this study were infected by DENV-1. Very few had primary DHF (4 of 248). RT-PCR measurements of viremia in plasma may not be an entirely accurate surrogate of the infected cell mass *in vivo*, although it is certainly a better surrogate than NS1 antigenemia, which persists well after the febrile period and is heavily influenced by the immune status of the host (i.e. primary versus secondary). Viremia measurements assessed by RT-PCR encompass both infectious and non-infectious viral particles, and the relative proportions may vary between the different serological responses and/or serotypes [40]. Assessment of plasma virus titers based on plaque titration would have been a valid alternative approach to measuring virus concentrations in plasma, however this biological assay is more difficult to standardize and validate relative to a RT-PCR assay. Another general limitation of measuring virus concentrations in plasma is that viruses might be sequestered in other tissues but inaccessible to measurement while still playing a role in disease pathogenesis.

Our study emphasizes the importance of the period before and just after the onset of fever. This and other studies have established that early viremia levels are associated with disease severity, although they are very clearly not the only determinant of outcome. Very little is known of the virological events in the hours preceding and shortly after fever onset, mainly because this is very difficult to investigate without a good experimental model. An interesting insight was provided by clinical trials of DENV-1, -3 and -4 monovalent live attenuated vaccines in the 1980s [41–43]. These vaccines were not sufficiently attenuated and some volunteers developed dengue fever. These studies suggest that viremic period starts several days before the onset of symptoms. This presymptomatic viremic period should not be underestimated because of its possible contribution to DENV transmission to uninfected mosquitoes. In our study, we also observed prolonged times of virus clearance in primary dengue, and long-lived higher viremia levels in DENV-1 infections. These might lead to a higher

possibility of human to mosquito virus transmission by maintaining viremia above the infectious level over a longer period of time.

Collectively, our findings reveal important patterns of the viremia and NS1 antigenemia kinetics according to the patient immune response, disease severity and virus serotype, and may help for the rational design of clinical trials of therapeutic interventions, especially antivirals.

Supporting Information

Table S1 The characteristics of the study population by serotype.

(DOC)

Table S2 Levels of viremia among the study population by illness day and serotype.

(DOC)

Table S3 Peak viremia analysis.

(DOC)

Table S4 Maximum of hemoconcentration, platelet and WBC nadirs and their occurrence times by disease severity. All these markers were determined in serial plasma samples from DENV-1 infected patients with primary DF (n = 15), primary DHF (n = 3), secondary DF (n = 91) and secondary DHF (n = 33).

(DOC)

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Author Contributions

Conceived and designed the experiments: VT NNM JF HTT CPS. Performed the experiments: VT NNM. Analyzed the data: VT NNM CPS. Wrote the paper: VT CPS.

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